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Prospective, historically controlled study to evaluate the efficacy and safety of a new pediatric formulation of nifurtimox in children aged 0 to 17 years with Chagas' disease

Prospective study of a pediatric nifurtimox formulation for Chagas' disease

Bayer study drug	BAY No. A2502/Nifurtimox		
Study purpose:	To evaluate efficacy and safety, and characterize the pharmacokinetics, of nifurtimox in children with Chagas' disease using 30-day and 60-day treatment durations		
Clinical study phase:	3	**Date:**	26 JAN 2020
Study No.:	16027	**Version:**	3.0
Author:	PPD		

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The approval of the Statistical Analysis Plan is documented in a separate Signature Document.

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Abbreviations

AE	Adverse event
ATC	Anatomical Therapeutic Chemical
CI	Confidence interval
C_{max}	maximum concentration
D	Day
DMC	Data Monitoring Committee
ECG	electrocardiogram
ELISA	Enzyme-linked immunosorbent assay
EOT	End of treatment
FAS	Full analysis set
IgG	Immunoglobulin G
IHA	Indirect hemagglutination assay
IVRS/IWRS	Interactive Voice Response System/Interactive Web Response System
MedDRA	Medical Dictionary for Regulatory Activities
OD	Optical density
PD	pharmacodynamics
PK	pharmacokinetics
PPS	Per-protocol set
qPCR	Quantitative polymerase chain reaction
SAE	Serious adverse event
SAP	Statistical Analysis Plan
SAS	Statistical Analysis System
T. cruzi	*Trypanosoma cruzi*
TEAE	Treatment-emergent adverse event
V	Visit
WHO	World Health Organization
WHO-DD	World Health Organization – Drug dictionary

1. Introduction

This statistical analysis plan (SAP) describes the study objectives, study design, study population, efficacy and safety variables, statistical analysis methods, and study tables to be used in this study. The original protocol, Version 1.0, is dated 04 NOV 2014. This SAP v3.0 , dated 26 JAN 2020, is based on the integrated clinical study protocol Version 11.0, dated 03 NOV 2020, which includes the CHICO SECURE part of the study.

This study consists of two parts:

- Part 1 comprises the treatment with nifurtimox including the 1-year follow up (CHICO)
- Part 2 comprises the 3-year long-term follow-up (LTFU) of all subjects who were randomized and received at least one dose of their assigned nifurtimox treatment regimen in CHICO (CHICO SECURE)

Part 1 (CHICO) was designed to develop a better understanding of the efficacy, safety/tolerability, and pharmacokinetics (PK) of nifurtimox in children with a diagnosis of Chagas' disease using pediatric formulations. Results from this study will demonstrate parasitological cure using conventional serological results to allow optimization of treatment of Chagas' disease in this most vulnerable population. In turn, this will improve the outlook for children by reducing mortality and long-term complications. This knowledge will allow for better and more appropriate approaches to the treatment of Chagas' disease. It will also provide more up-to-date information on the use of nifurtimox, and has the potential to increase our understanding of treatment response in children.

Part 2 (CHICO SECURE) was designed, at the request of the US Food and Drug Administration (FDA), to assess the incidence of seronegative conversion in subjects who were randomized and received at least one dose of their assigned 60- or 30-day nifurtimox treatment regimen. In part 2 (CHICO SECURE), subjects will be followed up for 3 years totaling to a follow-up period of 4 years after end of nifurtimox treatment, the first year of follow up is included in the design of part 1 (CHICO).

2. Study Objectives

2.1 Objectives for part 1 (CHICO)

The primary objective of this Phase 3 clinical study is:

- To assess the superiority of a 60-day regimen of nifurtimox to historical untreated control at the 12-month follow-up (360 days from end of treatment [EOT]) as sero-

reduction (defined as a ≥ 20% reduction in optical density [OD] measured by conventional enzyme-linked immunosorbent assay [ELISA]) compared to baseline in subjects ≥ 8 months to < 18 years of age at randomization or sero-conversion (defined as negative Immunoglobulin G [IgG] concentration) in all subjects

Secondary objectives of the study are:

- To assess the comparability of a 30-day regimen of nifurtimox to a 60-day regimen of nifurtimox as sero-reduction or sero-conversion at the 12-month follow-up (360 days from EOT)

- To evaluate the safety/tolerability profile of nifurtimox by laboratory parameters (hematology, blood chemistry, urinalysis), electrocardiogram (ECG) monitoring, vital sign measurements (blood pressure, heart rate, respiratory rate, temperature), adverse event (AE) monitoring, and physical examinations, including neurological examinations

- To evaluate the pharmacokinetics (PK)/pharmacodynamics (PD) of nifurtimox in children receiving the drug for treatment of Chagas' disease

Exploratory objectives of the study are:

- To assess the comparability of a 60-day regimen of nifurtimox to historical active control (benznidazole) as sero-reduction or sero-conversion at the 12-month follow-up (360 days from EOT)

- To assess the comparability of a 30-day regimen of nifurtimox to a 60-day regimen of nifurtimox using qualitative polymerase chain reaction (qPCR) at the 12-month follow-up (360 days from EOT)

- To evaluate the relationship of conventional serology (as sero-reduction or sero-conversion) to qPCR using frequencies of matches and mismatches to assess agreement

- To evaluate the relationship of non-conventional serology to conventional serology

- To evaluate the relationship of conventional serology to indirect haemagglutination assay (IHA)

2.2 Objectives for part 2 (CHICO SECURE)

The primary objective of the long-term follow-up (LTFU) part of the study is:

- To assess the incidence of seronegative conversion as confirmed by two types of assay, recombinant ELISA and indirect hemagglutination assay (IHA), in subjects who were randomized and received at least one dose of the 60-day nifurtimox treatment regimen, compared to an external control group of untreated patients with Chagas' disease at the 4-year follow-up.

The secondary objectives are:

- To assess the incidence of seronegative conversion, as confirmed by two types of assay (recombinant ELISA and IHA), in subjects who were randomized and received at least one dose of the 30-day nifurtimox treatment regimen at the 4-year follow-up.

- To evaluate the proportion of responders who show both seronegative conversion as confirmed by two types of assay (recombinant ELISA and IHA) and no evidence of established cardiomyopathy as evaluated in electrocardiogram (ECG) recordings.

- To assess serial reduction of antibody titers, as measured by recombinant ELISA and total purified antigen ELISA, in subjects who were randomized and received at least one dose of either the 60- or 30-day nifurtimox treatment regimen compared to Visit 1 in part 1 of the study

The exploratory objectives are:

- To assess the incidence of seronegative conversion as confirmed by two types of assay (recombinant ELISA and IHA) in subjects by age categories (≤2 years, >2 years to ≤6 years, >6 to ≤12 years, >12 to <18 years; age is defined as subject's age at randomization).

- Assess the proportion of responders and the incidence of seronegative conversion as confirmed by non-conventional serology in subjects who were randomized and received at least one dose of either 60- or 30-day nifurtimox treatment regimens.

- To assess the occurrence of congenital infection with T. cruzi in children born of female subjects who were randomized and received at least one dose of 60- or 30-day nifurtimox treatment regimen. The congenital infection will be confirmed by parasitological method in children ≤8 months of age and by serological method in children >8 months of age.

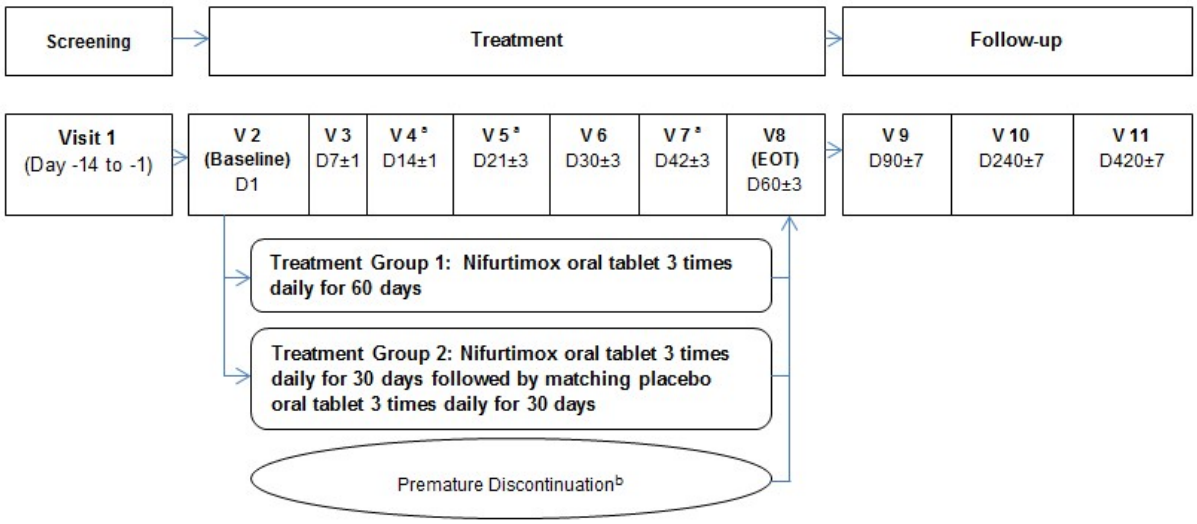
3. Study Design

3.1 Part 1 (CHICO)

Design overview

This is a Phase 3, prospective, randomized (to dosing regimen), age-stratified, double-blind, parallel-group study to evaluate the efficacy, safety/tolerability, and PK of oral administration of nifurtimox in children with a diagnosis of Chagas' disease. A schematic of the study design is presented in Figure 3–1.

Figure 3–1. Study Design of Part 1



D = Day, EOT = end-of-treatment, V = Visit.

a At Visits 4, 5, and 7, study site personnel will contact the subject/legally authorized representative via telephone to assess for the occurrence of AEs, use of concomitant medications, and compliance with study drug administration. If any safety concern arises, subjects may return to the study site for an Unscheduled Visit.

b Subjects who discontinue prematurely from study drug administration will continue to return to the investigational site for study assessments at Visits 3, 6, and 8 (EOT), and undergo telephone assessments as described for Visits 4, 5, and 7. If subjects are unable/unwilling to do so, they must return to the investigational site 30 (±3) days after the last dose of study drug for EOT (Visit 8) assessments, and undergo telephone assessments as described for Visits 4, 5, and 7. If the subject is unable/unwilling to return to the clinic for the EOT Visit (Visit 8), then a telephone assessment as described for Visits 4, 5, and 7 may be performed in lieu of Visit 8 assessments.

In this study, approximately 300 pediatric subjects will be randomized (2:1 randomization, 60-day regimen vs. 30-day regimen). Subjects will be stratified by age at randomization into four strata as specified below:

Stratum 1: 0 to 27 days

Stratum 2: 28 days to younger than 8 months

Stratum 3: 8 months to younger than 2 years

Stratum 4: 2 years to younger than 18 years

A minimum of 38 subjects in each age stratum is targeted, but not required, in order to be able to derive meaningful safety conclusions. Enrollment will continue until this specification is

met, unless it is determined that such a target would be unlikely to be reached in a reasonable time.

After study eligibility has been confirmed and safety assessments have been performed at Visit 2 (Day 1), subjects will be randomized via Interactive Voice Response System/Interactive Web Response System (IVRS/IWRS) in a 2:1 ratio (60-day regimen vs. 30-day regimen) to one of two treatment groups:

- Nifurtimox tablets administered three times daily for 60 days (Days 1 – 60, active nifurtimox treatment, Treatment Group 1) or
- Nifurtimox tablets administered three times daily for 30 days, followed by nifurtimox placebo administered three times daily for 30 days (Days 1 – 30, active nifurtimox treatment; Days 31 – 60, placebo; Treatment Group 2).

The study is composed of 3 periods:

- Screening period
- Treatment period
- Follow-up period

Treatment period

The first dose of study drug will be administered at Visit 2. Pre- and post-dose PK blood samples will be obtained at specified time points from those subjects consenting to PK assessments. At the 2 – 4 hour time point of PK blood sampling (i.e., at the time of maximum concentration [C_{max}]), an ECG (optional for subjects < 5 years of age at the discretion of the investigator) will be obtained to allow for PK/PD investigations. Study drug will be dispensed, and instructions for study drug administration will be provided to all subjects.

Subjects will return to the investigational site for efficacy and safety assessments at Visit 3 (Day 7±1) and Visit 6 (Day 30). Subjects who have not consented to PK assessments will take that day's doses of study drug as instructed. Subjects consenting to PK assessments must hold the morning dose of study drug; for these subjects, a pre-dose PK blood sample will be obtained, then the morning dose of study drug will be administered. Post-dose blood samples will be obtained at specified time points, and ECGs will be obtained at the 2 – 4 hour post-dose time point. A pre-paid phone card will be provided to all subjects to facilitate contact between study site personnel and the subjects/subjects' authorized representatives.

At Visit 6 (Day 30), subjects will return all remaining study drug and empty packaging, and study drug for the remaining 30 days of treatment will be dispensed.

Visits 4 (Day 14), 5 (Day 21), and 7 (Day 42) will be telephone assessments where study site personnel will contact the subject/legally authorized representative via telephone to assess the

occurrence of AEs, use of concomitant medications, and compliance with study drug administration. A Phone Contact Form for the telephone assessments will be provided to all sites.

Subjects will return to the investigational site for efficacy and safety assessments on Day 60 (Visit 8), which will be the EOT for both treatment groups. Subjects who have not consented to PK assessments will take that day's doses of study drug as instructed. Subjects consenting to PK assessments must hold the morning dose of study drug. A pre-dose PK blood sample will be collected, then the morning dose of study drug will be administered. Post-dose PK blood samples will be obtained at specified time points, and ECGs will be obtained at the 2 – 4 hour post-dose time point. Study drug will be collected, and no additional study drug will be dispensed.

Follow-up period

After the EOT visit (Visit 8), subjects will return to the site on Days 90 (Visit 9), 240 (Visit 10), and 420 (Visit 11) for additional efficacy and safety assessments. The total duration of each subject's participation is expected to be approximately 14 months.

Primary variable(s)

The primary efficacy variable will be sero-reduction or sero-conversion at 12 months post-treatment using two conventional ELISA serology tests as the measure of efficacy.

End of part 1 of the study

The end of part 1 of the study as a whole will be reached as soon as the last visit of the last subject has been reached in all centers in all participating countries.

3.2 Part 2 (CHICO SECURE)

Design overview

This is a prospective, long-term follow-up of subjects, who were randomized and received at least one dose of their assigned 60- or 30-day nifurtimox treatment. The study is designed to evaluate seronegative conversion and serial reduction in OD measured by ELISA.

Subjects will be followed up for a total of 4 years after end of nifurtimox treatment. The first year of follow-up period is included in part 1 and 3 years of follow-up are included in part 2 of the study.

The following age categories will be considered for part 2 of the study: ≤2 years, >2 years to ≤6 years, >6 to ≤12 years, >12 to <18 years (age is defined as subject's age at randomization).

Subjects enrolled will be followed annually with visits to the investigational site. Seronegative conversion will be measured by two types of assay, recombinant ELISA and IHA. For assessment of serial reduction compared to baseline defined as screening visit of part 1 (Visit 1), OD will be measured by recombinant ELISA and total purified antigen ELISA.

The first visit should be performed as soon as possible after availability of regulatory and ethics committee approval for all subjects who have already completed part 1 of the study for 1 year ± 6 weeks or longer. Subjects who are entering the study beyond the time window for FU Visit 1 (2 years ± 6 weeks) will have their eligibility assessments as soon as possible provided that consents/assents have been obtained. Subjects will have annual visits at the investigational site for efficacy and safety assessments for 3 years. Timing of study visits is relative to end of treatment in part 1 of the study.

Between the annual visits at the investigational site, subjects and/or his or her parent(s) or legally authorized representative(s) will be contacted by qualified personnel by a method agreed (eg. phone call) about 6 months after a visit at the investigational site to identify the occurrence of potential clinical symptoms of Chagas' disease or any pathology mentioned in the exclusion criteria using a structured interview. In case of suspected findings, an unscheduled visit at the investigational site to initiate appropriate diagnostic evaluations will be requested.

Primary variable

The primary efficacy variable will be the incidence rate of seronegative conversion measured by two types of assay (recombinant ELISA and IHA) in subjects who were randomized and received at least one dose of the 60-day nifurtimox treatment regimen. Both test results should be negative for the subject to be considered as seroconverted.

End of part 2 of the study

The end of part 2 of the study as a whole will be reached as soon as the last visit of the last subject has been reached in all centers in all participating countries.

Primary completion

The primary completion event for part 2 of the study is last subject last visit (LSLV).

4. General Statistical Considerations

4.1 General Principles

The statistical evaluation will be performed using the software package SAS release 9.2 or higher (SAS Institute Inc., Cary, NC, USA). Unless otherwise specified, all significance tests will be conducted using a 2-sided alpha level of 0.05, and confidence intervals (CIs) will be 2-sided 95% intervals. All variables collected in this study will be summarized with descriptive statistics at each assessment time. For continuous variables, descriptive statistics will include the number of data available and missing data, means, standard deviations, medians, minimums, and maximums. For categorical variables, frequency counts and percentages will be provided. These will be calculated for each age stratum by treatment regimen, as well as the overall set of study subjects.

4.2 Handling of Dropouts

A subject who discontinues study participation prematurely for any reason is defined as a "dropout" if the subject has already been randomized, assigned to treatment, and administered at least one dose of study drug.

A subject who, for any reason (e.g., failure to satisfy the selection criteria), terminates the study before the time point used for the definition of "dropout" is regarded as a "screening failure".

Depending on the time point of withdrawal, a withdrawn subject is referred to as either "screening failure" or "dropout". A subject who is withdrawn from the study will not be replaced.

Subjects who discontinue prematurely from study drug administration will continue to return to the investigational site for study assessments at Visits 3, 6, and 8 (EOT), and undergo telephone assessments as described for Visits 4, 5, and 7. If subjects are unable/unwilling to do so, they must return to the investigational site 30 (±3) days after the last dose of study drug for EOT (Visit 8) assessments, and undergo telephone assessments as described for Visits 4, 5, and 7. If the subject is unable/unwilling to return to the clinic for the EOT Visit (Visit 8), then a telephone assessment as described for Visits 4, 5, and 7 may be performed in lieu of Visit 8 assessments.

Premature discontinuations from study drug will be summarized by treatment regimen, both for any type of discontinuation, and for each specific reason for discontinuation.

4.3 Handling of Missing Data

Part 1 (CHICO)

No imputations will be made for missing values occurring in the safety and background variables. Background variables include demographics (sex, age), height/length, weight, medical/surgical history, and medication history.

All efforts will be taken to collect all data. However, despite best efforts, it may be inevitable that missing data are reported. In the primary efficacy analysis done on the full analysis set (FAS), subjects who have missing conventional serology results at the 12 month time point will be treated as failures (i.e. no cure).

For the secondary analysis using the per-protocol set (PPS), all subjects who do not have both conventional serology determinations to assess the 12-month time point will be excluded from the analysis.

Part 2 (CHICO SECURE)

No imputations will be made for missing data due to dropouts or other causes. For the primary efficacy analysis done on the FAS, drop-outs will be included in the analysis using a Poisson distribution with person-year.

4.4 Interim Analyses and Data Monitoring

No interim analysis is planned.

Part 1 (CHICO)

An independent Data Monitoring Committee (DMC) will meet periodically to review the safety data of enrolled subjects, as well as the continuing scientific merit of the trial.

The DMC may recommend stopping enrollment in the case of a negative risk/benefit assessment. The DMC will be comprised of a minimum of two clinicians with expertise in relevant clinical specialties and at least one statistician knowledgeable about statistical methods for clinical trials. Each committee member will be screened for evidence of an absence of serious conflicts of interest. The operation of the DMC will be governed by a charter that will describe the group's frequency of meetings, procedures (including, but not limited to, periodic safety monitoring), and requirements for reporting its observations to the Sponsor.

Part 2 (CHICO SECURE)

No DMC is planned.

4.5 Data Rules

Baseline

Baseline is defined as the last measurements performed prior to the first study drug administration, which is either in Visit 1 (e.g. parameters of serum hematology, chemistry, coagulation, and urinalysis) or Visit 2 (e.g. vital signs and ECG), unless otherwise specified. If Visit 2 is missing, use data from Visit 1 instead.

Repeated measures

If there are repeated measurements per time point (e.g. laboratory values, vital signs, etc.), the following rules will be used:

- Before the start of the study drug administration (i.e., for screening and baseline value), the latest measurement will be used, in the event these latest measurements are measurements at scheduled visits. Unscheduled visits will be used, if there are no measurements at scheduled visits. If the latter is the case, the last unscheduled visit will be used.

- In case of repeated measurements at any post-screening time point, the first value of the scheduled measurements at that time point will be used. No unscheduled measurements will be used for any time points beside screening / baseline even where measurements at scheduled visits are missing.

In case of non-adherence to time windows, data will be assigned to the nearest nominal visit according to assessment schedule.

4.6 Validity Review

The results of the validity review meeting will be documented in the Validity Review Report and may comprise decisions and details relevant for statistical evaluation. Any changes to the statistical analysis prompted by the results of the validity review meeting will be documented in an amendment and, if applicable, in a supplement to this SAP.

5. Analysis Sets

5.1 Assignment of analysis sets

Part 1 (CHICO)

Full analysis set (FAS)

The efficacy analyses will be done using the full analysis set (FAS), which is the set of randomized subjects who received at least one dose of study drug.

Analyses of safety and background data will be performed on the FAS.

Per protocol set (PPS)

The primary analysis will also be done using the per-protocol set, which is comprised of randomized subjects treated with study drug who have no major protocol deviations. Major protocol deviations defined in this study will include:

- Subjects who have both conventional serology results missing at the 12-month time point
- Subjects who do not meet in-/exclusion criteria but entered treatment phase
- Subjects who take less than 80% or more than 120% of total assigned doses of study drug
- Subjects whose treatment codes are unblinded during the study
- Subjects who have taken prohibited medicine during the study

Part 2 (CHICO SECURE)

Full analysis set (FAS)

The efficacy analyses will be done using the full analysis set (FAS), as defined in part 1. Subjects will be analyzed as randomized in part 1 (CHICO) of the study.

Analyses of safety and background data will be performed on the FAS. For safety analyses, subjects will be analyzed as treated in part 1 of the study.

6. Statistical Methodology

6.1 Population characteristics

6.1.1 Disposition

Disposition will be summarized descriptively for the study phases of screening, treatment, and follow-up for FAS. The number of subjects discontinuing the screening phase together with the primary reason for discontinuation will be presented overall. The number of subjects discontinuing the treatment and follow-up phases together with the primary reason for discontinuation will be presented overall and by treatment regimen. In addition, the number of subjects with major/important protocol deviations will be presented overall and by treatment regimen. Subjects discontinuing the study due to COVID-19 and any protocol deviation caused by COVID-19 will be summarized accordingly.

6.1.2 Demographic and Other Baseline Characteristics

Demographics (sex, ethnicity, race, age at randomization, weight, height/length) and baseline characteristics (i.e., ELISA, IHA and qPCR test results, Chagas' disease signs and symptoms) will be summarized overall and by treatment regimen. The same summary will also be performed for each age stratum.

6.1.3 Medical History

For data coding, Medical Dictionary for Regulatory Activities (MedDRA) [version 18.0 or most recent version] will be used for medical history. Medical history (i.e., previous diagnoses, diseases or surgeries) not pertaining to the study indication, start before signing of the informed consent and considered relevant to the study will be presented for each MedDRA primary system organ class and high level term by treatment regimen and overall using frequency counts.

Concomitant medications will be tabulated by treatment regimen using ATC codes (WHO-DD); frequencies of subjects having received each drug category will be provided.

6.1.4 Extent of Exposure and Compliance

Part 1 (CHICO)

All summaries related to intake of study medication will be by treatment regimen based on FAS.

The treatment duration will be summarized descriptively overall and for each age stratum. It will be calculated as follows:

Treatment duration = (drug stop date - drug start date + 1 day).

If the end date of the first drug distribution and the start date of the second drug distribution are the same, the calculation is:

Treatment duration = (drug stop date– drug start date for the 1st distribution) + (drug stop date– drug start date for the 2nd distribution + 1)

The number of tablets and corresponding extent of exposure (by dose) will be summarized descriptively. Compliance is defined as 100 * number of tablets taken / number of tablets per protocol. The compliance will be summarized descriptively by treatment regimen and overall. In addition, compliance will be categorized into three groups (<80%, 80-120%, >120%) and summarized by treatment regimen and overall.

Part 2 (CHICO SECURE)

Not applicable.

6.2 Efficacy

6.2.1 Part 1 (CHICO)

6.2.1.1 Primary efficacy variable and analysis

The primary efficacy variable will be sero-reduction (defined as a ≥ 20% reduction in optical density [OD]) or sero-conversion (defined as negative Immunoglobulin G [IgG] concentration) in the 60-day regimen group at 12 months post-treatment using two conventional ELISA serology tests. This sero-reduction (in subjects ≥ 8 months to < 18 years of age at randomization) or sero-conversion (in all subjects) is considered cure, and the primary variable is binary (cure, no cure). In the event of discordancy between the two conventional ELISA test results, the following will be considered:

- For sero-reduction, the average percentage of OD reductions for the two conventional ELISA tests will be used (e.g., Test #1 = 15% and Test #2 = 25%; average = 20% and, hence, cure).

- For sero-conversion, only when both test results are negative that the subject is considered as negative (i.e., cure).
- If one of the test results is missing, the subject is considered as "no cure".

Derivation of the rate for sero-conversion for the historical controls

Historical controls are estimated from cure rates presented in two publications [1][2]. The historical placebo cure rate used is shown in Table 6–1:

Table 6–1: Historical Cure Rates for Placebo

Publication	Age range (in years)	Sero-conversion rate (95%CI) in placebo patients
de Andrade et al 1996	7-12	3/65 = 5% (1%,13%)
Sosa et al 1998	6 -12	2/44= 5% (1%,16%)

Both publications show a 5% cure rate for the placebo group. For the primary objective in our study, superiority over placebo will be confirmed if the lower limit of the 95% CI for the nifurtimox (60-day regimen) cure rate is greater than 16%, the larger of the upper limits of the 95% CIs from the two publications.

Primary analysis of the primary efficacy variable

The difference in the proportion of nifurtimox subjects with sero-reduction or sero-conversion (60-day regimen) and the proportion estimated from historical data will be tested using an asymptotic 2-sided 95% CI with a continuity correction of 1/2n for a single proportion.

The null hypothesis H_0 is $p_{nifurtimox} = p_{placebo}$

The alternative hypothesis H_1 is $p_{nifurtimox} \neq p_{placebo}$, where p_t is the cure rate for treatment t.

Superiority will be confirmed if the lower limit of the CI for the proportion of nifurtimox subjects (60-day regimen) with sero-reduction or sero-conversion is greater than 16%. The proportion and asymptotic 2-sided limits (p_l, p_u) of the 95% CI for $p_{nifurtimox}$ can be computed by [3]

$$\hat{p}_{nifurtimox} = x/n$$

$$p_u = \hat{p}_{nifurtimox} + 1.96\sqrt{\hat{p}_{nifurtimox}(1-\hat{p}_{nifurtimox})/n} + 1/2n$$

$$p_l = \hat{p}_{nifurtimox} - 1.96\sqrt{\hat{p}_{nifurtimox}(1-\hat{p}_{nifurtimox})/n} - 1/2n$$

where *x* denotes the number of nifurtimox subjects with sero-reduction or sero-conversion and *n* the total number of subjects in 60-day regimen group. The SAS code will be:

PROC FREQ;

TABLES cured / BINOMIALC;

WEIGHT counts;

RUN;

Secondary analysis of the primary efficacy variable

A secondary analysis will be done to compare the proportion of subjects with sero-reduction or sero-conversion for the 60-day and 30-day nifurtimox regimens. This will be performed using an asymptotic 2-sided 95% CI for the difference of two independent proportions.

The null hypothesis H_0 is $p_{nifurtimox,\ 60d} = p_{nifurtimox,\ 30d}$

The alternative hypothesis H_1 is $p_{nifurtimox,\ 60d} \neq p_{nifurtimox,\ 30d}$, where p_t is the cure rate for treatment *t*.

Difference in efficacy between the two groups will be confirmed if the CI for the difference of two proportions of nifurtimox subjects (60-day regimen *versus* 30-day regimen) with sero-reduction or sero-conversion does not include 0. The proportions and asymptotic 2-sided limits (l_l, l_u) of the 95% CI for $p_{nifurtimox,\ 60d}$ - $p_{nifurtimox,\ 30d}$ can be obtained by [3]

$$\hat{p}_i = \frac{x_i}{n_i}, \qquad i = 1, 2.$$

$$l_u = (\hat{p}_1 - \hat{p}_2) + 1.96\sqrt{(\hat{p}_1(1-\hat{p}_1)/n_1) + (\hat{p}_2(1-\hat{p}_2)/n_2)} + \frac{1}{2}\left(\frac{1}{n_1} + \frac{1}{n_2}\right)$$

$$l_l = (\hat{p}_1 - \hat{p}_2) - 1.96\sqrt{(\hat{p}_1(1-\hat{p}_1)/n_1) + (\hat{p}_2(1-\hat{p}_2)/n_2)} - \frac{1}{2}\left(\frac{1}{n_1} + \frac{1}{n_2}\right)$$

where p_i, x_i and n_i denote the cure rate, the number of nifurtimox subjects with sero-reduction or sero-conversion, and the total number of subjects, for treatment *i* respectively. Treatment *i=1, 2* represents 60-day and 30-day nifurtimox regimens, respectively.

The SAS code will be:

PROC FREQ;

TABLES group*cured / RISKDIFFC;

WEIGHT counts;

RUN;

The analyses specified above (the proportion of subjects with sero-reduction or sero-conversion and an asymptotic 95% CI for the 60-day nifurtimox regimen, and the difference of two independent proportions for the 60-day and 30-day regimens and an asymptotic 95% CI) will also be done using data from Days 7 (Visit 3), Days 30 (Visit 6), the EOT visit (Visit 8), and Days 240 (Visit 10). In the event of small sample sizes for the subgroups, an exact 95% CI will be calculated instead of an asymptotic one.

Sensitivity analyses of the primary efficacy variable

The following sensitivity analyses for the primary efficacy variable will be performed:

- Missing values due to the following reasons treated as treatment failures and other missing values excluded from analysis:

  1. the reason for discontinuation is known or suspected to be treatment ineffectiveness or an AE,

  2. a subject has not had a negative serology at any visit and has no serology determination for the primary analysis visit,

  3. an indeterminate or incorrectly performed serology determination occurs at the primary analysis visit (e.g. missing values due to lab procedure, not enough sample materials to perform the test, performance error, etc.).

- Observed data only

- Analysis on PPS

Subgroup analyses of the primary efficacy variable

The proportion of nifurtimox subjects with sero-reduction or sero-conversion (60-day regimen) and an asymptotic 2-sided 95% CI for the proportion will be calculated by country and for each age stratum.

The same proportion and an asymptotic 2-sided 95% CI for the difference of the 60-day and 30-day nifurtimox regimens will also be summarized by country and for each age stratum.

6.2.1.2 Secondary efficacy variables and analysis

Secondary efficacy variables include clinical signs/symptoms of Chagas' disease (see Appendix 9.1), concentration test for *T. cruzi* (for subjects < 8 months of age), non-conventional serologic testing, and disease state determined by qPCR ("cure" defined as Not Detectable / "no cure" defined as Detectable).

All secondary efficacy variables, except signs/symptoms of Chagas' disease, will be summarized overall and by treatment regimen at each assessment time for each age stratum.

Signs/Symptoms of Chagas' disease will be summarized for each item (see Appendix 9.1).

The relationship of conventional serology results to qPCR results will be done using frequencies of matches and mismatches on the determination of disease status, and phi-correlation and kappa coefficient to assess the degree of agreement. The relationship of non-conventional serology to conventional serology will be analyzed in the same way. The following SAS code will be used:

PROC FREQ;

TABLES group*cured / CHISQ AGREE;

WEIGHT counts;

RUN;

6.2.1.3 Exploratory efficacy analysis and analysis

For the exploratory objective to assess the comparability of nifurtimox with benznidazole, the cure rate of 58% with a 95% CI (45%, 70%) from publication of de Andrade et al. in 1996 [2] will be referenced. If the lower limit of the same 95% CI for the primary efficacy variable (the 60-day nifurtimox regimen cure rate) is greater than 45%, nifurtimox will be deemed as comparable to benznidazole.

The comparison of conventional serology to IHA results will be done using a 3-by-3 table (Table 6–2) with the following categories:

- ELISA tests: sero-conversion and sero-positive (sub-categories: sero-reduction and others), where "others" refers to any sero-positive results that do not satisfy the definition of "sero-reduction".

- IHA test: negative and positive (sub-categories: titers decreasing and titers non-decreasing).

Table 6–2 Summary table of comparison between serology and IHA results

				Conventional ELISA Tests				
				Sero-negative	Sero-positive		Missing	Total
					Sero-reduction	Others		
IHA Testing	60-Day (N=x)	Negative		xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)
		Positive	Decreasing in titers	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)
			Non-decreasing in titers	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)
		Missing		xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)
		Total		xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)
	30-Day (N=x)	Negative		xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)
		Positive	Decreasing in titers	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)
			Non-decreasing in titers	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)
		Missing		xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)
		Total		xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)

In addition, McNemar's test will be performed by treatment regimen for all patients in FAS (Table 6–3) with the following categories:

- ELISA tests: cure (sero-conversion and sero-reduction) versus no cure;
- IHA test: negative or positive but decreasing in titers versus positive but non-decreasing in titers.

Table 6–3 McNemar's test on comparison between serology and IHA results for all patients in FAS

			Conventional ELISA Tests					
			Cure (sero-negative or sero-reduction)	No cure	Missing	Total	χ^2	P-Value
	60-Day (N=x)	Negative or positive but decreasing in titers	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)		
		Positive but non-decreasing in titers	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)		
		Missing	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)		
		Total	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	x.xx	x.xx
IHA Testing	30-Day (N=x)	Negative or positive but decreasing in titers	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)		
		Positive but non-decreasing in titers	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)		
		Missing	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)		
		Total	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	x.xx	x.xx

The same test would also be performed by treatment regimen in sero-positive subjects (Table 6–4). The categories for this test are

- ELISA tests: sero-reduction versus others (sero-positive that are not sero-reduction);
- IHA test: negative or positive but decreasing in titers versus positive but non-decreasing in titers.

Table 6–4 McNemar's test on comparison between serology and IHA results for all sero-positive patients in FAS

			Conventional ELISA Tests					
			Sero-reduction	Others	Missing	Total	χ^2	P-Value
	60-Day (N=x)	Negative or positive but decreasing in titers	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)		
		Positive but non-decreasing in titers	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)		
		Missing	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)		
		Total	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	x.xx	x.xx
IHA Testing	30-Day (N=x)	Negative or positive but decreasing in titers	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)		
		Positive but non-decreasing in titers	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)		
		Missing	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)		
		Total	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	xx (xx.x%)	x.xx	x.xx

6.2.2 Part 2 (CHICO SECURE)

6.2.2.1 Primary efficacy variable and analysis

Primary efficacy variable

The primary efficacy variable will be the incidence rate of seronegative conversion measured and confirmed by two types of assay (recombinant ELISA and IHA) in subjects who were randomized and received at least one dose of the 60-day nifurtimox treatment regimen. Both test results should be negative for the subject to be considered as seroconverted. Incidence rate is the number of new cases of seronegative conversion over the study period (i.e. 4 years after end of nifurtimox treatment) divided by the person-time at risk.

Subjects used antitrypanosomal drugs during the study before they were seroconverted will not be considered as sero-convertion, but their time of observation till when they took antitrypanosomal drugs will be factored into the person time calculation.

Primary analysis of the primary efficacy variable

The difference in the incidence rate of nifurtimox subjects with seronegative conversion (60-day regimen) and the seronegative rate estimated from historical data will be tested using a Poisson two-sided 95% exact confidence interval (CI).

The incidence rate of seronegative conversion for the primary efficacy variable will be modelled using a Poisson distribution with a 2-sided 95% exact CI. The rate will be estimated as the number of seronegative conversion divided by the total time at risk (person-year) during the study. The rate of seronegative conversion (R) and a 2-sided limits (R_l, R_u) of the 95% CI for the rate can be calculated using the following formula [4]

$$R = \frac{n}{\sum_i N_i * V_i}, i = 1, 2, \ldots K$$

$$R_l = \chi^2_{2n,\alpha/2} \Big/ 2\sum_i N_i * V_i, i = 1, 2, \ldots K$$

$$R_u = \chi^2_{2(n+1),1-\alpha/2} \Big/ 2\sum_i N_i * V_i, i = 1, 2, \ldots K$$

where n is the number of seronegative conversion observed , N the total subjects at risk in annual visit i, V_i the ith annual visit, K the total number of visits, R_l and R_u are lower and upper confidence limits for the rate of seronegative conversion respectively, $\chi^2_{v,a}$ is the chi-square quantile for upper tail probability on v degrees of freedom.

This study uses an external control group of untreated patients with Chagas' disease in the 4-year follow-up period presented in Sosa et al. [7, 7]. In the reference, seroconversion to a negative result in the placebo group after 4-year follow-up was detected in 2 subjects with conventional serology and in 0 subjects with IHA test. Thus, none of the subjects in the placebo group was considered as seroconverted by both test results. The person time at risk was on average 44 * 4 = 176 person years. The incidence rate estimate was 0% (0 / 176).

Superiority will be confirmed if the lower limit of the CI for the incidence rate of seronegative conversion for subjects in 60-day regimen is greater than the rate of historical placebo subjects with sero-conversion (i.e. 0 %).

Frequency counts and percentages of seronegative conversion will be provided by visit.

6.2.2.2 Secondary efficacy variables and analysis

Secondary efficacy variables include:

- Incidence rate of seronegative conversion measured and confirmed by two types of assay (recombinant ELISA and IHA) in subjects who were randomized and received at least one dose of the 30-day nifurtimox treatment regimen

- Proportion of responders who show both seronegative conversion by two types of assays (recombinant ELISA and IHA) and no evidence of established cardiomyopathy as measured by ECG

- Serial reduction of OD measured by recombinant ELISA and total purified antigen ELISA

Analysis of secondary efficacy variables

The OD values from individual conventional ELISA tests (recombinant ELISA and total purified antigen ELISA) will be analyzed descriptively. Changes from baseline will be summarized to show any serial reduction of OD values. Baseline is defined as the OD values from the same ELISA tests measured at Visit 1 in part 1 of the study.

The proportion of subjects who show both seronegative conversion as confirmed by two assays (recombinant ELISA and IHA) and no evidence of established cardiomyopathy as measured by ECG will be calculated overall and by treatment regimen.

The same method for calculating incidence rate and its 2-sided 95% CI of the primary efficacy variable will be applied on the following variable:

- Incidence rate of seronegative conversion measured and confirmed by two types of assay (recombinant ELISA and IHA) in subjects who were randomized and received at least one dose of the 30-day nifurtimox treatment regimen

6.2.2.3 Exploratory efficacy analysis and analysis

Exploratory efficacy variables include:

- Incidence rate of seroconversion measured and confirmed by two types of assay (recombinant ELISA and IHA) in subjects by age categories (≤2 years, >2 years to ≤6 years, >6 to ≤12 years, >12 to <18 years; age is defined as subject's age at randomization)

- Proportion of presence of parasite or positive serology in babies born of mothers who were randomized and received at least one dose of either the 60- or 30-day nifurtimox treatment regimen

- Proportion of responders and incidence rate of seronegative conversion as confirmed by non-conventional serology in subjects who were randomized and received at least one dose of either 60- or 30-day nifurtimox treatment regimens

Analysis of exploratory efficacy variables

The same method for calculating incidence rate and its 2-sided 95% CI of the primary efficacy variable will be applied on the following variable:

- Incidence rate of nifurtimox subjects with seronegative conversion in all subjects by age categories. The age categories are grouped as ≤2 years, >2 years to ≤6 years, >6 to ≤12 years, >12 to <18; age is defined as subject's age at randomization

- Incidence rate of seronegative conversion as confirmed by non-conventional serology in subjects who were randomized and received at least one dose of either 60- or 30-day nifurtimox treatment regimens

The proportion of subjects with seronegative conversion as confirmed by non-conventional serology will be calculated overall and by treatment regimen. The numeric results of non-conventional serology will be summarized overall and by treatment regimen.

The proportion of presence of parasite or positive serology in babies born of mothers who were randomized and received at least one dose of either the 60- or 30-day nifurtimox treatment regimen will be calculated overall and by treatment regimen.

The results from qPCR and IHA will be summarized overall and by treatment regimen.

Signs/Symptoms of Chagas' disease will be summarized for each item (see Appendix 9.1).

6.3 Safety

Part 1 (CHICO)

Safety Variables

Safety variables include adverse events (AEs), physical examination abnormalities, vital signs, ECG, hematology and blood chemistry, coagulation, and urinalysis. All analyses of safety variables will be descriptive only, no formal testing will be performed.

Adverse Events

All adverse events occurring during the study (i.e., from signing of informed consent until last visit) will be reported. The MedDRA coding system will be used to code the adverse events.

An overview of frequencies of subjects who died, experienced any adverse event, experienced any drug-related adverse event, experienced a serious adverse event (SAE), experienced a drug-related serious adverse event, or discontinued due to an adverse event will be summarized by treatment regimen.

Adverse events will be considered treatment emergent, if they first occurred or worsened at or after first application of study medication during the course of the study up to and including 7 days after last application of study medication.

Incidence of treatment emergent AEs (TEAEs), drug-related TEAEs, serious TEAEs, drug-related serious TEAEs, as well as TEAEs leading to discontinuation will be summarized by primary system organ class and high level term by treatment regimen.

The definitions of incidence are as follows:

- Incidence of all events: # of subjects reporting the event with a start date during or after treatment/# of subjects valid for safety.
- Incidence of drug-related events: # of subjects reporting a drug-related event with a start date during or after treatment/# of subjects valid for safety.
- Incidence of events by maximum intensity: # of subjects reporting the event at the indicated intensity with a start date during or after treatment/# of subjects valid for safety.

Adverse events leading to death, premature discontinuation and serious adverse events will be listed. In this study, all AEs of urticarial, weight loss of > 20%, severe rash, and severe polyneuropathy will be considered AEs of special safety interest. Adverse events of special safety interest will be summarized by treatment regimen.

AEs occurring between the time of consent and the first dose of study drug will be summarized separately.

Laboratory Data

Quantitative data will be summarized by means of descriptive statistics (arithmetic mean, standard deviation, median, minimum and maximum) by visit and treatment regimen for the original data as well as for the difference to baseline. Frequency tables will be provided for qualitative data. Summaries will be given by treatment regimen. The incidence of laboratory data outside the reference range (low, high) and clinically significant laboratory changes will be summarized by treatment regimen using frequency tables. The definitions of incidence are as follows:

- Incidence of high lab abnormalities: # of subjects with at least one high laboratory assessment after the start of treatment who had a normal or lower than normal laboratory assessment at baseline / # of subjects at baseline with a normal or lower than normal laboratory assessment who also had at least one valid laboratory value after start of treatment. Subjects with missing or high abnormal values at baseline are not included in the denominator.
- Incidence of low lab abnormalities: # of subjects with at least one low laboratory assessment after the start of treatment who had a normal or higher than normal

laboratory assessment at baseline / # of subjects at baseline with a normal or higher than normal laboratory assessment who also had at least one valid laboratory value after start of treatment. Subjects with missing or low abnormal values at baseline are not included in the denominator.

ECG and Vital Signs Data

Quantitative ECG data, both original values and change from baseline, will be summarized by visit and treatment regimen. ECG interpretations will also be summarized by visit and treatment regimen. ECG data will be analyzed for the FAS and by age stratum.

Vital signs data will be analyzed descriptively. The original values and changes from baseline will be summarized by visit and treatment regimen.

Pregnancy Data

Pregnancy data will be listed by visit and treatment regimen.

Part 2 (CHICO SECURE)

Safety variables include AEs, physical examination abnormalities, ECG and vital signs. All analyses of safety variables will be descriptive only, no formal testing will be performed.

Adverse events will be recorded when considered at least possibly related to nifurtimox and adverse events caused by protocol-required procedures.

Quantitative ECG data, both original values and change from baseline, will be summarized by visit and treatment regimen. ECG interpretations will also be summarized by visit and treatment regimen. ECG data will be analyzed for the FAS and by age stratum.

Vital signs data will be analyzed descriptively. The original values and changes from baseline will be summarized by visit and treatment regimen.

Pregnancy data will be listed by visit and treatment regimen.

6.4 Pharmacokinetics / pharmacodynamics

Part 1 (CHICO)

Plasma concentration time courses will be analyzed and reported within the study report. In addition, a full PK evaluation of the data will be performed and reported in a separate study

report. Population PK methods will be used to provide parameter estimates describing the PK behavior of nifurtimox and to identify possible covariates related to age.

Part 2 (CHICO SECURE)

Not applicable.

6.5 Determination of sample size

Part 1 (CHICO)

According to Guhl et al. [5], nifurtimox 12 months post-treatment cure rate is about 55%, with a sample size of 260 for the 60-day regimen, the power is 99% for the lower limit of the 95% CI to be greater than 16%.

The number of subjects in the 30-day treatment regimen is based on the width of a 95% CI for the difference of two proportions. A sample size of 130 subjects for the 30-day subjects together with 260 subjects for the 60-day subjects will produce a CI with a width of approximately 20%.

Part 2 (CHICO SECURE)

According to Sosa-Estani [7, 7], the 48 months post treatment cure rate for benznidazole is about 5% as confirmed by two types of assay. For the primary objective, assuming a drop-out rate of 15%, the expected number of patients to be observed at the end of Part 2 (i.e. 48 months post treatment) in the 60-day regimen would be 170 out of the 200 patients in Part 1. Using the assumptions of 170 nifurtimox 60-day regimen patients in Part 2, a 5% cure rate, and that seronegative conversion events follow Poisson distribution, the estimated incidence rate is calculated to be 1.32% and the 95% CI of the incidence rate is (0.61%, 1.51%).

7. Document history and changes in the planned statistical analysis

Figure 7–1 Version History

Version	Date	Action
Version 1.0	21 OCT 2015	Signed initial version
Version 2.0	16 FEB 2018	Signed version 2.0

Version 3.0	26 JAN 2020	Signed version 3.0

8. References

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3. Fleiss, J. L., Levin, B., and Paik, M. C. (2003), Statistical Methods for Rates and Proportions, Third Edition, New York: John Wiley & Sons.
4. Ulm K. Simple method to calculate the confidence interval of a standardized mortality ratio (SMR). American journal of epidemiology. 1990;131(2):373-5.
5. Guhl F, Nicholls R, Montoya R, Rosas F, Velasco VM, et al. (2004) Rapid negativization of serology after treatment with benznidazole for chagas disease in a group of colombian schoolchildren. Medimond International Proceedings. Spain, Vol. 1. pp. 107–114
6. Sosa Estani S, Segura EL, Ruiz AM, Velazquez E, Porcel BM, Yampotis C. Efficacy of chemotherapy with benznidazole in children in the indeterminate phase of Chagas'Disease. Am J Trop Med Hyg. 1998 Oct;59(4):526-529
7. FDA. 2017. Benznidazole tablets. Company: Chemo Research, S.L. Available from https://www.accessdata.fda.gov/drugsatfda_docs/nda/2017/209570Orig1s000TOC.cfm

9. Appendices

9.1 Assessment of Chagas' disease signs and symptoms

Assessments of the presence of Chagas' disease signs and symptoms will be performed during physical examinations at Screening, Visits 3, 6, and 8 (EOT), and Visits 9, 10, and 11 (follow-up) in part 1 and at FU Visits 1, 3, and 5 in part 2 of the study, and will be entered into the eCRF. Signs and symptoms of Chagas' disease include but are not limited to the following:

Acute Chagas' disease:

- Fever: usually prolonged

- malaise
- lymphadenopathy
- hepatomegaly
- splenomegaly
- subcutaneous edema (localized or generalized)
- signs of portal of entry of T. cruzi:
    - through the skin – chagoma
    - via the ocular mucous membranes - Romaña sign
- hypotonicity
- anemia
- myocarditis
- meningoencephalitis
- pneumonitis
- ECG abnormality, may include but not limited to:
    - sinus tachycardia
    - first-degree atrioventricular block
    - low QRS voltage
    - primary T-wave changes
- Chest radiograph abnormality, include variable degrees of cardiomegaly