

This is a repository copy of *Natural Resistance of Leishmania infantum to Miltefosine Contributes to the Low Efficacy in the Treatment of Visceral Leishmaniasis in Brazil.*

White Rose Research Online URL for this paper:
<https://eprints.whiterose.ac.uk/147054/>

Version: Published Version

Article:

Brambilla Carnielli Trindade, Juliana, Monti-Rocha, Renata, Costa, Dorcas Lamounier. et al. (7 more authors) (2019) Natural Resistance of Leishmania infantum to Miltefosine Contributes to the Low Efficacy in the Treatment of Visceral Leishmaniasis in Brazil. American journal of tropical medicine and hygiene. pp. 789-794. ISSN 1476-1645

<https://doi.org/10.4269/ajtmh.18-0949>

Reuse

Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

Natural Resistance of *Leishmania infantum* to Miltefosine Contributes to the Low Efficacy in the Treatment of Visceral Leishmaniasis in Brazil

Juliana B. T. Carnielli,^{1,2*}† Renata Monti-Rocha,¹† Dorcas Lamounier Costa,³ Aretha Molina Sesana,¹ Laura N. N. Pansini,¹ Marcela Segatto,¹ Jeremy C. Mottram,² Carlos Henrique Nery Costa,³ Sílvia F. G. Carvalho,⁴ and Reynaldo Dietze^{1,5*}

¹Núcleo de Doenças Infecciosas, Universidade Federal do Espírito Santo, Vitória, Brazil; ²York Biomedical Research Institute, Department of Biology, University of York, United Kingdom; ³Instituto Natan Portella para Doenças Tropicais, Universidade Federal do Piauí, Teresina, Brazil; ⁴Centro de Ciências Biológicas e da Saúde, Universidade Estadual de Montes Claros, Montes Claros, Brazil; ⁵Global Health and Tropical Medicine, Instituto de Higiene e Medicina Tropical, Universidade NOVA de Lisboa, Lisbon, Portugal

Abstract. In India, visceral leishmaniasis (VL) caused by *Leishmania donovani* has been successfully treated with miltefosine with a cure rate of > 90%. To assess the efficacy and safety of oral miltefosine against Brazilian VL, which is caused by *Leishmania infantum*, a phase II, open-label, dose-escalation study of oral miltefosine was conducted in children (aged 2–12 years) and adolescent-adults (aged 13–60 years). Definitive cure was assessed at a 6-month follow-up visit. The cure rate was only 42% (6 of 14 patients) with a recommended treatment of 28 days and 68% (19 of 28 patients) with an extended treatment of 42 days. The in vitro miltefosine susceptibility profile of intracellular amastigote stages of the pretreatment isolates, from cured and relapsed patients, showed a positive correlation with the clinical outcome. The IC₅₀ mean (SEM) of eventual cures was 5.1 (0.4) μM, whereas that of eventual failures was 12.8 (1.9) μM ($P = 0.0002$). An IC₅₀ above 8.0 μM predicts failure with 82% sensitivity and 100% specificity. The finding of *L. infantum* amastigotes resistant to miltefosine in isolates from patients who eventually failed treatment strongly suggests natural resistance to this drug, as miltefosine had never been used in Brazil before this trial was carried out.

INTRODUCTION

American visceral leishmaniasis (VL), caused by *Leishmania infantum* (synonymous with *Leishmania chagasi* in Brazil), is a major health problem in many parts of Brazil. The disease is usually fatal if untreated and is characterized clinically by fever, gradual weight loss, splenomegaly, hypergammaglobulinemia, and pancytopenia.¹

Visceral leishmaniasis treatment has been challenging because it relies on a few classic agents (pentavalent antimony, amphotericin B deoxycholate), all of which are parenteral and poorly tolerated. In Brazil, the standard therapeutic regimen is 20 mg Sb^V/kg/day, for a minimum of 20 days. Amphotericin B deoxycholate is the second-line drug of choice. Lipid formulations of amphotericin B are less toxic but more expensive and, as a result, only available under request to the Ministry of Health for patients with severe disease with complications, for example, bleeding, and for children younger than 1 year and patients older than 50 years.²

In this scenario of scarcity of efficacious drugs, in the late 1990s, an oral drug, miltefosine (hexadecylphosphocholine), a phospholipid analogue, was considered an important advance in leishmaniasis therapy. Although developed originally as an anticancer drug, miltefosine is relatively safe and became the first-line therapy in India, where VL is caused by *Leishmania donovani*. The reported cured rates were high both in adults and in children, 94–95%.^{3,4} However, efficacy decreased after extensive use in the Indian subcontinent over the ensuing decade.^{5–7}

There are no data on miltefosine effectiveness against VL caused by *L. infantum* in South America. This study was designed to evaluate the efficacy and safety of miltefosine in a phase II trial, in Brazilian patients with VL and investigate whether the clinical outcome could be associated with in vitro susceptibility of the parasites to miltefosine.

MATERIALS AND METHODS

Study design. This was a phase II, open-label, dose-escalation study of oral miltefosine (Impavido[®], supplied by AEterna Zentaris) in children (aged 2–12 years) and in adolescent-adults (aged 13–60 years) at two sites in Brazil, Montes Claros and Teresina. The objective was to investigate if efficacy and safety of oral miltefosine in Brazilian VL patients were similar to that already published for Indian VL patients.

Patients. The patients were enrolled and treated in 2005. Inclusion criteria were newly diagnosed (untreated) VL, with parasitological confirmation via visualization of amastigotes in tissue samples or a positive culture, and either gender. Exclusion criteria were severe decreases in the formed elements of the blood or host biochemical abnormalities: platelet count < 30 × 10⁹/L; white blood count (WBC) < 1 × 10⁹/L; hemoglobin < 5 g/100 mL; liver enzymes ≥ 3 times upper limit of normal range; serum creatinine or BUN ≥ 1.5 times upper limit of normal range. Other exclusion criteria were evidence of serious underlying disease (cardiac, renal, hepatic, or pulmonary); immunodeficiency or antibody to HIV; severe protein and/or caloric malnutrition (Kwashiorkor, Marasmus); any non-compensated or uncontrolled condition; and lactation, pregnancy (to be determined by adequate test), or inadequate contraception in females of childbearing potential for treatment period plus 2 months.

Treatment. The first patients were treated at Montes Claros with the recommended regimen of 2.5 mg miltefosine/kg/day, using the 10 mg formulation for 28 days. Adolescent-adults received 100 mg/day (one 50 mg capsule twice a day with meals), the same dose used in India. When cure rates for

* Address correspondence to Juliana B. T. Carnielli, York Biomedical Research Institute, Department of Biology, University of York, Wentworth Way, Heslington, York, YO10 5DD, E-mail: juliana.carnielli@york.ac.uk or Reynaldo Dietze, Global Health and Tropical Medicine, Instituto de Higiene e Medicina Tropical, Universidade NOVA de Lisboa, Rua da Junqueira 100, Lisbon 1349-008, Portugal, E-mail: reynaldo.dietze@ihmt.unl.pt.

† These authors contributed equally to this work.

the 14 patients at Montes Claros were seen to be low, subsequent patients at Teresina were administered drug at the same daily dose (2.5 mg/kg/day for children, 100 mg/day for adults) for 42 days in an attempt to increase cure rates.

Follow-up. Patients were seen in follow-up at the end of treatment to ascertain initial cure and for 6 months after the end of therapy to ascertain final cure.

Endpoints. Failure was defined by clinical and parasitological criteria: signs or symptoms suggestive of leishmaniasis, accompanied by confirmation of the presence of *Leishmania* in a bone marrow aspirate at that time. Cure was lack of failure. The primary endpoint was efficacy: the rate of cure by the end of follow-up 6 months after treatment. Other endpoints were adverse events and correlation of in vitro susceptibility with cure.

Sample size. The sample size was chosen for convenience: up to 40 pediatric and 40 adolescent-adult patients (Figure 1).

In vitro susceptibility of amastigotes to miltefosine. *Leishmania* isolates from both sites were obtained before treatment by bone marrow aspiration from 26 of the final total of 40 patients. Promastigotes were grown in liver infusion tryptose (LIT) medium supplemented with 10% heat-inactivated fetal calf serum (Hi-FCS). All isolates were previously identified as *L. infantum* using polymerase chain reaction–restriction fragment length polymorphism.⁸ The miltefosine susceptibility of intracellular amastigote was performed as follows: adherent macrophages from peritoneal fluid of Swiss mice were infected with late-log phase promastigotes at a ratio of seven parasites to one macrophage, using the 16-well Lab-Tek tissue culture slides (Nunc, NY). After 24 hours of incubation in Roswell Park Memorial Institute (RPMI-1640) supplemented with 10% Hi-FCS in 5% CO₂ at 37°C, free promastigotes were removed and the culture was then exposed to different concentrations of miltefosine (0, 0.55, 1.67, 5, and 15 μM) in triplicate. Higher concentrations were not tested because of drug toxicity to the

infected macrophage. After 72 hours of additional incubation, the slides were stained with Diff-Quick solutions and 100 cells in each well were counted to determine the percentage of infected macrophages. Drug activity was determined from the percentage of infected cells in drug-treated cultures relative to nontreated cultures. The half-maximal inhibitory concentrations (IC₅₀) of miltefosine for amastigotes were calculated from nonlinear regression analysis, using GraphPad Prism v.7.0a software (GraphPad Software, San Diego, CA). The results were expressed as the mean of three independent experiments. All assays were carried out blind regarding isolate identity and clinical outcome.

Ethical review board. Informed consent was obtained from all study participants and/or guardians before enrollment. The study protocol was reviewed and approved by the Institutional Review Board of the Universidade Estadual de Montes Claros, Universidade Federal do Piauí, and by the Brazilian National Review Board (CONEP D-18506-Z019). ClinicalTrials.gov Identifier: NCT00378495.

RESULTS

Montes Claros site. Fourteen patients were enrolled in the clinical trial. Of these, 8 (57%), all children, failed to respond to treatment. The time of clinical and parasitological failure occurred at 1 month (one patient), 2 months (five patients), and 5 months (two patients) after the end of treatment (Figure 1). Laboratory and clinical data of cured and not cured patients are shown in Table 1. Adverse events were minor and included vomiting (four patients), nausea (three patients), and abdominal pain (two patients). These symptoms resolved when drug was given with the main meals.

Because of the high failure rates for these first 14 patients, the protocol was amended for subsequent patients such that

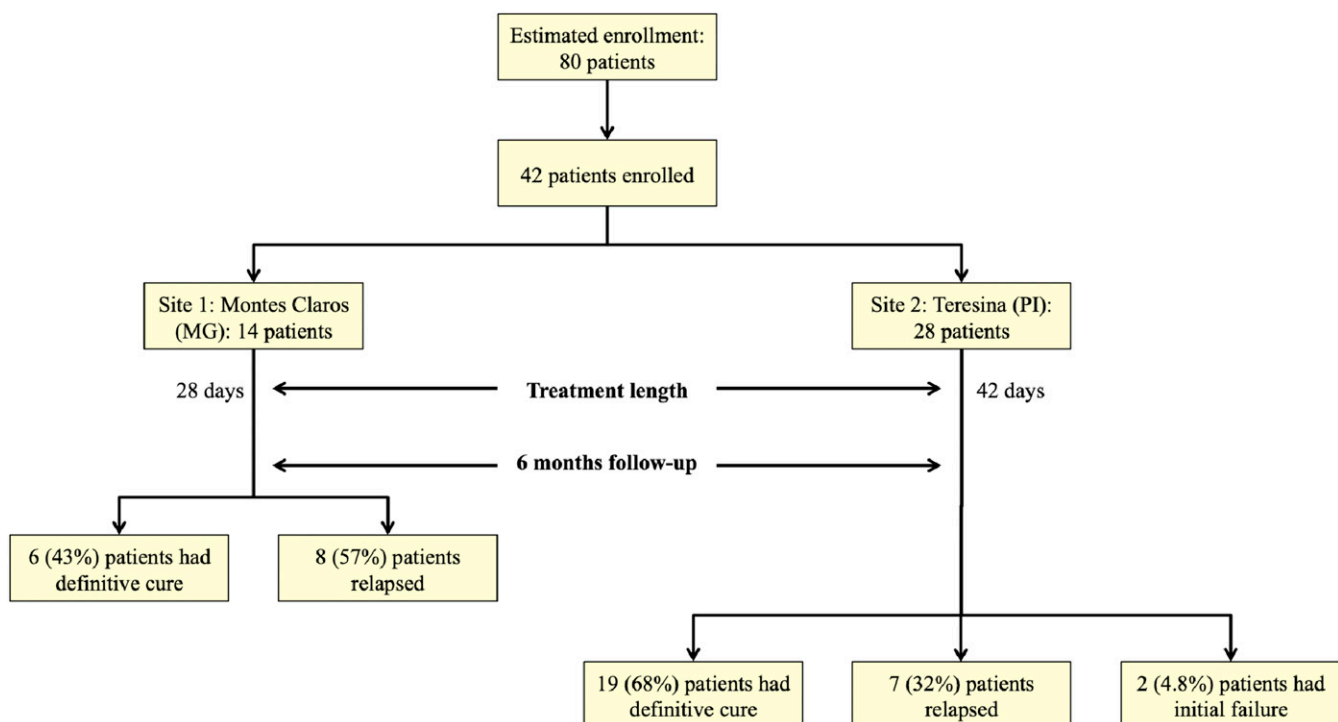


FIGURE 1. Flow charts of phase-II Brazilian miltefosine trial. This figure appears in color at www.ajtmh.org.

TABLE 1
Entrance and end-of-treatment data

Site	1: Montes Claros		2: Teresina	
	28 days		42 days	
	Day 0*	Day 28*	Day 0*	Day 42*
Treatment duration				
Parameter	Day 0*	Day 28*	Day 0*	Day 42*
Age (years)				
Cured	17 (2–43)	NA	19 (2–53)	NA
Failed	5 (2–11)	NA	17 (2–41)	NA
Duration of illness (days)				
Cured	28 (5–60)	NA	38 (9–60)	NA
Failed	32 (15–90)	NA	50 (21–120)	NA
Spleen size (cm)				
Cured	8.3 (4–15)	2.8 (0–4.5)	8.8 (2–14.5)	1.8 (0–5.5)
Failed	10.5 (7–12.5)	4.1 (2–4.5)	8.5 (0–12)	3.1 (0–7)
Hemoglobin (g/dL)				
Cured	8.8 (6.6–12)	11.6 (10–13.7)	8.5 (5.2–10.8)	11.5 (7.5–14.6)
Failed	8.3 (6.7–10.6)	10.0 (8.7–12.3)	8.4 (6–11)	11.3 (10.2–13.5)
WBC ($\times 1,000/\text{mm}^3$)				
Cured	3.6 (1.8–7.3)	6.8 (2.7–14.4)	3.2 (1.3–8.9)	7.8 (2.4–15.2)
Failed	3.5 (2.7–5.3)	7.7 (6.1–12.9)	2.6 (1.6–3.6)	7.5 (3.6–11.8)
Albumin (g/dL)				
Cured	3.1 (2.6–3.9)	4.3 (3.7–4.9)	3.3 (2.6–3.9)	4.0 (3.5–4.8)
Failed	3.6 (3–4)	4.2 (3.9–4.9)	3.3 (2.4–4.3)	4.1 (3.6–4.8)

* Data represent mean (range) of values.

Laboratory reference values: hemoglobin, 12–17 g/dL; WBC, 5,000–10,000/mm³; albumin, 3.5–5.5 g/dL.

they received the same daily dose but for an extended period of 42 days. These subsequent patients were enrolled at the site in Teresina.

Teresina site. Thirty patients were enrolled. Four patients prematurely terminated treatment: one patient because HIV infection was detected and three patients because of drug toxicity. A 56-year-old male was removed on day 12 because of renal toxicity and suspected treatment failure; a 4-year-old female was removed on day 6 because of vomiting; a 26-year-old male was removed on day 12 because of liver toxicity and suspected treatment failure. Thus, 26 patients (12 children and 14 adolescent-adults) completed the 42 days of treatment of whom 7 (three children and four adolescent-adults) failed to respond to treatment. The time of clinical and parasitological failure was at 1 month (one patient), 3 months (three patients), 4 months (two patients), and 6 months (one patient) after the end of treatment. Considering all 28 evaluable patients (26 per protocol patients plus two patients who may have failed at the time they were removed for reasons of intolerance), there were nine failures (32%) consisting of three children and six adolescent-adults. If we use an intention-to-treat analysis, considering all 30 enrolled patients, there were 11 failures (36%). Laboratory and clinical data of cured and not cured patients are shown in Table 1 and Figure 1.

Both sites. A summary of the outcomes for all patients enrolled at both clinical sites is shown in Table 2. For children, extension of treatment period from 28 days (Montes Claros site) to 42 days (Teresina site) improved the cure rate from 43% to 67%, although with the small number of patients in this phase II study, this difference was not statistically significant ($P = 0.0995$: Fischer's exact test). For adolescent-adults, the cure rate at the Teresina site, the only site with appreciable numbers of patients, was 69%. Inspection of the entrance characteristics does not reveal obvious differences between parameter values for patients destined to cure or to fail treatment with miltefosine (Table 1).

Miltefosine susceptibility of *L. infantum* isolates. To test if the differences in clinical outcome to miltefosine treatment

could be explained by variance in miltefosine susceptibility of the infecting parasite *L. infantum*, the in vitro susceptibility of intracellular amastigote of *L. infantum* to miltefosine was assessed for pretreatment parasites from 26 patients: 14 parasite isolates came from patients who cured and 12 parasite isolates from patients who failed. There was no significant difference in *L. infantum* infectivity on a peritoneal mouse macrophage model when pretreatment isolates from cured patients and those who failed were compared (Figure 2A). Moreover, no differences were observed in *L. infantum* infectivity ratio between parasites containing or not the miltefosine sensitivity locus (MSL), which we previously associated with miltefosine treatment outcomes⁹ (Figure 2B). Miltefosine concentration that reduces 50% of infected macrophages (IC_{50}) was calculated and the in vitro IC_{50} values of these pretreatment isolates showed a significant difference between isolates from patients who were cured (mean $IC_{50} = 5.1 \mu\text{M}$; SEM = 0.4 μM) and those who failed (mean $IC_{50} = 12.8 \mu\text{M}$; SEM = 1.9 μM): $P = 0.0002$ via *t*-test (Figure 2C). The in vitro susceptibility assay also showed a significant difference between isolates MSL⁺ (mean $IC_{50} = 5.9 \mu\text{M}$; SEM = 1.0 μM) and MSL⁻ (mean $IC_{50} = 10.9 \mu\text{M}$; SEM = 1.8 μM), which corroborates our previous association between MSL and treatment outcome (Figure 2D). By ROC curve analysis, the IC_{50} of 8.0 μM was highlighted as the best discrimination point

TABLE 2
Final efficacy data

	Children	Adolescent-adults	All ages
Montes Claros*	11	3	14
Cure	3 (27%)	3 (100%)	6
Failure	8 (73%)	0 (0%)	8
% Cure (95% CI)	27%	100%	43% (18–71)
Teresina†	12	16	28
Cure	8 (67%)	11 (69%)	19
Failure	4 (33%)	3 (19%)	7
Probable failure	0 (0%)	2 (12%)	2
% Cure (95% CI)	67%	69%	68% (48–84)

* Patients from Montes Claros were treated for 28 days.

† Patients from Teresina were treated for 42 days.

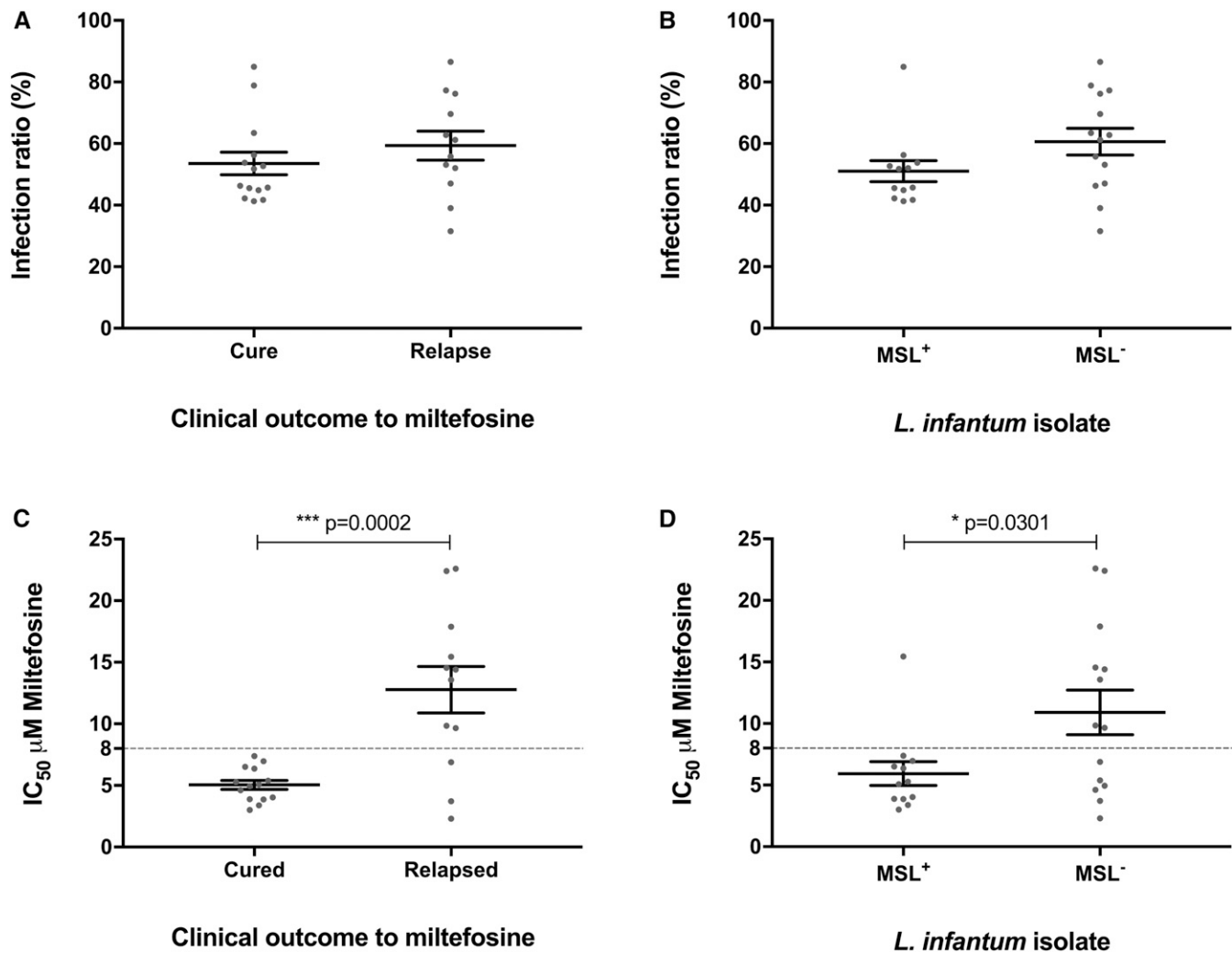


FIGURE 2. Infectivity and in vitro miltefosine susceptibility of *L. infantum* isolates collected before treatment for cured and failed patients. (A) and (B) Infection ratio, percentage of infected macrophage 96 hours post infection with *L. infantum* isolates. (C) and (D) In vitro miltefosine susceptibility of intracellular amastigote stage of *L. infantum* isolates. Each symbol represents the value for an individual *L. infantum* isolate. The horizontal bars in panels indicate the mean values for the groups and the standard errors of the means. Asterisk indicates significant difference between groups (t-test). MSL⁻ = homogeneous population for absence of MSL; MSL⁺ = homogeneous/heterogeneous population for presence of MSL.

to predict treatment outcome, such that isolates with lower IC₅₀ would be predicted to cure and higher IC₅₀ would be predicted to fail. Based on the IC₅₀ values of the 26 *L. infantum* isolates, cure was predicted for 17 patients, but only 14 patients were cured. Among nine isolates for which failure was predicted, all patients relapsed, resulting in a sensitivity of 82% and specificity of 100%.

DISCUSSION

The efficacy of miltefosine in the treatment of South American (Brazilian) VL was lower than expected. At the first site, where most patients were pediatric, miltefosine was administered for the standard 28 days, resulting in a cure rate of 43% (6 of 14 patients). Previous pharmacokinetic studies showed that miltefosine exposure was lower in pediatric patients compared with adult patients with VL, which has been linked with treatment failure.^{10–12} However, a comparable pediatric and adolescent-adult cure rate (67% and 69%, respectively) was observed at the second site, where an

extended period of treatment (42 days) was performed. In a nonrandomized comparison, the extended treatment course improved slightly the cure rate compared with the 28-day standard regimen (68% or 64% in an intention to treat analysis). Nevertheless, this cure rate is far below the > 90% cure rate for miltefosine reported in Indian VL before its widespread use.^{2–4,13}

Because the values of entrance clinical and laboratory parameters did not predict eventual cure and confounding concomitant diseases were ruled out by exclusion criteria, we hypothesized that inherent differences in parasite susceptibility to drug might be predictive of therapeutic success. To this end, we cultured parasites from 26 of the 40 enrolled patients, with approximately equal numbers of parasites from eventual cures (14 parasites) and failures (12 parasites). The in vitro miltefosine susceptibility phenotype of intracellular amastigotes showed that IC₅₀ values of the pretreatment isolates were able to distinguish eventual cures from eventual failures, strongly suggesting that treatment failure observed in Brazil is associated with natural resistance of *L. infantum* parasite to miltefosine.

Killing of parasites by drugs depends on pharmacokinetics and pharmacodynamics, which may vary in different VL patients in different parts of the world. For Indian subcontinent VL, the lower rate of efficacy of miltefosine in children versus adolescent-adults¹⁴ may be linked to lower exposure of the drug in children.¹⁵ With respect to activity of drug against the parasite, Prajapati et al.¹⁶ found similar pretreatment IC₅₀ in promastigotes from patients destined to cure (5.8 µM) compared with patients destined to fail (4.5 µM). However, there was a slight increase in parasite resistance to miltefosine as shown by an increase in IC₅₀ posttreatment (6.1 µM) compared with pretreatment (3.7 µM). Bhandari et al.¹⁷ showed an increase in IC₅₀ in isolates at the time of failure (4.7 µM) compared with pretreatment (1.9 µM), and Deep et al.¹⁸ confirmed that IC₅₀ increase on failure (11 µM) compared with pretreatment (3.9 µM). Our present report appears unique in finding significant differences between the activity of miltefosine against VL pretreatment that correlate with eventual treatment outcome. Our findings are of particular interest because, as mentioned previously, the different susceptibility of *Leishmania* to miltefosine that correlates with clinical outcome is inherent to Brazilian *L. infantum* because these data from 2005 predates any use of this drug in Brazil.

Our recent genomic analysis showed a strong association ($P = 0.0005$) between the deletion of the *L. infantum* MSL and miltefosine treatment failure. The absence of MSL in the parasite increases the risk of treatment failure 9.4-fold and predicts miltefosine failure with 92% sensitivity and 78% specificity, which highlights MSL as a potential molecular marker to predict miltefosine treatment outcome in VL.⁹ Our data suggest that neither the presence nor absence of the MSL influences the ability of the parasites to infect macrophages in vitro. However, there is a strong correlation between the presence of the MSL and the in vitro susceptibility of amastigote stage to miltefosine ($P = 0.0301$). This result highlights that screening for the presence of the MSL by polymerase chain reaction could be a convenient prognostic marker in clinical practice to predict efficacy of miltefosine because it can be performed directly on biological samples in a much shorter time than the in vitro characterization of the susceptibility of the parasite to miltefosine. The molecular mechanism involved in the resistance of *L. infantum* to miltefosine in these strains, however, is still unknown and subject to further investigation.

Finally, some limitations of our study should be taken into account: (1) the lack of a randomized controlled trial can limit inferences from our results; (2) this trial was carried out at only two sites, one in the northeast and the other in the southeast part of Brazil; (3) the trial had to be interrupted before it reached the planned sample size, because of ethical reasons (low efficacy), resulting in only 44 patients enrolled; (4) the trial was split into two different dosing regimens. Moreover, considering that the *L. infantum* studied here were isolated over a decade ago, it is also important to know the miltefosine-resistant phenotype throughout Brazil now. We have started to address this point by screening for the MSL frequency in different areas of Brazil and found that MSL frequency changes geographically (data not shown), indicating that miltefosine could be successfully used after MSL stratification in some parts of Brazil.⁹

Published online August 19, 2019.

Acknowledgments: We are grateful to Fausto Edmundo Lima Pereira for critical reading of the manuscript and to Elenice Moreira Lemos (in memoriam) for her substantial contribution for the progress of this project.

Disclosures: The clinical trial was supported by WHO/TDR [Study D-18506-2019, Project A30831]. This work was also supported by Fundação de Pesquisa do Estado do Espírito Santo-FAPES, Brazil [grant number 36316326/2007 and 70984379/2015], Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq, Brazil [grant number 478080/2009], and the Global Challenges Research Fund and MRC [grant number MR/P024483]. The funders had no role in study design, data collection, data analysis, interpretation, and writing of the manuscript.

Authors' addresses: Juliana B. T. Carnielli and Jeremy C. Mottram, York Biomedical Research Institute, Department of Biology, University of York, United Kingdom, E-mails: juliana.carnielli@york.ac.uk and jeremy.mottram@york.ac.uk. Renata Monti-Rocha, Simile Medicina Diagnóstica, Belo Horizonte - Minas Gerais, Brazil, e-mail: mrenata@hotmail.com. Aretha Molina Sesana and Laura N. N. Pansini, Núcleo de Doenças Infeciosas, Universidade Federal do Espírito Santo, Vitória, Brazil, E-mail: laurapansini@gmail.com. Marcela Segato, Faculdade Brasileira UNIVIX, Vitória - Espírito Santo, Brazil, E-mail: marcelasegatto@gmail.com. Dorcas Lamounier Costa and Carlos Henrique Nery Costa, Instituto Natan Portella para Doenças Tropicais, Universidade Federal do Piauí, Teresina, Brazil, E-mails: dorcas.lc@gmail.com and chncosta@gmail.com. Sílvio F. G. Carvalho, Centro de Ciências Biológicas e da Saúde, Universidade Estadual de Montes Claros, Montes Claros, Brazil, E-mail: guimaraescarvalho@yahoo.com.br. Reynaldo Dietze, Global Health and Tropical Medicine, Instituto de Higiene e Medicina Tropical, Universidade NOVA de Lisboa, Lisbon, Portugal, E-mail: rdietze@ndi.ufes.br.

This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

REFERENCES

1. Werneck GL, Batista MS, Gomes JR, Costa DL, Costa CH, 2003. Prognostic factors for death from visceral leishmaniasis in Teresina, Brazil. *Infection* 31: 174-177.
2. Ministério da Saúde, 2011. *Leishmaniose Visceral: Recomendações Clínicas para Redução da Letalidade*. Brasília-DF, Brasil: Ministério da Saúde, 78.
3. Jha TK, Sundar S, Thakur CP, Bachmann P, Karbwang J, Fischer C, Voss A, Berman J, 1999. Miltefosine, an oral agent, for the treatment of Indian visceral leishmaniasis. *N Engl J Med* 341: 1795-1800.
4. Sundar S, Jha TK, Thakur CP, Engel J, Sindermann H, Fischer C, Junge K, Bryceson A, Berman J, 2002. Oral miltefosine for Indian visceral leishmaniasis. *N Engl J Med* 347: 1739-1746.
5. Sundar S, Singh A, Rai M, Prajapati VK, Singh AK, Ostyn B, Boelaert M, Dujardin JC, Chakravarty J, 2012. Efficacy of miltefosine in the treatment of visceral leishmaniasis in India after a decade of use. *Clin Infect Dis* 55: 543-550.
6. Rijal S et al., 2013. Increasing failure of miltefosine in the treatment of Kala-azar in Nepal and the potential role of parasite drug resistance, reinfection, or noncompliance. *Clin Infect Dis* 56: 1530-1538.
7. Rahman M et al., 2011. Phase IV trial of miltefosine in adults and children for treatment of visceral leishmaniasis (kala-azar) in Bangladesh. *Am J Trop Med Hyg* 85: 66-69.
8. Segatto M et al., 2012. Genetic diversity of *Leishmania infantum* field populations from Brazil. *Mem Inst Oswaldo Cruz* 107: 39-47.
9. Carnielli JBT et al., 2018. A *Leishmania infantum* genetic marker associated with miltefosine treatment failure for visceral leishmaniasis. *EBioMedicine* 36: 83-91.
10. Dorlo TPC et al., 2017. Visceral leishmaniasis relapse hazard is linked to reduced miltefosine exposure in patients from Eastern Africa: a

- population pharmacokinetic/pharmacodynamic study. *J Antimicrob Chemother* 72: 3131–3140.
11. Dorlo TP et al., 2014. Failure of miltefosine in visceral leishmaniasis is associated with low drug exposure. *J Infect Dis* 210: 146–153.
 12. Mbui J et al., 2019. Pharmacokinetics, safety and efficacy of an allometric miltefosine regimen for the treatment of visceral leishmaniasis in Eastern African children: an open-label, phase-II clinical trial. *Clin Infect Dis* 68: 1530–1538.
 13. Bhattacharya SK et al., 2007. Phase 4 trial of miltefosine for the treatment of Indian visceral leishmaniasis. *J Infect Dis* 196: 591–598.
 14. Ostyn B, Hasker E, Dorlo TP, Rijal S, Sundar S, Dujardin JC, Boelaert M, 2014. Failure of miltefosine treatment for visceral leishmaniasis in children and men in South-East Asia. *PLoS One* 9: e100220.
 15. Dorlo TP, Huitema AD, Beijnen JH, de Vries PJ, 2012. Optimal dosing of miltefosine in children and adults with visceral leishmaniasis. *Antimicrob Agents Chemother* 56: 3864–3872.
 16. Prajapati VK, Sharma S, Rai M, Ostyn B, Salotra P, Vanaerschot M, Dujardin JC, Sundar S, 2013. In vitro susceptibility of *Leishmania donovani* to miltefosine in Indian visceral leishmaniasis. *Am J Trop Med Hyg* 89: 750–754.
 17. Bhandari V, Kulshrestha A, Deep DK, Stark O, Prajapati VK, Ramesh V, Sundar S, Schonian G, Dujardin JC, Salotra P, 2012. Drug susceptibility in *Leishmania* isolates following miltefosine treatment in cases of visceral leishmaniasis and post kala-azar dermal leishmaniasis. *PLoS Negl Trop Dis* 6: e1657.
 18. Deep DK, Singh R, Bhandari V, Verma A, Sharma V, Wajid S, Sundar S, Ramesh V, Dujardin JC, Salotra P, 2017. Increased miltefosine tolerance in clinical isolates of *Leishmania donovani* is associated with reduced drug accumulation, increased infectivity and resistance to oxidative stress. *PLoS Negl Trop Dis* 11: e0005641.