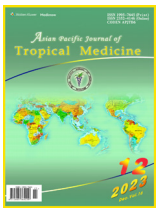

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Evaluation of parasitemia by qPCR in patients with chronic Chagas disease treated with benznidazole

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ABSTRACT

Objective: To evaluate parasitemia by qPCR in patients undergoing etiological treatment and followed in a Brazilian reference center.

Methods: Parasite load was quantified by qPCR in 32 participants with chronic Chagas disease who were treated with benznidazole. Serological analyses were performed before and after the treatment and parasite loads were compared prior and 12/18 months post the treatment.

Results: Thirty-two participants were recruited and treated with benznidazole, and 20 were followed-up. Adverse events (AE) were observed in 22 out of 29 participants that had safety data (76%), and dermatological alterations were the most frequently observed AE. Of the 20 participants analyzed, 13 and 7 completed 12 and 18 months follow-up after the treatment, respectively. 12 Months after the final treatment, *Trypanosoma cruzi* was detectable in 3 patients by qPCR; 18 months after the final treatment, *Trypanosoma cruzi* was detectable per qPCR in 4 of the 7 participants. Thus, between 12 and 18 months, 7 participants of the 20 initial follow-up cases showed positive qPCR, indicating treatment failures.

Conclusions: qPCR can be used as an alternative method for evaluating the effectiveness of the etiological treatment of CD, and can be applied to analyze early therapeutic failures. The study showed that benznidazole therapy had limited effectiveness in treating chronic CD patients, thus emphasizing the importance of conducting continued research for developing more effective therapies and diagnosis for CD.

KEYWORDS: *Trypanosoma cruzi*; Chagas disease; Antitrypanosomal therapy; Benznidazole; qPCR; Therapeutic failure

1. Introduction

Chagas disease (CD) is an infectious disease caused by the protozoan *Trypanosoma* (*T.*) *cruzi*, characterized by acute and chronic phases, and is classified by the World Health Organization (WHO) as one of the most neglected diseases in the world[1]. In the

Significance

Chagas disease is a neglected and endemic tropical disease that is present in Latin America, south of the North America and in countries that receive migrants of the endemic region. It is caused by the *Trypanosoma cruzi* parasite and the benznidazole is recommended as the first-choice drug in Brazil for treating this disease. This study demonstrated the therapeutic failure of benznidazole by qPCR within 18 months after the end of treatment, as well as the maintenance of reactive serologies during follow-up. qPCR can be used as a diagnostic method to evaluate the effectiveness of the etiological treatment of Chagas disease, and can be applied to analyse therapeutic failures, emphasizing the importance of continued research to improve therapeutic and diagnostic protocols for Chagas disease.

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chronic phase, the parasites persist in organic tissues, resulting in low parasitemia. After decades of parasitic infection, approximately 30% of participants develop cardiac alterations, arrhythmias, heart failure, or sudden death, and up to 10% of individuals may experience digestive changes, *e.g.*, megaesophagus, or megacolon[2]. It is estimated that 6 to 7 million people worldwide are infected with *T. cruzi*. CD is endemic to many areas of Latin America. However, in recent years, CD has spread across the American continent and continues to spread to countries where CD was not previously endemic, due to migratory flows[3].

For carriers of chronic-phase CD, antitrypanosomal therapy is recommended for up to 50 years for individuals with an undetermined clinical form, and with low disease severity in the cardiac region[4,5]. According to observational studies of these chronic patients, the effects of antitrypanosomal drugs can prevent or slow the progression of the disease, particularly in the development of heart disease[6,7]. There is evidence that up to 20% of all CD cases treated in the chronic phase can be cured, and this is confirmed by a decrease in serological levels after decades of therapy[8].

In the 1980s, considerable advancements in techniques for diagnosing parasites and infectious diseases were made with the introduction of molecular biology. Later, in 1989, a kDNA sequence of *T. cruzi* was amplified, allowing for the qualitative detection and classification of a small number of cells[9]. The main breakthrough occurred in 2007 with the development of quantitative Polymerase Chain Reaction (qPCR), where specific primer pairs were used to target the DNA satellite of *T. cruzi*, making it possible to detect a minimum value of 0.8 parasites/mL in chronic CD participants. This technique was initially used to complement or even replace previous diagnostic methods, and as a criterion for defining cure after antitrypanosomal therapy for CD[10].

Serological tests applied after antitrypanosomal therapy do not give fast responses, since antibody levels can vary according to the age and immune status of the host, while qPCR can detect a very low parasite load of *T. cruzi*[10,11] and is possibly the best tool for monitoring after treatment responses. A recent study demonstrated the importance of qPCR to monitor parasitemia in chronic CD patients treated with benznidazole[12]. Given the above, this study aims to assess the parasite load after etiological treatment in eligible people living with chronic CD.

2. Subjects and methods

2.1. Study design

A descriptive and prospective study was conducted within a cohort of chronic CD participants evaluated between 2014 and 2017, by Chagas Disease Study Group, at the State University of Campinas, (GEDoCh/Unicamp) São Paulo, Brazil.

2.2. Ethical consideration

The study was approved by the Research Ethics Committee of the Medical Sciences School (opinion no. 1266/2011). All procedures followed the guidelines and normative standards for research involving human beings, as established in Resolution 466/2012 of the National Health Council, and were conducted according to the principles established in Helsinki Declaration.

2.3. Eligibility criteria

The patients inclusion criteria were: (1) over 18 years old; (2) two serological tests confirming a positive CD diagnosis (chemiluminescence and immunofluorescence tests); (3) the exclusive use of benznidazole for 60 consecutive days; and (4) no severe heart or digestive diseases. The exclusion criteria were: (1) use of another antitrypanosomal therapy; (2) severe clinical form of CD; or (3) pregnancy.

2.4. Etiological treatment with benznidazole

All participants received indications for specific antitrypanosomal therapy for CD in accordance with the indications established by the 2nd Brazilian Consensus on Chagas Disease, 2015[1]. Benznidazole was used for the antitrypanosomal therapy of *T. cruzi* at a dosage of 5 mg/kg/day for 60 days, not exceeding a daily dose of 300 mg. Data on sex, age, location of possible parasite infection, and clinical form of CD, were collected from medical records. Adverse events (AE) were reported by participants and observed by the medical team at the time of occurrence. AE were evaluated according to the National Health Surveillance Agency of Brazil, acronym ANVISA[13].

2.5. Sample collection

Peripheral blood samples were collected at a volume of 10 mL for qPCR and 4 mL for the serological tests (chemiluminescence and immunofluorescence tests). Both blood collections were taken at the same time, and according to the following periodicity: (1) prior to antitrypanosomal therapy; (2) at the end of treatment (60 days); (3) 12, and 18 months after the last dosage of treatment. Blood samples were also collected for the following biochemical tests which were performed on the 30th and 60th days after a treatment: (1) complete blood count; (2) serum aspartate aminotransferase (AST); (3) alanine aminotransferase (ALT); (4) gamma-glutamyl transferase (γGT); (5) bilirubin; (6) alkaline phosphatase; (7) albumin; (8) uric acid; (9) creatinine; (10) triglycerides; (11) total and fractional cholesterol (LDL and HDL); and (12) fasting blood glucose.

2.6. Molecular diagnosis

The genetic material of *T. cruzi* was extracted from peripheral blood samples using a High Pure PCR Template Preparation kit (Roche, Mannheim, Germany), and elution was performed in 100 µL of elution buffer (70 ℃) in the last step of the DNA extraction, according to the manufacturer's recommendations. To build the standard curve for qPCR amplification, we used a *T. cruzi* culture kept in a liver infusion tryptose (LIT) medium. After counting and calculation in a Neubauer chamber, the final concentration was 7.2×10^6 epimastigotes per millilitres. Serial dilutions (10×) up to 7.2×10^{-3} epimastigotes per milliliters were carried out, and after DNA extraction, the dilution points considered of the standard curve were 7.2×10^5 to 7.2×10^0 parasites equivalents per milliliters (par.Eq./mL).

The amplification reactions (performed in triplicate, supplementary Figure 1 and 2) were carried out in a Rotor-Gene 6000 thermocycler (Corbett LifeScience, California, USA) following protocols described[10]. The intermediate point of a randomly chosen standard curve was used as a positive control. We used the human *RNaseP* gene (TaqMan Human *RNaseP* detection reagent, Applied Biosystems) for the internal control of the reaction. A sample was taken as being valid when the internal control was efficiently amplified, and was positive for *T. cruzi* when the cycle threshold (Ct) for the *T. cruzi* target was <45. Thus, samples with Ct ≤ 32 (greater than 0.1 par.Eq./mL) were taken as being detectable and quantifiable, and samples above this value were considered to be detectable but not quantifiable.

2.7. Statistical analysis

We used the SAS System for Windows (Statistical Analysis System; SAS Institute Inc., Cary, NC, USA) software program, version 9.4, for the data calculations. For the serologies analysis before and after treatment, data are expressed as as median (IQR), and comparisons prior and 12/18 months after the treatment were conducted with Wilcoxon test and significance level adopted was 5% (P<0.05), using the Wilcoxon test.

3. Results

3.1. Epidemiological data

The 32 participants with chronic CD and medical follow-ups GEDoCh/Unicamp. were evaluated from July/2015 to December/2017. Nineteen participants were male (59%). The minimum age was 39 years old, and the maximum age was 56 years old [(48.21±12.02) years], while the median age was 48 years old. Thirteen participants were female (41%), and the minimum age was 42 years old, the maximum age was 54 years old [(46.9±10.6) years], and the median age 45 years old. Regarding the clinical forms, the most frequent form was indeterminate (defined as clinical form without cardiac and/or digestive involvement), which was observed in 16 cases (50%), followed by cardiac forms in 14 cases (44%), and megaesophagus in two cases (6%).

Of the 32 participants who were initially included in the study, therapy was discontinued for 12 patients (38%) for the following reasons: Among the sample universe, nine participants experienced severe AE to benznidazole, and three participants did not adhere to the treatment procedures. Of the 20 participants who received a complete follow-up evaluation, thirteen participants were monitored for 12 months, and seven participants were monitored for 18 months after completing the antitrypanosomal therapy (Figure 1).

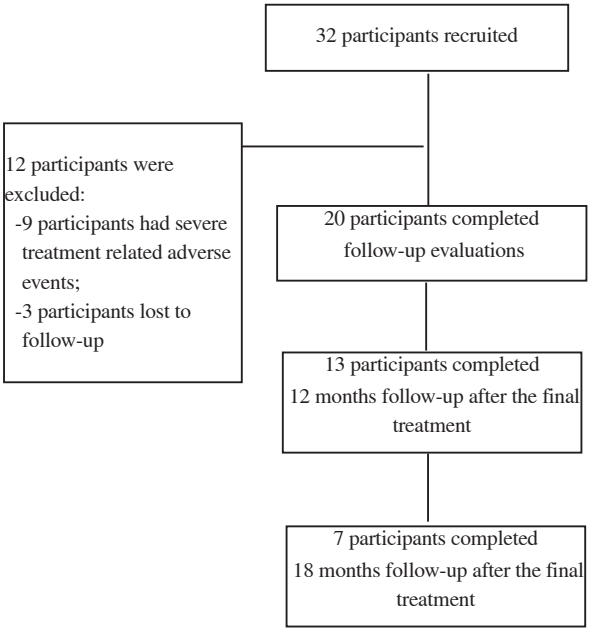

Figure 1. Flowchart of participant recruitment.

3.2. Molecular performance

The Rotor-Gene 6000 instrument (Qiagen) generated information on the efficiency of the reaction, E=1.04 (0.9-1.1) and the value of the coefficient of correlation R^2=0.99 (>0.99), which confirmed the effectiveness of the reaction. The standard curve of absolute amplification for qPCR was performed in quintuplicate for each diluted point. The mean Ct values were (36.40±1.11) for the *T. cruzi* target. No contaminations of negative controls were observed from carry-over during either the DNA extractions or the qPCR. Statistical tests to compare the detectable and quantifiable results of the qPCR before and after therapy with benznidazole were not possible due to null values after treatment.

The results of the qPCR prior to the antitrypanosomal therapy for the 20 participants who complete the follow-up were as follows: Four had a detectable parasite load, albeit not quantifiable. Five

Table 1. Test results, adverse effects, and participant characteristics.

Participants	Sex and age*	Clinical form	qPCR results (par. Eq/mL) and Ct				Initial and final serology titration (12/18 months after the treatment)	AE and day observed
			Prior treatment	60 days of the treatment	12 months after the treatment	18 months after the treatment		
P1	M / 56	Cardiac	0.00 and 41.62	ND	ND	ND	1:160	-
P2	M / 43	Indeterminate	ND	ND	ND	ND	1:320 and R	Cutaneous alterations (8th day)
P3	M / 60	Indeterminate	ND	ND	ND	0.01 and 39.03	1:1 280 and R	-
P4	M / 39	Indeterminate	ND	ND	ND	0.00 and 42.01	1:160 and R	Cutaneous alterations (2nd day)
P5	F / 49	Indeterminate	2.36 and 30.84	ND	ND	0.00 and 41.56	1:1 280 and 1:320**	Cutaneous alterations (6th day); hepatic disorders (~30th day)
P6	M / 48	Indeterminate	ND	ND	ND	ND	1:80 and 1:320**	Digestive intolerance; hepatic disorders (~30th day)
P8	M / 52	Megadigestive	4.11 and 31.32	ND	ND	NC	1:320 and 1:320	Cutaneous alterations (13th day); digestive intolerance
P9	F / 41	Indeterminate	6.05 and 28.12	ND	ND	NC	1:160 and R	Digestive intolerance
P10	F / 45	Cardiac	0.01 and 38.17	ND	ND	NC	1:320 and R	-
P11	M / 47	Cardiac	367 883 and 23.8	ND	ND	NC	1:1 280 and R	-
P12	M / 42	Cardiac	ND	ND	ND	NC	1:320 and 1:320	Cutaneous alterations (10th day)
P13	M / 58	Cardiac	ND	ND	0.004 585 and 39.55	NC	1:320 and R	Peripheral neuropathy (58th day)
P14	M / 50	Cardiac	ND	ND	0.003 242 and 40.03	NC	1:80 and 1:80	Cutaneous alterations (4th day)
P15	F / 48	Cardiac	0.14 and 34.77	ND	ND	NC	1:320 and 1:640**	Cutaneous alterations (10th day)
P16	M / 47	Indeterminate	0.00 and 42.68	ND	0.005 049 and 39.42	NC	1:320 and 1:640**	Cutaneous alterations (5th day)
P17	M / 52	Indeterminate	0.00 and 40.04	ND	ND	NC	1:160 and R	-
P18	M / 50	Indeterminate	ND	ND	ND	NC	1:320 and 1:640**	-
P19	M / 43	Megadigestive	ND	ND	ND	NC	1:640 and 1:320**	-
P20	M / 49	Cardiac	ND	ND	ND	NC	1:640 and 1:320**	-

*years at the initial moment of study; **follow-up for 18 months; AE: BNZ treatment related adverse events; M: male; F: female; Ct = Cycle threshold; qPCR: quantitative PCR; par. Eq/mL: parasites equivalents per milliliters; BNZ: benznidazole; ND = No detectable; R=Reagent (reference values 1:40); NC: not collected.

Table 2. Measures of titlers and comparison results between prior and 12/18 months post-treatment.

Variables		Titlers		*Z*	*P*
		Median (IQR)	Range		
Stage 1 follow-up (*n*=13)	Prior treatment	320.00 (320.00, 320.00)	80.00, 1280.00	-0.21	0.99
	12 months post-treatment	320.00 (160.00, 320.00)	80.00, 1280.00		
Stage 2 follow-up (*n*=7)	Prior treatment	240.00 (160.00, 1280.00)	80.00, 1280.00	- 0.33	0.87
	18 months post-treatment	320.00 (160.00, 640.00)	80.00, 1280.00		

Wilcoxon test was used for comparision between the two groups.

participants had a quantifiable parasite load. Eleven cases showed no detectable results (Table 1). After the antitrypanosomal therapy (60 days), the qPCR results were as follows: Nineteen participants had no detectable parasite load, while blood samples were not collected for one case. 12 Months after the final treatment, three of the 20 cases analyzed showed detectable qPCR (P13, P14, P16). We found that 18 months after the final treatment, four of the seven participants (P3, P4, P5, P7) had quantifiable or detectable qPCR. Thus, between 12 and 18 months, seven participants (35%) of the 20 initial follow-up cases showed positive qPCR, indicating treatment failures.

3.3. Serological follow–up

Before the benznidazole therapy for the 32 initially selected participants, the serology was titrated for 23 participants (72%), and there was no titration for the rest of nine (28%), confirming only a CD-reactive serology. Of the 20 participants analysed who completed 12 and 18 months follow-up after the treatment, all had reactive serologies prior of the treatment. While for 10 cases that had initial titration, the result remained reactive without titration at the end of the follow-ups. In the other 10 cases with titrated serology before and at the end of the treatment, we observed that there was an increase in the titer in four participants (P6, P15, P16, P18), while the titer was maintained in three participants (P8, P12, P14), and three participants had decreased titer values (P5, P19, P20) (Table 1).

3.4. Comparison of serology test results prior and 12/18 months after the treatment

Due to non-titration at the end of treatment in 50% of the analysed cases, we could not infer a relationship between qPCR and serology. In the 20 consecutive cases, there was no statistical significance when comparing CD serology results before and after the benznidazole therapy (Table 2).

3.5. AE to benznidazole

Of the 32 participants initially recruited, 22 had benznidazole treatment related AE (76%). Seven of them (24%) had no AE, and three subjects (9%) did not have follow-ups. The most important event was a dermatological alteration in 18/29 participants (62%). These reactions resulted in the interruption of antitrypanosomal therapy in nine cases (9/18, 50%), and in the other two cases (2/18, 11%), they were controlled with corticosteroids and antihistamines, allowing treatment to be completed. Other AE observed were gastrointestinal disorders in four cases (4/29, 14%), hematological alterations in three cases (3/29, 10%) (decrease in the neutrophil rate), liver function alterations in two cases (2/29, 7%) (increased AST, ALT), and neurological alterations related to peripheral neuropathy in one case (1/29, 3%). Most AE occurred between the 3rd and 30th day after starting the treatment. Only peripheral neuropathy was reported in 1 case (1/29, 3%) on day 58.

4. Discussion

Currently, serology is used, with a progressive reduction in antibody titers to nonreactive values[1]. However, a negative serological result may take decades to achieve or may be never achieved, making it impractical for assessing public health interventions. In this study, chronic chagasic subjects treated with benznidazole were evaluated using qPCR and serologies. Unfortunately, titration was not detectable in some cases, but the persistence of reactive serology before and after treatment validates other studies[16] and demonstrates that it is not an interesting technique for monitoring parasitemia during etiological treatment for CD[12,18]. Thus, our results on serology are similar to the results cited in literature, thereby requiring a follow-up period greater than 18 months to conclude the parasitological cure from serology after *T. cruzi* therapy with benznidazole[19]. In this period, positive serology results are compatible with therapeutic failures, requiring continued follow-ups over longer periods to make conclusive confirmations[11,20,21].

In this study, participants were in the chronic phase of Chagas disease. Pre-treatment qPCR results revealed that 45% of the 20 patients had a quantifiable or detectable parasite load. However, in 55% (11/20) participants, no parasite load was detected. This could make after-treatment evaluations difficult, since the lack of detectable results before treatment would be an indicator of treatment effectiveness. In literature, one can observe that PCR results before treatment identify up to 60% of undetected parasite loads for chronic

cases of *T. cruzi* infections[19]. Moreover, results from previous studies that evaluated individuals aged between 36 and 47 years old, similar to the participants evaluated in this study, reported that qPCR can be used to verify the therapeutic failure of specific treatments for CD in the chronic phase, and also found that a significant portion of the participants had no detectable parasite load before treatment. Some of these studies found that the percentage of patients with no detectable parasite load before treatment ranged from 30% to 56%[20–22].

Thus, no previous detection of parasite load does not invalidate the treatment, since negative qPCR results do not indicate the absence of parasites. The parasite load may have been below the qPCR detection limit because the samples were from chronic individuals. Another possibility is that these individuals lived in non-endemic areas and had no chance of reinfection, which could also explain the low and fluctuating pattern of parasitemia[23]. Our results suggest that the low and near-limit parasite loads were due to the lower detection limit of qPCR. Regarding the cases in which the parasite load was detected prior to treatment, compared with other cases that evaluated chronic heart disease patients from different regions of Latin America (Brazil, Argentina, and Colombia), the observed quantification was 20 times greater than what was observed for Brazilian individuals[11].

In this study, samples P5, P8, P9, and P11, showed parasitemia greater than 2.36 par. Eq/mL before antitrypanosomal therapy, different from literature, which may suggest immune system modulation in these individuals, and even DC reactivation[12,20], as occurred in case P11 (367 883 par. Eq/mL and Ct=23.8). This participant had received a heart transplant and confirmed successive infections by *Klebsiella pneumoniae* carbapenemase and cytomegalovirus, due to the use of immunosuppressants. Given that their origin came from an endemic CD area, they requested a smear (direct parasitological test) every three days, as per the Clinical Hospital's protocol. In the third collection, the *T. cruzi* parasite was verified, confirming the exacerbation of CD, and antitrypanosomal therapy with benznidazole was initiated (unpublished data).

Regarding the end of treatment with BZN after 60 days, qPCR did not detect parasite load in all the 20 follow-up cases[18]. This finding was also verified by the Benefit Study, which reported that, during the treatment period, molecular assays were also negative for all patients after the 14th day until the end of treatment (on the 60th complete day)[5]. It has been reported in literature that parasitemia can be detected during follow-ups after increased treatment time, because, during treatment, or up to one year after administration, parasites can be eliminated by up to 100%[11,12,24].

The 12 months after antitrypanosomal therapy, it was observed that there was no detection by qPCR in 17 samples, and in cases P13, P14, and P16 there was detection, but without quantifications as to the parasite load. One year after treatment, the negative qPCR rate was much higher in the group that received the drug combination, reaching a 96% frequency level as reported[25]. In this study, the qPCR results for the end of treatment and at 12 months after treatment may have been due to the performance of benznidazole at its maximum dosage, acting positively on the immune response of the participants, and may even draw into question a possible parasitological cure that could be achieved *via* benznidazole. We do recognize the limitations inherent to molecular methods, *e.g.*, the volume of the sample collected, the conditions for amplification of the molecular reaction, and different discrete typing units (DTUs)[19].

When qPCR is applied for 18 months after antitrypanosomal therapy with benznidazole, qPCR was positive in three samples, and sample P7 presented detectable qPCR. This result was very important, especially given about the socioeconomic conditions of the participants, who often travel long distances to the GEDoCh/ Unicamp to be attended to once a year, making consultations difficult not only in this study but also for other Brazilian studies, where financial remuneration is questionable according to the Center for Research Ethics. Studies pointed out that treatment abandonment and segments can vary depending on how the study was implemented[26–28]. Thus, if at 12 months treatment failure can be seen for 15% of the cases treated with benznidazole, at 18 months, treatment failure can also be observed in another four cases (samples P3, P4, P5, and P7). Different studies demonstrated that only 2.1%[28] and 3.3%[15] of treated Spanish subjects had positive qPCR after antitrypanosomal therapy with benznidazole, and were followed up for four and seven years, respectively. However, just over half of these individuals were able to continue with clinical and laboratory follow-ups. According to our study, only 9% (3/32) of the participant's missed follow-ups, meaning that our study could overcome the aforementioned barriers.

The 38% (12/32) of the initially recruited participants did not complete treatment, of which 28% (9/32 individuals) were unable to complete the therapy with benznidazole due to serious AE and gastrointestinal changes even after initiating corticosteroid or antihistamine treatments.

In this study, serology was not able to show a significant drop in titer levels after antitrypanosomal therapy with benznidazole for the 32 cases, regardless of the therapy time. However, qPCR was an alternative and additional option to the criterion of therapeutic failures for the study population, and was validated at 18 months for 4 of 7 cases (P3, P4, P5, and P7). In turn, the confirmation of therapeutic failures by qPCR was possible within 12 months after treatment for 3 of all 20 cases (P13, P14, and P16).

The limitation of this study inherent to molecular methods are the quantity (mL) of the samples collecteds, the conditions for amplification of the qPCR, and the possible different discrete typing units (DTUs).

In conclusion, we can preliminarily state that qPCR showed effectiveness in detecting the parasitic load for 15% of the samples, even if this was not quantifiable, thereby confirming therapeutic failure after using BZN with only 12 months of follow-ups.

Conflict of interest statement

We declare that we have no conflict of interest.

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Authors' contributions

TBSP, EAA and GEBM developed the conception or design of the work, data collection, data analysis and interpretation, drafting the article, critical revision of the article, final approval of the version to be published. TBSP, EAA, RGL, JSW, LCM, SCBC and GEBM developed the critical revision of the article, final approval of the version to be published.

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