BMJ Open

New chemotherapy regimens and biomarkers for Chagas disease: the rationale and design of the TESEO study, an open-label, randomised, prospective, phase-2 clinical trial in the Plurinational State of Bolivia

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ABSTRACT

Introduction Chagas disease (CD) affects ~7 million people worldwide. Benznidazole (BZN) and nifurtimox (NFX) are the only approved drugs for CD chemotherapy. Although both drugs are highly effective in acute and paediatric infections, their efficacy in adults with chronic CD (CCD) is lower and variable. Moreover, the high incidence of adverse events (AEs) with both drugs has hampered their widespread use. Trials in CCD adults showed that quantitative PCR (qPCR) assays remain negative for 12 months after standard-of-care (SoC) BZN treatment in ~80% patients. BZN pharmacokinetic data and the nonsynchronous nature of the proliferative mammal-dwelling parasite stage suggested that a lower BZN/NFX dosing frequency, combined with standard or extended treatment duration, might have the same or better efficacy than either drug SoC, with fewer AEs.

Methods and analysis New ThErapies and Biomarkers for ChagaS infEctiOn (TESEO) is an open-label, randomised, prospective, phase-2 clinical trial, with six treatment arms (75 patients/arm, 450 patients). Primary objectives are to compare the safety and efficacy of two new proposed chemotherapy regimens of BZN and NFX in adults with CCD with the current SoC for BZN and NFX, evaluated by qPCR and biomarkers for 36 months posttreatment and correlated with CD conventional serology. Recruitment of patients was initiated on 18 December 2019 and on 20 May 2021, 450 patients (study goal) were randomised among the six treatment arms. The treatment phase was finalised on 18 August 2021. Secondary objectives include evaluation of population pharmacokinetics of both drugs in all treatment arms, the incidence of AEs, and parasite genotyping.

Ethics and dissemination The TESEO study was approved by the National Institutes of Health (NIH), U.S. Food and Drug Administration (FDA), federal regulatory agency of the

Strengths and limitations of this study

► This study will evaluate the safety and efficacy of new regimens of benznidazole and nifurtimox, with lower frequency of dosing combined with reduced or extended treatment duration, vis-à-vis the standards of care of both drugs.

► The assessment of parasitic load reduction will be performed during treatment and 36 months of systematic follow-up by quantitative PCR (qPCR), conventional serology, and novel host-derived and parasite-derived biomarkers.

► Basic population pharmacokinetics of both benznidazole and nifurtimox will be performed in all treatment arms.

► Parasite genotyping will be performed in all patients before treatment and in patients with positive qPCR results during the 36 months of follow-up.

► One of limitations is that the study will only include adult chronic Chagas disease patients in the indeterminate or early cardiac compromised (Kuschnir stage I), looking for the effectiveness of the different aetiological treatments on the progression to severe cardiac and/or gastrointestinal disease stages.

Plurinational State of Bolivia and the Ethics Committees of the participating institutions. The results will be disseminated via publications in peer-reviewed journals, conferences and reports to the NIH, FDA and participating institutions.
Trial registration number NCT03981523.

INTRODUCTION

Chagas disease (CD) or American trypanosomiasis is a tissue and blood parasitic disease caused by the kinetoplastid protozoan

parasite *Trypanosoma cruzi*. It can be transmitted by the faeces of reduviid bugs, congenital transmission, blood transfusion, tissue transplant and oral transmission.[1] CD reportedly affects ~7 million people mostly in Latin America and is responsible for an estimated 14 000 deaths yearly. It is also currently considered an emerging worldwide public health problem due to increasing international migrations from endemic regions.[2] The economic impact of CD is also very significant. According to a study conducted in 2013, the global costs for CD are US$7–US$19 billion per year, similar or higher to those of other widespread diseases, such as rotavirus infection.[3] Despite its important regional and international health impact, CD is considered by WHO one of the most neglected tropical diseases (NTDs) associated with socioeconomical inequality and thus exclusion and stigmatisation, both in endemic and nonendemic countries.[4] Currently, no safe and efficacious vaccines for this disease are available, and the existing drugs and regimens for the aetiological treatment of this condition have toxicity and efficacy issues.[5] Moreover, the aetiological treatment of CD in its chronic stage was questioned for decades by the hypothesis that autoimmunity was the major mechanism leading to chronic CD (CCD) pathology.[6] This hypothesis contributed negatively to the development of aetiological treatments for the chronic disease until very recently.[7] As a result, several generations of health professionals were trained in the belief that CCD had no possible treatment.[8] Consequently, it is estimated that currently less than 1% of confirmed CD patients receive aetiological treatment.[9] However, the role of the persistence of *T. cruzi* as a trigger for tissue damage and the pathophysiology of CD is now recognised, providing the rationale for antiparasitic treatment for all seropositive patients.[7 10–14]

To date, only two drugs have been approved for the treatment of CD, the nitroheterocyclic compounds benznidazole (BZN) and nifurtimox (NFX).[13] Such treatments are currently indicated for acute cases, congenital infections, reactivations and patients in the chronic phase without symptomatology or with mild cardiac or digestive involvement. The dosing regimens currently recommended (standard of care, SoC) are 5 mg/kg/day divided into two doses for 60 days for BZN, and 8 mg/kg/day divided into two or three doses for 60 days for NFX. Both treatments are known to be associated with adverse events (AEs) up to 70% of the patients and with 10%–27% of serious AEs (SAEs), leading to permanent treatment discontinuation in 9%–31% of the cases.[15–20] Moreover, the efficacy of these treatments is highly variable, and it has been shown that it depends on multiple factors: age of the patient, disease stage, drug dose and treatment duration, and the infecting *T. cruzi* strain or genotype, among other factors.

On the other hand, for patients in the chronic stage the efficacy of the treatment is difficult to assess. Using quantitative PCR (qPCR) as an efficacy parameter, it can be estimated that parasitological clearance (defined as the parasitic load in the blood below the detectable limit of qPCR) would occur in 60%–90% of the treated cases, at 12 months of follow-up.[7 21] However, the Pan-American Health Organization (PAHO) guidelines for the diagnosis and treatment of CD state that only the seroconversion by conventional serology (CS) can be interpreted as an indicator of parasitological cure,[22] but it is well known that in chronically infected adults with successful parasitological cure, CS seroconversion can take 10–20 years to be confirmed following chemotherapy.[23–25] Thus, the lack of biomarkers (BMKs) of early response to treatment and eventual parasitological cure is a main roadblock to evaluate the true efficacy of currently available and novel chemotherapeutic approaches.[26 27]

Recent studies suggest that the current BZN dose of 5 mg/kg/day divided into two doses (SoC) can lead to an overdosing of the patients while using half of the daily dose could be enough to reach and sustain anti-*T. cruzi* therapeutic plasma levels.[28] In the case of NFX, a recent study showed that 3 mg/kg two times per day given for 60 days showed an efficacy of 70%, which is comparable to the results with the SoC.[29] A fundamental insight in this respect was provided by a study in a murine model of the disease,[30] which showed that reducing the dosing frequency of BZN or NFX from daily (continuous) to every 5 days (intermittent) provided the same parasiticidal efficacy, using a much lower total dose of the drug. Such findings were reported by authors to indicate that both drugs act on the parasite through a critical peak serum concentration (C_{max} effect), rather than a continuous exposure (area under the curve-AUC effect).[31] However, carefully designed translational pharmacokinetics–pharmacodynamic modelling and population pharmacokinetics (popPK) studies of both drugs in humans, as those to be performed in the New ThErapies and Biomarkers for ChagaS infEctiOn (TESEO) study and future clinical studies, will be critical to define the precise pharmacodynamic drivers for these drugs.

Based on these antecedents, our first hypothesis is that a lower frequency of BZN or NFX dosing, with standard or extended treatment duration, might have the same or better efficacy than the SoC of either drug, with fewer AEs. Our second hypothesis is that, in those patients who respond to BZN or NFX treatment, the serum levels of one or more potential BMKs proposed in this study will be significantly reduced or become negative within 3 years of post-treatment follow-up. The 3-year follow-up is designed to investigate whether the high level of parasitaemia suppression induced by BZN 1 year after the end of treatment (EOT) found in recent clinical studies[32 33] is sustained after longer follow-up times, or whether parasitaemia relapse occurs, as reported in the BENEFIT trial,[34] previous observational studies ≥2 years after the EOT,[35–40] and in a canine model of CCD.[41]

Therefore, the TESEO study aims to assess the safety and efficacy of new dosing regimens of BZN and NFX for the treatment of CCD patients, combining a reduction of the frequency of dosage with reduced or extended treatment duration, as well as a 3-year follow-up, and

Table 1 Treatment arms in TESEO study

Arm	Drug*	Regimen	Arm abbreviation
1	BZN	150 mg b.i.d., 60 days	BZN-60 (SoC)
2	BZN	150 mg q.d., 30 days	BZN-30
3	BZN	150 mg q.d., 90 days	BZN-90
4	NFX	240 mg b.i.d., 60 days	NFX-60 (SoC)
5	NFX	240 mg b.i.d., 30 days	NFX-30
6	NFX	240 mg q.d., 90 days	NFX-90

*Both drugs are taken orally.
b.i.d, two times a day; BZN, benznidazole; NFX, nifurtimox; q.d., once a day; SoC, standard of care; TESEO, New ThErapies and Biomarkers for ChagaS infEctiOn.

evaluation by qPCR plus a panel of novel potential host-derived and parasite-derived BMKs of early assessment of therapeutic response to aetiological treatment and eventual parasitological cure.

METHODS AND ANALYSIS
Study design
This clinical trial is an open-label, blinded allocation, randomised, prospective, phase-2, observational study. A total of 450 patients will be randomly assigned to one of the six treatment arms (75 in each arm) (table 1). The treatment arms include the SoC of BZN (150 mg two times per day for 60 days) and NFX (240 mg two times per day for 60 days), while the four experimental groups will test a lower frequency of dosing, combined with shorter or extended treatment duration (table 1). With the proposed new

regimens, the aim is to reduce AEs, while maintaining or increasing antiparasitic efficacy. The detailed clinical trial design is shown in figure 1.

In this study, the basic popPK parameters of BZN and NFX for all treatment regimens, including the SoC with both drugs, will also be evaluated. Patients will be followed up for 36 months. This extended follow-up is aimed at evaluating the sustainability of the antiparasitic effect, when compared with the results of recent clinical trials with a 12-month follow-up.[32 33 42] The study also aims to generate information concerning qPCR and novel potential BMKs for the early assessment of antiparasitic response to the current and novel chemotherapeutic interventions proposed in this study.

The study design was approved by the National Institutes of Health (NIH) and US Food and Drug Administration (FDA) without requiring a placebo arm, based on proving the non-inferiority of the novel treatment schemes when compared with the SoC and the use of a historical placebo from a recent study in the same geographical locations and populations in the Plurinational State of Bolivia.[32]

Objectives
Primary objectives
The primary objectives of the TESEO study are to determine the safety and efficacy of new proposed chemotherapy regimens of BZN or NFX in adults with CCD are comparable or superior to SoC (table 1), evaluating the relevant time response of qPCR and novel BMKs for the assessment of parasitaemia and systemic parasite clearance. Parasitic loads (by qPCR) and levels of *T. cruzi*-specific BMKs will be evaluated at EOT and at 4, 6, 12, 18,
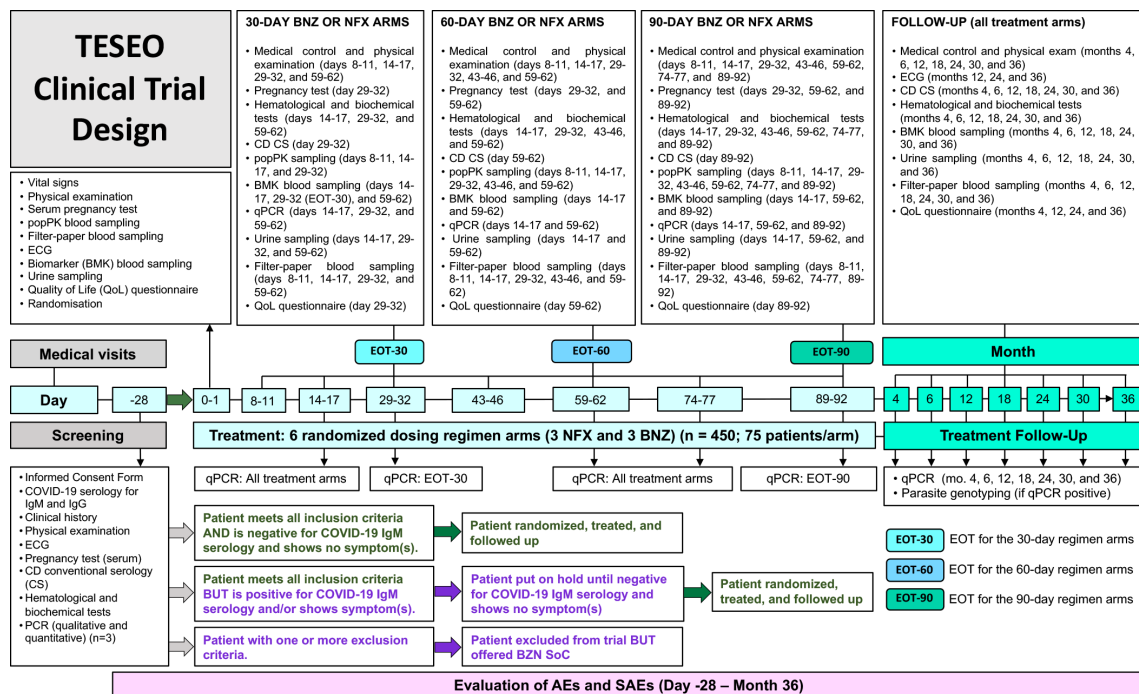
Figure 1 TESEO clinical trial design. AEs, adverse events; BMKs, biomarkers; BZN, benznidazole; CD, Chagas disease; EOT, end of treatment; NFX, nifurtimox; popPK, population pharmacokinetic; qPCR, quantitative PCR; SAEs, serious AEs; SoC, standard-of-care; TESEO, New ThErapies and Biomarkers for ChagaS infEctiOn.

24 and 36 months of follow-up, as compared with historical placebo control for BZN in the same population.[32] The EOTs are defined by the duration of the treatment arm (table 1, figure 1).

BMKs for early assessment of therapeutic outcomes

To address the need of BMKs for early assessment of therapeutic responses and eventual parasitological cure, various host-derived and parasite-derived BMKs, which have shown promising results in recent preclinical or clinical studies, will be evaluated in the TESEO study. The selected BMKs belong to two different classes: host-derived and parasite-derived BMKs.[26] The host-derived BMKs include: (1) lytic, protective CD-specific anti-α-galactopyranosyl antibodies (Ch anti-α-Gal Abs),[43–46] as evaluated by chemiluminescent ELISA (CL-ELISA), using as antigens purified glycosylphosphatidylinositol (GPI)-anchored trypomastigote-derived mucins (tGPI-mucins),[44 47] or synthetic α-Gal-containing neoglycoproteins (α-Gal-NGPs)[47–50] and (2) antibodies to the specific parasite-derived recombinant proteins KMP11, HSP70 and PFR2, and the synthetic peptide 3973, as assessed by ELISA.[51–55] The parasite-derived BMK involves the detection of trypomastigote excreted/secreted antigens (TESA) by an aptamer-based assay.[56–58] BMKs will be evaluated at baseline, during treatment, at EOT, and through a 3-year follow-up interval at the times indicated above in Primary Objectives and figure 1. Changes in BMKs' levels will be correlated with parasitic load clearance, as measured by qPCR.

Rationale for the selection of host-derived and parasite-derived BMKs

Host-derived BMKs

Lytic anti-α-Gal antibodies to tGPI-mucins and α-Gal-NGPs

The major lytic, protective antibodies (Abs) in CD patients are produced against highly immunogenic, immunodominant α-Gal epitopes abundantly expressed on O-glycans of tGPI-mucins of the infective mammalian host-dwelling or tissue-culture-derived trypomastigote (TCT) stage,[44 59–63] and on complex phosphoglycans (P-glycans) of gp72 glycoprotein of the infective insect-derived metacyclic trypomastigote (MT) stage[64]; such glycans are absent in humans and nonhuman primates.[65 66] Thus, very high levels of trypanolytic anti-α-Gal Abs are found in both acute (IgM) and chronic (IgG) stages of CD,[43 45 67–69] which induce complement-independent lysis of TCT stage,[44 59 61 67] or complement-dependent lysis of MT stage.[45] tGPI-Mucins (in TCTs) and gp72 glycoprotein (in MTs) are the main molecular targets of lytic anti-α-Gal Abs from CCD patients (Ch anti-α-Gal Abs).[44 59 60 63 70] Although tGPI-mucins exhibit intraspecies polymorphism in their O-linked glycans, the expression of highly immunogenic, nonreducing, terminal α-Gal residues seems to be very conserved on tGPI-mucins from at least four major *T. cruzi* discrete typing units (DTUs) or genotypes[71 72] causing infections in humans (TcI, TcII, TcV and TcVI).[44 60 73 74] Corroborating these results, we and others

have demonstrated the ubiquitous presence of high levels of Ch anti-α-Gal Abs in CCD patients from different endemic regions in Latin America,[43 44 46 60 67–69 75–79] and in a non-endemic country, Spain.[74 80] We have previously shown that tGPI-mucins[81] (also known as TcMUC II, F2, F2/3, or AT antigens),[44 46 60 76 82] in a CL-ELISA (or AT CL-ELISA), could be used to evaluate lytic Ch anti-α-Gal Ab titres to confirm the efficacy of BZN treatment in children and adolescents with recent chronic infection, in a placebo-controlled randomised trial in Brazil.[46 76] After a 3-year follow-up, 58% (37/64) of BZN-treated children were considered cured by intention-to-treat (ITT) analysis, as measured by negative seroconversion with AT CL-ELISA, although all CS tests remained positive.[46] After a 6-year follow-up of that trial, 65% and 85% of the patients were considered cured by ITT and by per-protocol (PP) analysis, respectively, as measured by AT CL-ELISA.[76] In fact, these studies were the first to confirm the efficacy of BZN chemotherapy for CCD, by using Ch anti-α-Gal Abs as BMKs of parasitological cure. Based on those successful studies[46 76] and a subsequent clinical trial in children in Argentina with similar BZN treatment outcomes using the *T. cruzi* recombinant protein F29 (also known as flagellar calcium-binding protein, FCaBP) as a BMK for early assessment of therapeutic efficacy,[83] PAHO and WHO recommended BZN for the treatment of early CCD in children up to 12 years of age in 1999.[81] In 2017, the FDA approved the BZN treatment in the U.S. for children (2–12 years of age), based on those clinical studies in Brazil and Argentina.[46 76 83 84] In a recent clinical study with 56 adult patients with CCD subjected to BZN SoC treatment, we observed a significant decrease (p=0.0052) in the titres of lytic anti-α-Gal Abs using tGPI-mucins in the AT CL-ELISA, starting at 12 months and up to 36 months (EOT).[80] Taken together, such findings strongly justify the use of Ch anti-α-Gal Abs as host BMKs for early assessment of chemotherapeutic outcomes in the TESEO clinical trial. In this study, we also aim to validate a series of synthetic α-Gal-NGPs[47–50] to eventually replace the tGPI-mucins as antigens in the AT CL-ELISA. These synthetic α-Gal-NGPs will overcome technical difficulties in purifying and obtaining high quantities of consistently homogenous parasite-derived tGPI-mucins, for large-scale application in clinical settings.[47]

Antibodies to T. cruzi-derived recombinant proteins KMP11, Hsp70, and PFR2, and peptide 3973

Although most conventional serological tests are very sensitive for the diagnosis of CD, the evaluation of patients under or following treatment is ambiguous, since some anti-*T. cruzi* antibodies are long-lasting and a significant seroconversion occurs only decades following chemotherapy.[24 25 85] Remarkably, a significant decrease in the reactivity of sera from CCD patients in the indeterminate (asymptomatic) phase against *T. cruzi*-derived recombinant proteins KMP11, HSP70, and PFR2, and peptide 3973 was detected 9 months after BZN treatment in 67%, 50%, 34% and 49% of the patients, respectively.[26 52 55]

Moreover, these *T. cruzi* antigens were recognised with high sensitivity (90%, 70%, 75% and 90%, respectively) and specificity (85%, 85%, 92% and 98%, respectively) by sera from CCD patients,[52 86] while they were not recognised by sera from healthy donors and patients with heterologous infections. It was also reported that the decrease in the reactivity of sera of CCD patients to a set of 14 recombinant *T. cruzi* proteins of the parasite, included in a multiplex serologic assay,[87] was associated with a substantial reduction in the parasitic load and with an improvement in the clinical status of treated CCD patients.[88] Recently, it has been reported that patients with demonstrated treatment efficacy based on analysis of these four serological BMKs as a set, showed an enhanced antigen-specific CD8$^+$ T cell multifunctional responsiveness.[54]

Parasite-derived BMKs
Detection of TESA by aptamers
T. cruzi is an intracellular parasite and was shown to secrete proteins (ie, TESA) into the host milieu.[89] The detection of circulating parasite antigens can demonstrate that live organisms are present in the host even if direct detection of trypomastigotes in blood is negative.[90] TESA BMK levels were also shown to significantly decrease in CCD patients following BZN SoC treatment.[58] It was recently reported that an aptamer-based, non-serological, non-PCR assay could detect TESA BMKs circulating in the blood of infected mice.[56 57] RNA aptamers selected to bind with high specificity and affinity to TESA were used in enzyme-linked aptamer assays to detect TESA BMKs in plasma from infected mice, including chronically infected mice that failed BZN treatment. These treated animals still contained significant levels of TESA, as compared with controls.[56]

Secondary objectives
The secondary objectives will be the evaluation of the parasitic load reduction during the treatment and 36 months of follow-up by qPCR and serological response, as measured by CS with commercial ELISA kits (as described below), and non-CS (NCS) with the BMKs described above.

The basic popPK parameters of all BZN and NFX treatment arms will be characterised and correlated with parasitological response as measured by qPCR.

The incidence of AEs, especially SAEs, AEs of Special Interest (AESI) and AEs leading to discontinuation of treatment are evaluated and correlated with the levels of the drug in the moment of appearance of the AE(s).

Parasite genotyping will be performed at the end of the follow-up period in all patients prior to treatment and in patients with positive qPCR result(s). Such genotyping will provide important information concerning the *T. cruzi* genotypes[72] and strains circulating in the study region and their potential correlation with treatment response.

Endpoints (outcome measures)
Efficacy endpoints
The primary efficacy endpoint is sustained parasitological clearance determined by negative blood qPCR at EOT and along the 3 years of follow-up. The secondary efficacy endpoints are changes in the circulating parasitic load during treatment and along the follow-up interval by qPCR, as well as CS and NCS responses, and changes in the levels of the BMKs.

Safety endpoints
The safety endpoints will consider the following evaluations: incidence, severity, and seriousness of AEs, either clinical, laboratory or ECG, and incidence of AEs leading to treatment discontinuation. All safety analyses will be performed blind to the treatment allocation. The AEs reporting period for the TESEO study begins on administration of the first dose of trial medication for non-serious events and after the signature of informed consent for serious events. The reporting period concludes at the end of patient participation in the study.

popPK endpoints
The popPK parameters (clearance–CL and volume of distribution–Vd) will be obtained by non-linear mixed-effects modelling (NONMEM) (ICON, Cambridge, Massachusetts, USA). Other PK measures, such as AUC, C_{max}, and C_{min}, will also be calculated, as previously described for BZN.[28] Blood samples will be drawn from patients receiving aetiological treatment with BZN or NFX at predose and postdose during the treatment, and at EOT. BZN and NFX popPK data will be correlated with the efficacy and safety endpoints, parasitological response; the frequency and timing of AEs will be correlated with the serum levels of the drugs.

Parasite genotyping
At the end of the 3-year follow-up period, *T. cruzi* genotyping and restriction fragment length polymorphism (RFLP)-PCR fingerprint of mitochondrial kinetoplast DNA (kDNA) products of parasite populations will be determined in the processed blood samples of all patients prior to the study treatment, and in samples from patients with positive qPCR result(s) during the 3-year follow-up.[91]

Patient eligibility and exclusion criteria
Adult patients (18–50 years old), infected with *T. cruzi,* as diagnosed by CS (two positive tests with different antigens) and a positive qPCR result during the screening period, will be eligible. The patient must be in the indeterminate form (no clinical manifestations) or early cardiac stage (Kuschnir stage I) of CCD (table 2).[92 93] To have a homogeneous population without pathologies linked to age, that could be confounders, patients older than 50 years old were excluded from participating in this study. Additionally, the patients must comply with all inclusion criteria and none of the exclusion criteria, summarised in box 1.

Table 2 Chagas disease cardiomyopathy classification for TESEO study, following the Kuschnir criteria*

| | Kuschnir classification | | | |
	Kuschnir stage I	Kuschnir stage II	Kuschnir stage III	Kuschnir stage IV
Symptoms	Asymptomatic	Asymptomatic or mild symptomatology	NYHA I/II	NYHA III/IV
Chest Rx	Normal	Normal	Mild cardiomegaly	Moderate or severe cardiomegaly
ECG	▶ Non-specific findings ▶ Incomplete right branch bundle block ▶ Upper left RBBB ▶ Non-severe bradycardia ▶ Long PR interval, without AV block criteria. ▶ Minor alterations of ST-T waves	▶ Complete RBBB±upper LAH ▶ AV block (first and second grade) ▶ Low-voltage QRS ▶ Diffuse changes in ST-T. ▶ Monomorphic isolated ventricular extrasystoles	▶ Q waves ▶ Third degree AV block ▶ Severe bradycardia ▶ Polymorphic PVC	▶ Atrial flutter. ▶ Atrial fibrillation

*Modified from Rassi et al.[93]
AV, atrioventricular; LAH, left bundle branch hemiblock; NYHA, New York Heart Association; PVC, premature ventricular contractions; RBBB, right branch bundle block; TESEO, New ThErapies and Biomarkers for ChagaS infEctiOn.

Recruitment, randomisation, treatment and follow-up of patients

Potential participants will be recruited from the surrounding communities and the usual patients visiting the Platform for the Comprehensive Care of Patients with CD (Chagas Platforms; https://www.ceadesbolivia.org/plataformaChagas.aspx), Plurinational State of Bolivia, in three study sites: Cochabamba, Tarija and Sucre. Review of the rate of patient accrual and compliance with inclusion/exclusion criteria will occur monthly during the recruitment phase to ensure that enough participants are being enrolled and that they meet eligibility criteria and the targeted goals outlined in study protocol.

The eligible patients will be allocated in equal proportions to one of the six treatment arms on day 1 (table 1 and figure 1). Treatment allocation will be designated by a computer-generated randomised list produced by an independent statistician. Randomisation envelopes are provided to the designated study team members to be opened at the time of recruitment. Master randomisation list will be kept stored and sealed by the study's Steering Committee until the end of the study. This study is an open-label trial, thus, both patients and field study teams will be aware of treatment allocation. However, clinical and safety assessments and laboratory assessments will be performed blinded to treatment allocation.

Adherence to treatment will be monitored with the Morisky-Green test[94] and pill count, and AEs will be monitored by clinical and laboratory controls (basic haematology and biochemistry) pursuant to the particular protocols of each of the three study sites. Data on adherence to the treatment protocol will be collected weekly by research staff and reviewed quarterly by the three PIs and the study clinical coordinator. Adherence of participants will be evaluated by performing pill counts at each visit. Pharmacokinetic data with documentation of patient exposure will also be available at the end of the trial. Available data on the use of BZN and NFX suggest an overall compliance rate of 75%. If adherence falls below the suggested rate of 75%, which might inhibit the ability of the study to test its primary hypotheses, the clinical coordinator will suggest a conference call for study PIs, co-investigators (Co-Is) and consultants to discuss methods for improving adherence.

Once the patient starts the study treatment, scheduled follow-up visits will occur on day 8 and then, biweekly until the EOT, according to treatment arm, and up to 4, 6, 12, 18, 24, 30 and 36 months after treatment initiation. In the study visits, the patient will undergo a physical exam, ECG and pregnancy test (in selected visits); blood samples will be drawn to assess safety and efficacy according to the scheme of the clinical study design in figure 1. Inclusion and exclusion criteria for the study are given in box 1.

If a patient does not return to a scheduled visit, all necessary steps must be taken to contact the patient and document the patient's situation. Before declaring 'follow-up loss', the medical investigator must do everything possible to contact him/her, to establish the reason for the interruption of the treatment, and all contact attempts must be properly documented. To minimise lost to follow-up, during the screening the detailed contact information will be recorded, including the patient's mobile and/or landline phone number and address and/or the contact information of a family member. This information will remain in each study site (Chagas Platform). If the patient does not appear at the Chagas Platform and the telephone number is not available, the field team will follow up with the patient or their family directly. It will be considered lost to follow-up of a patient only if he/she does not return to the follow-up visit of 36 months.

As part of the study, we will evaluate if after the treatment the participant is free of the parasite that causes

Box 1 Inclusion and exclusion criteria of TESEO clinical trial.

Inclusion criteria
1. Adults, 18–50 years old.
2. Weight: 40–90 kg.
3. Individuals diagnosed with CD by conventional serology (two positive tests with different antigens) and with at least one positive qualitative PCR assay out of three during the screening.
4. Patients classified as being in the indeterminate form (without clinical manifestations) or early cardiac form (Kuschnir I) of CCD (table 2).
5. Signed informed consent form.

Exclusion criteria
1. Clinical signs of dilated cardiomyopathy: Dyspnoea, legs' oedema, syncope, pulmonary crackles). Patients with an electrocardiogram (ECG or EKG) showing the following characteristics: sinus tachycardia or atrial fibrillation, ventricular, arrhythmias, left atrial enlargement, left bundle-branch block accompanied by right axis deviation, and/or patients with Fridericia's corrected QT interval >450 ms, a formula for calculating the QT interval on an ECG.[118]
2. History of CD treatment with BZN or NFX or any triazole drug(s) in the last 5 years
3. Clinical signs and/or symptoms of a digestive form of CCD, which is characterised by the presence of two or more of the following criteria:* (A) Excessive exertion in at least 25% of bowel movements. (B) Hard stools in at least 25% of stools (Bristol types 1 and 2). (C) Feeling of incomplete evacuation in at least 25% of bowel movements. (D) Feeling of obstruction or anorectal block in at least 25% of bowel movements. (E) Manual manoeuvres to facilitate defecation in at least 25% of bowel movements. (F) Less than three complete spontaneous stools per week. *Criteria must be met for at least the last 3 months and symptoms must have been started for at least 6 months before diagnosis.
4. Hypersensitivity to the active substances (BZN or NFX) or the excipient.
5. Previous diagnosis of porphyria.
6. Any other acute or chronic health conditions that, in the opinion of the study's principal investigators (PIs), may interfere with the efficacy and/or safety evaluation of the study drug.
7. Formal contraindication to BZN or NFX.
8. Any concomitant or anticipated use of drugs that are contraindicated with the use of BZN or NFX, as defined by the study's Manual of Operations.
9. Individuals are currently known to abuse alcohol and/or drugs.
10. Pregnancy or breastfeeding.
11. Not using a highly effective contraceptive method (only for women in reproductive age).
12. Laboratory parameters outside the normal range or the acceptable range for the following parameters: (A) Transaminase levels (alanine transaminase, and aspartate transaminase) must be within the acceptable margin of 25% above the upper limit of normality. (B) Creatinine levels must be within the acceptable margin of 10% above the upper limit of normality. (C) Total bilirubin must be within the acceptable margin of 15% above the upper limit of normality. (D) Haemoglobin, platelets and leucocytes must be within the acceptable margin of ±5% of the normal range.

CD. In the case of persistence of *T. cruzi*, an alternative treatment and post-study follow-up care will be offered, at no cost to the individual by the regular patient care programme of the three Chagas Platforms involved in this study. Although not a guarantee, these alternative treatments can be successful when the previous treatment has failed, with lower side effects.

Study organisation

The TESEO clinical trial is being conducted in the Plurinational State of Bolivia (for simplicity, henceforth also referred as Bolivia), in the Chagas Platforms of Cochabamba, Tarija, and Sucre. The Chagas Platforms are part of a collaborative project between the Fundación Ciencia y Estudios Aplicados para el Desarrollo en Salud y Medio Ambiente (CEADES, Cochabamba, Bolivia) and the Barcelona Institute for Global Health (ISGlobal, Barcelona, Spain).[95] Bolivia was chosen as the study site because it has the highest relative percentage of chronically infected, asymptomatic individuals of any endemic country in the world, representing approximately 10% of the urban population and up to 30%–40% of the rural population.[96 97] Moreover, the selected sites in Bolivia have the necessary expertise in phase-2 clinical trials,[32 33] including high level of care to CD patients and the required infrastructure and personnel for the TESEO study. The three sites also have facilities to carry out all tests proposed in the study (ECG, biochemistry, haematology and qPCR), as well as adequate logistics infrastructure to properly collect and store samples and send them to laboratories in the USA and Spain for further analyses.

The biochemistry, haematology and CS tests are carried out at each study site. qPCR analysis is centralised at the BioMol Laboratory at CEADES. To that end, blood samples collected in 6 M guanidine hydrochloride, 0.2 M EDTA, pH 8.0[98 99] are sent from each site to the CEADES BioMol Laboratory. At the end of the 3-year follow-up, coded and frozen serum samples for analysis of BMKs will be sent from each site to the CEADES laboratory and stored at −20°C until shipment to UTEP. UTEP will carry out the analysis of BMKs for lytic anti-α-Gal Abs and genotyping of the parasite populations and will forward the serum samples to FDA (Silver Spring, Maryland, USA), and 'Instituto de Parasitología y Biomedicina López Neyra' (IPBLN, Granada, Spain), for specific BMK analysis (TESA detection by aptamers and NCS by ELISA, respectively).

Laboratory procedures

In this study, blood and urine samples are collected from patients for the laboratory procedures described below and future BMK studies. The maximal quantity of blood collected per patient during each visit will be 18–25 mL. Each urine sample to be collected per visit will be ~10 mL. Blood samples are separately processed for qPCR and parasite genotyping analysis, biochemical and safety laboratory parameters, pregnancy test, popPK analysis and conventional and NCS (BMK analysis) (figure 1 and appendix table 1).

Conventional serology

For patient eligibility, two different CS tests based on different antigens are used. To assess the changes over time by CS, the specific kits Chagatek ELISA (Lemos Laboratory, Buenos Aires, Argentina) and Chagatest ELISA recombinante 3.0 (Wiener Lab., Rosario, Argentina) are used. According to the kit inserts, the specificity and sensitivity of Chagatest ELISA recombinante 3.0 are 98.3% and 99.3%, respectively; the specificity and sensitivity of Chagatek ELISA are >99% and 100%, respectively. To avoid variability due to the technique, the serum samples collected at the various time points described in the protocol are kept frozen at −20°C and will be processed in parallel at the end of the study (3-year follow-up).

Safety laboratory parameters

Safety clinical laboratory parameters include blood cell counts, and liver and kidney function tests. These analyses are regularly performed in each laboratory site, in such a way that results will be available daily, facilitating a close follow-up of the patients.

qPCR assays

For the evaluation of the effect of the different treatment protocols on the patients' circulating parasitic load, a qPCR method, based on TaqMan technology and validated by an international panel for the standardisation and validation of *T. cruzi* PCR, supported by the PAHO/WHO/Special Programme for Research and Training in Tropical Diseases (PAHO/WHO/TDR), is used.[98 100] The qPCR assays are performed at the CEADES BioMol Central Laboratory, according to the procedures previously described.[98 99] Briefly, at each PCR time point, 5 mL of blood is collected in triplicate in EDTA blood sample tubes and mixed with equal parts of 6 M guanidine hydrochloride, 0.2 M EDTA, pH 8.0, at each Chagas Platform. The processed blood samples are sent to the CEADES BioMol Lab and stored at room temperature until processing. High-throughput automated isolation of DNA from the processed blood samples is performed in the KingFisher Duo Prime system (Thermo Fisher Scientific), using the MagMax DNA Multi-Sample Ultra 2.0 kit, according to the supplier's instructions (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0017325_MagMAX_MultiSampleUltra2_WholeBlood_UG.pdf, Applied Biosystems, Thermo Fisher Scientific). The limit of detection (LOD) for *T. cruzi* satellite DNA is 0.69 parasite equivalents/mL, and the limit of quantification (LOQ) is 1.5 parasite equivalents/mL.[98 100] The PCR amplification is carried out in triplicate from each of the extracted samples, using a *T. cruzi* satellite DNA region and the RNAse P as an endogenous amplification control. A PCR time point is considered positive if at least one of the 9 PCR amplifications results positive. The qPCR analyses will be performed during the study follow-up; nevertheless, the results will not be disclosed

to the study team and to the patients until the end of the patients' study participation.

BZN and NFX serum concentrations

Serum concentration of BZN and NFX will be quantified by ultrahigh performance high-performance liquid chromatography coupled to tandem mass spectrometry at UTEP, following the previously described methodology for BZN.[28 101] The same methodology will be validated for NFX.

Safety management

The study physician will evaluate all the AEs and classify them as serious/nonserious and related/non-related to the study drugs. The study treatment can be interrupted temporarily or permanently according to the study physician evaluation and/or according to the defined protocol rules (box 2). The interruption of the treatment does not imply withdrawal from the study. In such cases, the treatment may be temporarily interrupted; therefore, it will be considered incomplete or delayed. Treatment can be resumed, following evaluation by the site medical investigator and the study PI(s). In case of temporary or permanent treatment interruption, patient should continue with the study visits and evaluations as planned, but the reasons for the treatment interruption should be recorded in the appropriate original documentation and case report form (CRF).

The AEs' reporting period starts upon administration of the first dose of study drug for non-serious events and after the signature of informed consent form (ICF) for SAEs and ends at the end of patient participation in the trial. The AEs that are considered related to the study drug or the study procedures, will be followed until resolved or considered stable by the study physicians, or if needed, the patient care will be delegated to an adequate health facility.

> **Box 2 Rules for the permanent interruption of the study drug**
>
> 1. ALT or AST >8 times the upper limit of normal (ULN)
> 2. ALT or AST >5 times the ULN for more than 2 weeks
> 3. ALT or AST >3 times the ULN and total bilirubin >2 times the ULN or the international normalised ratio >1.5
> 4. ALT or AST >3 times the ULN with the appearance of any one of the following symptoms: fatigue, nausea, vomiting, pain or sensitivity in the upper right quadrant, fever, rash and/or eosinophilia (>500 cells/mL)
> 5. An adverse event or any other condition that, in the opinion of the CoI(s) and/or PI(s), may place the patient at severe risk if he/she continues with the study treatment.
> 6. Any condition that the investigators or PIs deem medically necessary to interrupt the treatment such as: (A) Significant leucopenia (<2500 cells/mm^3). (B) Severe gastrointestinal symptoms. (C) Severe allergic dermopathy. (D) Peripheral sensory neuropathy.
> 7. Pregnancy

The reporting procedures for the AEs vary depending on the serious/nonserious classification of the event. All SAEs must be reported within 24 hours of awareness of the event by the investigator to the study coordinator and the two pharmacovigilance (PVG) teams working in this study: Division of Microbiology and Infectious Disease (DMID)-CROMS PVG (Safety and Pharmacovigilance Technical Resources International, Bethesda, Maryland, USA) and Lat Research (Clinical Research Organisation, Buenos Aires, Argentina). If the SAE is considered related to the study drug and unexpected, it meets the category of Suspected Unexpected Serious Adverse Reaction (SUSAR). A SUSAR must be informed promptly to the FDA, the Agencia Estatal de Medicamentos y Tecnologías en Salud (AGEMED), the Data and Safety Monitoring Board (DSMB) and the Ethics Committees (ECs) of the participating institutions, within seven calendar days if the event is fatal or life-threatening and less than 15 days for any other SUSAR. The SAEs that do not meet the SUSAR characteristics, will be reported to the regulatory agencies in the annual study report, along with other safety data.

The study Steering Committee, composed by the three PIs (ICA, FT and JG) and one of the coinvestigators (IR), can determine whether to terminate the trial at any time prior to inclusion of the intended number of patients. However, it intends to exercise this right only for valid scientific or administrative reason(s). In terminating the trial, the study Steering Committee will assure that adequate consideration is given to the protection of the patients' interests. Reasons for early termination may include but not be limited to: (1) low enrolment rate; (2) high frequency of protocol violations; (3) inaccurate or incomplete data; (4) unsafe or unethical practices; (5) following the recommendation of the DSMB or the ECs of the participating institutions; (6) administrative decision; (7) insufficient time or resource to conduct the trial and (8) lack of eligible patients.

Data and safety monitoring board

A DSMB composed of a minimum of four members (including one statistician) independent of the research team and drug manufacturers, ELEA (BZN) and Bayer (NFX), was set up prior to trial initiation (18 December 2019). The DSMB for this protocol is convened by authority of the DMID, National Institute of Allergy and Infectious Diseases (NIAID), NIH, and is advisory to DMID and the study team. The DSMB will monitor the trial to ensure that harm is minimised, and benefits maximised for the trial patients. The DSMB will review efficacy and safety data on an ongoing basis and at predetermined intervals, review all information related to the occurrence of SAEs and AEs leading to treatment discontinuation, and issue recommendations about the trial if the existing benefit/risk of the patients in the trial seems compromised. The first DSMB meeting occurred on 10 June 2020, once 20% of recruited patients had completed the treatment.

Sample size

The sample size was determined following the generalised estimating equations (GEE)-based approach of Pan,[102] involving the following considerations: our preliminary studies yielded an average clearance rate $(0.91+0.81)/2=86\%$ for standard BZN (as opposed to $(0.255+0.085)/2=17\%$ for the historical placebo control group),[32] corresponding to an odds ratio (OR) of 29.99. We assume an first-order autoregressive AR(1) model correlation structure among repeated measures on each patient with rho=0.2; a total of four non-inferiority testing (new BZN/NFX regimens vs BZN/NFX SoC) lead to a Bonferroni adjusted significance level $\alpha=0.05/4=0.0125$. According to the formula in Pan,[102] a total of 75 patients per arm allows us to establish non-inferiority for all four comparisons with a margin as small as 9.5% in clearance rate difference (corresponding to 1.887 in OR) at the joint significance level of $\alpha=0.05$ with a power of 90%.

A total of 450 patients in the trial gives sufficient power to establish the efficacy of all therapies against the historical placebo control, based on the results from our previous study.[32] Additionally, this number of patients also allows at least a 90% probability of observing at least one event of peripheral neuropathy or paraesthesia, transaminase increase and hypersensitivity.

Data management and statistical analyses

Clinical and epidemiological data are collected from patients' visits during the study planned visits and entered the Data Management Programme. Laboratory data are uploaded to the system from records generated in the sites' laboratories. OpenClinica Community V.3.14 (OpenClinica, Waltham, Massachusetts, USA, www.OpenClinica.com), an open-source web-based application designed to support the data capture from study research, will be used in the TESEO study. The study PIs and the Biostatistics and Data Management Unit (DMU) at ISGlobal, Barcelona, Spain, are responsible for: (1) study-wide data management; (2) metadata creation; (3) data security and (4) quality assurance of data.

All analyses will be performed on the per-protocol (PP) and intention-to-treat (ITT) strategies. ITT's analysis will include all randomised patients and PP analysis will use an ITT population excluding people with any major protocol deviations such as patients who did not receive their assigned treatment, patients who substantially violated screening, inclusion, or exclusion criteria (boxes 1 and 2), or patients with a permanently discontinued treatment. Furthermore, the trial has a dataset that includes all randomised patients having received at least one dose of study therapy, to perform safety analyses.

Data will be described according to their characteristics. The categorical data will be presented as frequencies, and we will list the number of missing and will be compared using the χ^2 test or Fisher's Exact test. Moreover, the continuous data will be described as mean, standard deviation (SD) or median, and interquartile ranges (IQRs) in agreement with the data, following or not the

theoretical normal distribution. In this case, the data will be compared using analysis of variance or Kruskal-Wallis at the 0.05 two-sided significance level. Time-to-event data will be presented using Kaplan-Meier curves with a log-rank test (two-sided 0.05 significance level). The analysis of covariates on time-to-event data will be performed using a Cox regression mode.[103] Longitudinal data will be analysed using generalised linear mixed-effects models (GLMM) or GEE, using both Stata (release 16, 2019; https://www.stata.com/company/, StataCorp) and R Studio (https://rstudio.com/, Boston, Massachusetts, USA).

For the treatment compliance, a full course of treatment is defined by a minimum of 75% of prescribed treatment days. The primary efficacy endpoint is a binary (cured or non-cured) variable based on a total of eight qPCR time-points from EOT until 36 months (figure 1). Each of the timepoints includes qPCR examinations of three samples, by triplicate assays. A patient will be considered 'cured' if he/she shows a total of 24 negative qPCR results.

The primary non-inferiority assessment will be done with a GLMM or GEE.

To assess the efficacy of each treatment group vs the historical placebo control,[32] we will apply Gibbons and Bock's method[104] to test no trend among these correlated proportions, with a mean equal to 0.082 sustained negative clearance at 12 months in historical placebo control.

To depict the time to reappearance of parasitaemia for patients who have cleared parasitaemia at EOT, Kaplan-Meier survival curves will be drawn across treatment groups.

To determine how the different tested BMKs affect binary outcomes of parasite clearance, GLMM will be performed. All the BMKs variables available will be included in the model from the baseline, during treatment and during the follow-up. This modelling analysis will allow us to examine which BMK variables are significantly associated with blood parasitic load evolution. This latter analysis will be presented for only the PP population, since randomised (ITT) analysis is not considered to be pertinent for this exploratory analysis.

The popPK analysis will be performed by NONMEM (ICON, Cambridge, Massachusetts, USA) through a three-step strategy: (1) basic population model selection, (2) covariate selection, and final population model selection; and (3) model validation.

For safety analyses, AEs will be classified according to the MedDRA dictionary (V.17.0) and summarised for each treatment group by seriousness, severity, causality and action taken with the study drug. Besides, the total number of AEs, the number and the proportion of patients experiencing at least one AE during the treatment period will be summarised for each treatment group by body system and preferred term. In the same way, the proportion of patients in each treatment group withdrawn due to an AE and/or an SAE will be described. Safety laboratory parameters (haematology and biochemistry) and vital signs will be described using the summarised

statistics according to the variable. Moreover, the number of patients with normal and abnormal, and with clinically significant abnormalities will be summarised.

ECG abnormalities will be described per treatment arm as the proportion of patients per type of ECG finding and changes over time.

The significance level for any comparison is $\alpha = 0.05$ and the analysis will be carried out using different statistical packages, such us Stata (https://www.stata.com/company/, StataCorp) and R Studio (https://rstudio.com/).

Interim analyses will be performed when all patients have completed treatment. The interim analysis results will not be disclosed, under any circumstances, to the Steering Committee, the PIs and trial personnel at the study sites managing day-to-day activities of the trial prior to the end of the trial. The analysis will be performed by an independent statistician. A futility stopping rule is defined as no difference from the placebo (a historical placebo,[32] as to be used in this study), in sustained pblood parasitic load clearance at EOT. Regarding efficacy, with clearance of blood parasitic load at EOT as the parameter of interest and cut-off of 60%, the conditional power (CP) at the time of the interim analysis will be calculated so that, if the probability that CP of rejecting the alternative hypothesis (active arm superior to placebo) is higher than 60%, the treatment arm should be stopped. Patients will be considered as early treatment failures. This rule is rigid and if not followed in the DSMB recommendations, the type I error will be inflated as a result. For safety, on completion of treatment of 20% of recruited patients, a safety interim analysis will be performed. The interim analysis results/outcome will not interfere with the trial procedures unless they meet the criteria defined for the harm and futility stopping rules.

The Steering Committee and coinvestigators of each participating institution will have password-protected access to temporary and final trial datasets, housed at the DMU/ISGlobal. Password-protected access to temporary, individual and final datasets will also be provided to consultants, collaborators, and study investigators after prior authorisation from the Steering Committee. To safeguard confidentiality, data disseminated to authorised study investigators will be blinded to any identifying patient information.

Resources and data availability
The investigators of this study are aware of and agreed to abide by the principles for sharing research resources, as described by NIH in the 'Principles and Guidelines for Recipients of NIH Research Grants and Contracts on Obtaining and Disseminating Biomedical Research Programmes.' Accordingly, resources developed in this study will be available to the scientific community as soon as the intellectual property of these resources and/or research tools have been protected or disclosed in publications. If a specific research tool is requested from the

TESEO investigators and is available, it will be shared with members of the scientific community.

Data sharing not applicable as no datasets generated and/or analysed for this study. However, once the datasets resulting from this study are available, they will be disseminated via publications in peer-reviewed journals, national and international conferences, and reports to the NIH, FDA and participating institutions.

Ethics statement, patient confidentiality, and dissemination policy

This study is conducted according to the Declaration of Helsinki-Ethical Principles for Medical Research Involving Human Subjects, by the World Medical Association. The study was registered in ClinicalTrials.gov on July 11, 2019. The study protocol has been approved by the following institutions, Institutional Review Boards (IRBs) or Ethics Committees (ECs): FDA, Investigational New Drug (IND), IND-143169; NIH/NIAID/DMID, protocol number 18-0015; UTEP, IRB protocol version number 743474-1, originally approved on 20 April 2015, and amended on 17 June 2021 (version number 743474-20); Bolivian federal regulatory authority, AGEMED, protocol number MS/AGEMED/AUMyT/AAAyC/CE/41/2019, originally approved on 8 February 2019, and amended on 30 August 2021 (MS/AGEMED/AUMyT/AAAyC/CE/385/2021); CEADES, protocol number 743474-7, originally approved on 21 December 2019, and amended on 16 August 2021 (version 743474-20); and Comité Ético de Investigación Clínica, Hospital Clinic de Barcelona, originally approved on 7 November 2019, and amended on 27 July 2021. The other participating institutions (FDA and IPBLN) will follow the protocol approved UTEP IRB, through an Inter-Institutional Agreement (IIA).

Documented informed consent will be obtained by the assigned study site investigator only after the potential participant has been fully informed about all aspects of the study: its duration, study treatments, study procedures, potential risk and benefices, participant responsibilities and patient confidentiality. The participants will confirm their voluntary participation in the study after he/she has understood the explanation provided by the investigator, and any doubts or questions have been satisfactorily answered. A copy of the informed consent form (ICF) will be provided to the participant. To assure the patient confidentiality, the patients will be identified by an identification code on the CRFs and in all documents submitted to ethical committees, DSMB and manufacturers.

All modifications in the study IRB protocol and ICF will be reported and approved by the ECs of the participating institutions, NIH/NIAID/DMID and FDA.

The data generated in this project will be disseminated via publications in peer-reviewed journals, presentations at national or international conferences, as well as in reports to the NIH, FDA and participating institutions. Publication authorship will be determined based on the relative scientific contributions of the PIs and key personnel (coinvestigators, collaborators, consultants, staff and students), following the Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals from the International Committee of Medical Journal Editors (http://www.icmje.org/icmje-recommendations.pdf).

Patient and public involvement

Patients or the public were not involved in the design, or conduct, or reporting, or dissemination plans of our research.

Standard Protocol Items: Recommendations for Interventional Trials Reporting guidelines

This manuscript was elaborated following the Standard Protocol Items: Recommendations for Interventional Trials reporting guidelines (https://www.spirit-statement.org/).[105]

DISCUSSION

It is well established that there is an urgent need to find safer and more efficacious drugs or improved therapeutic regimens with the clinically available drugs for the aetiological treatment of CCD. Three key objectives remain: confirm the 20% treatment failure of parasitological 'cure' (sustained blood parasitic load clearance by qPCR) observed up to a 12-month follow-up in previous studies, evaluate the parasitological efficacy on an extended (36-month) interval, and reduce the frequent AEs associated with available drugs and current treatment schemes. In addition, the study aims to evaluate BMKs for early assessment of therapeutic outcomes, which remain as one of the main roadblocks in the development of new therapeutic options and also an important need for routine management of patients.

Posaconazole and the E1224 (a ravuconazole prodrug) were the most promising experimental anti-*T. cruzi* drugs, but they both failed when tested in clinical trials at the dose and duration of treatment used in these studies.[32 42] Efforts have been made in different fields, from in vitro assays (using high throughput screening, HTS) to drug repurposing, but plausibly it still will take considerable time and effort to register a new drug for the aetiological treatment of CD.[106–109]

The fact that CD belongs to the group of NTDs could explain that the accepted dosing treatments with BZN and NFX, which were based on non-randomised studies carried out in the '60s, have not been revised since their introduction. Lately, there is a trend in the study of reduced antiparasitic regimens. An observational non-randomised study, using the standard BZN dose (5 mg/kg/day) administered for 30 days in adults with CCD, showed that treated patients developed fewer ECG abnormalities when compared with untreated patients along a median follow-up of 9.8 years.[85] Moreover, in a pilot study using an intermittent BZN scheme of 5 mg/kg/day every 5 days for a total of 60 days in 20 CCD patients, showed a

good efficacy profile 1 week after the EOT and in 14 of the 17 patients (82%) at the end of follow-up (12 to 48 months).[110 111] However, none of these studies compared the effectiveness and safety of the shorter regimen with the BZN SoC.

Following the concept of dose reduction, the BENDITA trial (ClinicalTrials.gov, NCT03378661), was a placebo-controlled study that compared five new dose regimens of BZN alone or combined with E1224 with the BZN SoC. The BENDITA study results have shown that the dose regimen of 2 weeks had similar efficacy and fewer side effects than the 8 weeks of BZN SoC, as assessed by sustained qPCR negative results at 6-month and 12 month follow-up.[34] The BETTY ("Short-Course Benznidazole Treatment to Reduce *Trypanosoma cruzi* Parasitic Load in Women of Reproductive Age") study (ClinicalTrials. gov, NCT03672487) will compare the BZN SoC to a short course of 150 mg/day/30 days, in women of reproductive age; the efficacy will be assessed by qPCR and endpoint PCR tests at the EOT and 10 months of follow-up, and the safety will be assessed by the occurrence of SAEs in both treatment arms. The EQUITY ("CHICAMOCHA 3 - Equivalence of Usual Interventions for Trypanosomiasis") study (ClinicalTrials.gov, NCT02369978) is a placebo-controlled trial that will evaluate half standard doses of NFX and BZN for 120 days (240 mg/day for NFX and 150 mg/day for BZN) in comparison with the SoC of both drugs (480 mg/day/60 days for NFX and 300 mg/day/60 days for BZN). The efficacy will be evaluated by PCR at 12–18 months of follow-up and by changes in the levels of B-type natriuretic peptides, a suggested surrogated BMK for CD cure,[27] and also by CS.[112] The MULTIBENZ ("Evaluation of Different Benznidazole Regimens for the Treatment of Chronic Chagas Disease") study (ClinicalTrials. gov, NCT03191162) aims to compare the SoC of BZN with two lower dose regimens: 150 mg/day/60 days and 400 mg/day/15 days; the efficacy will be assessed by PCR during the 12-month follow-up and by CS.

A key strength of the TESEO study is the long-term evaluation of antiparasitic efficacy by qPCR and novel potential BMKs. The 3-year follow-up will allow us to detect therapeutic failure with six different BZN/NFX schemes in the medium term, beyond the 12 months used in recent CD clinical trials.[32 33] Moreover, no published popPK for NFX is available and we will use a previously described methodology for BZN[28 101] to validate the popPK for NFX.[113] The new data will provide useful information on the requirements of follow-up duration to assess the true efficacy of chemotherapy in CCD patients. Such efficacy will be evaluated using standard methodologies for CS and qPCR, as well as NCS, such as lytic anti-α-Gal antibodies,[43–48 60 76] antibodies against KMP11, HSP70, PFR2 and peptide 3973,[51–55] and parasite-derived BMK TESA, using an aptamer-based assay,[56–58] as described above. It is known that the efficacy of CCD treatment depends, among other factors, on the infecting *T. cruzi* strain or genotype.[72 114] In fact, *T. cruzi* genotypes or (DTUs, TcI-VII) exhibit marked differences regarding virulence and pathological traits in vitro and in vivo murine models of CD.[72] In the TESEO study we will determine the genotype of the parasites infecting all patients before the treatment, and in patients that exhibit positive qPCR result(s) during the 3-year follow-up. The results of genotyping in patients with therapeutic failure will allow us to evaluate the impact of polyclonal infections and possible drug tolerance on the outcome of aetiological treatments. When completed, this study could be included in a meta-analysis, as those currently published.[23–25 115 116]

Since the TESEO trial is being performed in Bolivia, we have been using the CS tests Chagatek ELISA and Wiener Chagatest ELISA recombinante 3.0, which that have previously shown high sensitivity and specificity for the diagnosis of CD in that population.[32] If other populations in North, Central, or South America, or other region(s) (eg, Europe) undergo a protocol similar to that of TESEO trial, the aforementioned tests and other commercial diagnostic tests for CD should be evaluated to determine those with highest sensitivity and specificity for each specific population. From our experience and from that of other research groups in South America, Central America and Mexico, there is a considerable variation in the diagnostic parameters with different kits, which is mainly due to the parasite genotype and strain diversity.[72] A recent article has described highly discordant results when three CS tests (including Wiener Chagatest ELISA recombinante V.3.0 and Chagatek ELISA used in this study) and two in-house tests were evaluated with 196 sera from Veracruz, Mexico.[117] That study underscores the need of a careful prescreening of the target population and use of a well-defined panel of positive (ideally, confirmed by PCR) and negative endemic controls from the studied region, prior to the adoption of any CS test as reliable diagnostic tool in any clinical trial.

In conclusion, the TESEO study aims to attain a wide landscape of results that could provide the basis for a new approach in the treatment of CCD. In comparing the safety and efficacy of four alternative regimens to the SoC of the two approved drugs for CD, BZN and NFX, the study turns out to be more comprehensive than past or ongoing clinical trials in terms of both its breath and duration of follow-up, using novel potential BMKs for early assessment of therapeutic response and eventual parasitological cure in direct comparison to qPCR and CS, as well as the genotypification of the infecting *T. cruzi* populations. Moreover, an improved knowledge of the efficacy of the currently available anti-*T. cruzi* drugs will allow us to better assess the potential of combining these with novel anti-*T. cruzi* drug candidates.

Trial status and its adaptation to the COVID-19 pandemic situation

The recruitment of patients was initiated on 18 December 2019, and the first patient was randomised on 7 January 2020. However, due to the SARS-CoV-2/COVID-19 pandemic, the recruitment was interrupted between March and September 2020, following the quarantine

restrictions established by the Bolivian Ministry of Health. Per the FDA Guidance on Conduct of Clinical Trials of Medical Products During the COVID-19 Public Health Emergency (https://www.fda.gov/media/136238/download) and the Ministry of Health of Bolivia, a set of mitigation measures were taken to safeguard the safety of the patients and the study personnel. During the strict lockdown, the study team made efforts to assure that all the patients randomised had continued with the study treatment and the follow-up visits; however, the scheduled visits had to be adapted according to the regional lockdown policies. On 20 April 2020, 20% of recruited patients completed their assigned treatment regimen. As stated in the study protocol, a DSMB virtual meeting took place to review the study status and the safety summary report. No safety concerns were identified but considering the current COVID-19 pandemic, the DSMB recommended to consider testing the study subjects for COVID-19 at screening and during the study follow-up. After the strict lockdown measures in Bolivia were temporarily suspended, the DSMB recommendation was implemented and the recruitment was resumed on 18 September 2020, with the screening of patients for COVID-19 before randomisation and during treatment and follow-up. To that end, we developed a serological testing algorithm, which included initial laboratory screening using lateral flow assay (LTA) commercial kits (approved by FDA for emergency use during the pandemic) for anti-SARS-CoV-2 IgM and IgG antibodies (CTK Biotech, Poway, California, USA). If the patient was positive for LTA-IgM and/or LTA-IgG, confirmatory ELISA tests (NovaLisa, NovaTec, Immunodiagnostic, Dietzenbach, Germany) for both antibody classes were performed. In the screening phase, patients with positive anti-SARS-CoV-2 IgM antibodies by LTA and ELISA were not randomised into the study, even if they met all inclusion criteria. They were put on hold until they become positive only for anti-SARS-CoV-2 IgG antibodies (by ELISA) and showed no COVID-19 symptoms, indicating that they were in the chronic phase of the disease and represented no transmission risk. The serological algorithm for COVID-19 is summarised in figure 1 (bottom left).

The study sites were successfully able to meet the recruitment goal of 450 participants with the final randomisation occurring on 20 May 2021. On 18 August 2021, the treatment phase was concluded in 86% (n=387) of the randomised patients. Of the remaining 14% (n=63), 13% (n=58) had to interrupt the treatment during early stages due to AEs. One per cent (n=5) withdrew the informed consent. Another DSMB Review Meeting was held virtually on 22 September 2021, after the study participants had finalised their treatment phase. No significant safety concerns were identified. The DSMB requested follow-up information regarding participants who were COVID-19 seropositive for the next DSMB meeting, expected to take place in September 2022.

Despite the considerable negative impact of the pandemic in the recruitment, thus far no patient has been lost during the treatment and/or follow-up as a direct consequence of COVID-19. As an additional pandemic mitigation measure, the same COVID-19 immunoassays were implemented to regularly screen the medical personnel, nurses, and laboratory and administration staff in the three study sites.

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