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MAJOR ARTICLE

Validation of Apolipoprotein A-1 and Fibronectin Fragments as Markers of Parasitological Cure for Congenital Chagas Disease in Children Treated With Benznidazole

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Background. No reliable tests or validated biomarkers exist to ensure parasitological cure following treatment of Chagas disease (CD) patients chronically infected with *Trypanosoma cruzi*. As seroreversion, the only marker of cure, happens more quickly in children, we investigated the correlation between previously identified biomarkers and seroreversion in children.

Methods. Thirty CD children (age 1 month to 10 years) diagnosed as *T. cruzi* positive (time point S0) were treated with benznidazole (BZ) 5–8 mg/kg/d for 60 days. At least 2 serological tests were used to evaluate treatment efficacy from the end of treatment (S1) until seroreversion (S2). Thirty children (age 1 month to 10 years) and 15 adults were used as healthy controls (HCs). Immunoblot and a proteomic-based assay were used to validate previously identified fragments of apolipoprotein A-1 (ApoA1) and fibronectin (FBN) as CD biomarkers.

Results. Correlation between seroreversion and absence of ApoA1 and FBN fragments by immunoblot was observed in 30/30 (100%) and 29/30 (96.6%) CD children, respectively. ApoA1 and FBN fragments were absent at the end of BZ treatment in 20/30 (66.6%) and 16/30 (53.3%) children, respectively. Absence of fragments in serum profiles was confirmed by mass spectrometry. Using intact protein analysis, a 28 109-Da protein identified as full-length ApoA1 by tandem mass spectrometry was detected in HC serum samples.

Conclusions. These data confirm that ApoA1 and FBN fragments can discriminate between healthy and *T. cruzi*–infected samples. Correlation with seroreversion was shown for the first time; results suggest predictive capacity potentially superior to serology, making them potentially useful as surrogate biomarkers.

Keywords. apolipoprotein A-1; biomarkers; Chagas disease; children; fibronectin; test of cure.

Chagas disease (CD), caused by the protozoan parasite *Trypanosoma cruzi*, is the most neglected tropical disease in Latin America [1, 2]. It is estimated that 8 million people are infected worldwide [3]. Vector-mediated transmission by triatomine bugs occurs in rural and suburban areas in Central and South America. In nonendemic countries, CD is no longer an exotic disease but has become an increasing public health concern [4]. This is the result of population migration, vertical transmission, organ transplantation, and blood transfusion, resulting in a high percentage of chronic asymptomatic patients in these populations [2, 5, 6].

Currently, there are no reliable tests or validated biomarkers to ensure parasitological cure in chronically infected patients. Polymerase chain reaction (PCR) techniques lack sensitivity when parasitemia is low and do not guarantee parasitological cure [7, 8]. Serological tests cannot discriminate between treated individuals and those with CD and have shown inconsistent results in adult studies [9]. Blood bank evaluations rely on serological testing, and confirmation assays must be performed to elucidate the status of non-negative results [10, 11]. In addition, seroreversion, the gold standard, can take decades in adults. This is a major barrier to determining the optimal treatment duration for currently available drugs or the assessment of potential new drugs in proof-of-concept clinical trials [12–15]. Clearly, there is an urgent need for reliable biomarkers [12, 13].

Of all the parasite biomarkers proposed over the last decade (reviewed in [13, 14, 16]), very few, if any, have been validated

Received 17 May 2018; editorial decision 6 September 2018; accepted 4 October 2018.

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DOI: 10.1093/ofid/ofy236

and can be used to determine treatment efficacy. Using a proteomic platform to compare serum protein profiles of asymptomatic chronically infected CD patients and healthy controls, we identified different host biomarkers [17]. Candidate biomarkers were further evaluated in a cohort of adult patients treated with nifurtimox (Nfx) and followed up for 3 years [18]. Among these, the most promising candidates were apolipoprotein A-I (ApoA1) and fibronectin (FBN) fragments, which were significantly upregulated in chronic or asymptomatic CD subjects compared with healthy controls (HCs) and returned to levels similar to those seen in HCs 3 years after Nfx treatment. Immunoblot assays for some of these proteomics-derived biomarkers were successfully developed and used to assess parasitological cure after Nfx treatment [18].

Our unexpected discovery prompted us to study *T. cruzi*–host interactions further. Indeed, several lines of evidence suggest that *T. cruzi* can take up low-density lipoprotein (LDL) particles, can be found in adipose tissue (a potential reservoir site of the parasite in man), and use LDL receptors during cell invasion, which leads to the increased accumulation of LDL cholesterol in host tissue in both acute and chronic CD [19–23]. In addition, truncated forms of ApoA1 have been shown to result from the digestion of native ApoA1 in high-density lipoproteins (HDLs) by cruzipain, a *T. cruzi* cysteine protease, in vitro and in vivo [24, 25]. Computational studies suggest that n-terminal and c-terminal ApoA1 peptides are important for its lipid association to form HDL particles [26]; however, the effect of ApoA1 truncations in HDL metabolism remains to be studied.

These findings suggest that ApoA1 fragments, among others, could be valid indicators of parasite signature and promising biomarkers to assess cure in Chagas patients. Taking advantage of the more rapid seroreversion in children (a few months to a few years), we investigated if the absence and/or reduction of ApoA1 and FBN fragments correlated with seroreversion in a cohort of children treated with benznidazole (BZ) and followed until seroreversion. This correlation, which has been impossible in adult samples, confirmed these fragments as signatures of parasite clearance and, consequently, validated them as biomarkers of parasitological cure.

METHODS

Ethical Statement

The protocol of this study was approved by the Ethical Boards for Medical Research of the Hospital de Niños Ricardo Gutierrez, Buenos Aires, Argentina (approval number CEI 14/14, institutional ethical committee). Written consent was required from patients' legal representatives and was obtained from all patients or legal guardians, as appropriate.

Study Design and Setting

The cohort study took place at the Hospital de Niños R. Gutierrez. The study included children between 1 month and 10 years old, born in Argentina, mainly infected by vertical transmission, and whose mothers were from Argentina, Bolivia, or Paraguay and were not treated before or during pregnancy. CD children <6 months were diagnosed by positive *T. cruzi* parasitemia (microhematocrit and PCR), serological tests (enzyme-linked immunosorbent assay [ELISA] and indirect hemagglutination antibody test [IHA]; Wiener Lab, Rosario, Argentina). Older children were diagnosed only by serological tests. PCR was performed as described by Duffy et al. [27]; serological tests were chosen for diagnosis and prognosis following Argentinian guidelines. CD children were treated with 5–8 mg/kg/d BZ for 60 days. BZ treatment efficacy in infants younger than 8 months was evaluated by parasitemia (microhematocrit and/or PCR) and serology at the end of BZ treatment. Older children were evaluated by PCR and serology. All children were followed up and evaluated by serology every 3 months during the first year post-treatment and then every 6 months until 2 consecutive negative serological results. Children were considered cured when at least 2 serological tests were negative, as recommended by the World Health Organization (WHO). For the proposes of this study, only children who reached seroreversion were included. Cardiac involvement due to CD was determined by electrocardiogram (ECG) and echocardiogram at diagnosis and every year after treatment. Thirty children within the same age range were used as healthy negative controls for serology, mass spectrometry analysis, and immunoblot. Samples from 10 infected, untreated mothers and 15 uninfected adults were evaluated using the same serological tests and included as positive controls and negative controls for immunoblots, respectively.

Sample Collection

Serum samples were collected in tubes without anticoagulant, centrifuged, and aliquoted for serological tests, immunoblot assay, and proteomics analysis. All samples were stored at –20°C within 1 hour of collection. Aliquots for each subject at diagnosis (S0), end of BZ treatment (S1), and seroreversion (S2) were sent to the National Reference Center for Parasitology (Montreal, Quebec) for immunoblotting and proteomics analysis.

Immunoblot Analysis

Rabbit antisera against the predicted neo-termini of a 24.7-KDa ApoA1 fragment and a 28.9-KDa fibronectin fragment were generated and used in immunoblots as described by Santamaria et al. [18]. Briefly, 1-µL serum samples were separated in 4%–12% Novex Bis-Tris Midi gradient gels (Life Technologies, Carlsbad, CA) under reducing conditions and transferred onto nitrocellulose membranes. Membranes were blocked in 5% milk-0.05% Tween-20 in phosphate buffer saline and incubated overnight at 4°C with rabbit anti-ApoA1 (24.7) 1:500 or rabbit anti-FBN (28) 1:1000 dilution. Then, they were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated antirabbit IgG (GE Healthcare Life Sciences, Uppsala,

Sweden) 1:100 000 dilution. Bands were developed by chemiluminescence using SuperSignal West Pico (Pierce, Rockford, IL) and exposed to x-ray film. Antibody specificity to new n-termini fragments was demonstrated by the detection of recombinant proteins of the respective fragments and the absence of detection of full-length proteins.

Serum Fractionation and Profiling

High-throughput serum fractionation by ion exchange chromatography was performed using a ProteinChip serum fractionation kit (Bio-Rad, Hercules, CA) as described by Ndao et al. [17]. All transfers and incubations were performed in an automatized liquid handling station (Biomek FX, Beckman-Coulter, Brea, CA). Our previous studies [17, 18] show that biomarkers of 28.9 and 24.7 KDa were present in fractions 1 (pH 9) and 3 (pH 5), respectively. These fractions were further analyzed using liquid chromatography–mass spectrometry (LC-MS).

Serum fractions were desalted, dried, and reconstituted in 30 µL of water–0.1% formic acid (FA) for mass spectrometry intact proteins analysis using a Maxis ESI/Q-TOF mass spectrometer (Bruker, Bellirica, MA) coupled with uHPLC (Dionex Ultimate 3000 RSLC nano-HPLC, Thermo Fisher Scientific, Waltham, MA). In brief, 5 µL of each fraction was loaded onto a C4 analytical column (5 µm–300 Å, 150*1 mm; Phenomenex, Torrance, CA). Proteins were separated on a linear gradient of 5%–90% of solvent B at a flow rate of 175 µL/min. Solvent A was water–0.1% FA, and solvent B was acetonitrile–0.8% FA.

Continuous mass spectra during the chromatogram run were acquired in MS mode between 400 and 3000 m/z. Scan speed was set at 1 Hz and ESI at 4200 V – N_2 7l/min – 220°C. Data files were analyzed using Compass Data Analysis 4.3 Software (Bruker, Bellirica, MA). Profiles from CD and HC children were compared using Bruker Compass Profile Analysis 2.1 software (Bruker, Bellirica, MA). An ANOVA *t* test model and a principal component analysis model were generated to identify peaks significantly different between HC and CD groups. Maximum entropy deconvolution was used to calculate protein peak masses for the biomarkers of 24.7 and 28.9 KDa discovered in our previous studies [17, 18].

ApoA1 Full-Length Identification by LC–Tandem MS

To establish the ApoA1 full-length identity in HCs, precursor ions for masses similar to full-length ApoA1 were submitted for a second analysis using a tandem mass spectrometry (MS/MS) acquisition method. MS/MS spectra were submitted to Bruker Daltonics BioTools 3.2 SR4 software (Bruker, Bellirica, MA) for protein identification using the Mascot Search Engine server, version 2.2.4 (Matrix Science, London, UK). Spectra were searched against human databases (Uniprot) with the following parameters: top-down analysis, no enzymatic digestion, and peptide and fragment tolerance set to 15 ppm and 0.05 Da, respectively.

RESULTS

Patients

All CD patients were asymptomatic with no cardiac involvement or other Chagas-associated pathology at enrollment. BZ treatment was well tolerated with mild adverse events [28]. Table 1 shows the sociodemographic and clinical characteristics of the 60 children included in the study. The parasitology and serology results at diagnosis (S0), end of BZ treatment (S1), and seroreversion (S2) are summarized in Supplementary Table 1. All children included in the study showed negative parasitemia by microhematocrit and/or PCR at S1 and remained negative until S2. ELISA and IHA titers were reduced or negative at S1 compared with S0 (Supplementary Table 1); titers continued to decrease until S2. Serological test results showed discrepancies (Supplementary Table 1), as observed in other studies [9, 10]; thus, children were only considered cured when at least 2 tests were negative, as recommended by the WHO.

Immunoblot of 24.7-KDa ApoA1 and 28.9-KDa FBN Fragments in Sera

ApoA1 and FBN digestion by cruzipain generates new N-termini fragments. Accordingly, antibodies against neo-peptides will recognize the protein fragments but not their full lengths, even if they are polyclonal antibodies; this specificity is demonstrated in Figure 1. When comparing infected (CD child) and noninfected samples (HC child and HC adult), the antisera selectively detected ApoA1 (24.7) (Figure 1A) and FBN (28.9) (Figure 1B) fragments in samples from CD children, whereas no band corresponding to the sizes of the fragments or full-length proteins was detected in HC children and adults, supporting previous observations [18]. Additionally, antibodies recognized specifically the

Table 1. Sociodemographic and Clinical Characteristics of 30 CD Child Patients and 30 HC Children

		CD children	HC children
Age at diagnosis	1 mo	7	None
	2–11 mo	11	15
	>1 y	12	15
Sex	Female	15	15
	Male	15	15
Origin	Buenos Aires	22	N/A
	Entre Rios	1	
	Capital Federal	7	
Infection route	Congenital	28	
	Unknown	2	
Origin of mother	Argentina	14	
	Bolivia	13	
	Paraguay	2	
	Unknown	1	
Benznidazole treatment (5–8 mg/kg/d)	60 d	22	
	<60 d	5	
	>60 d	3	

Abbreviations: CD, Chagas disease; HC, healthy control.

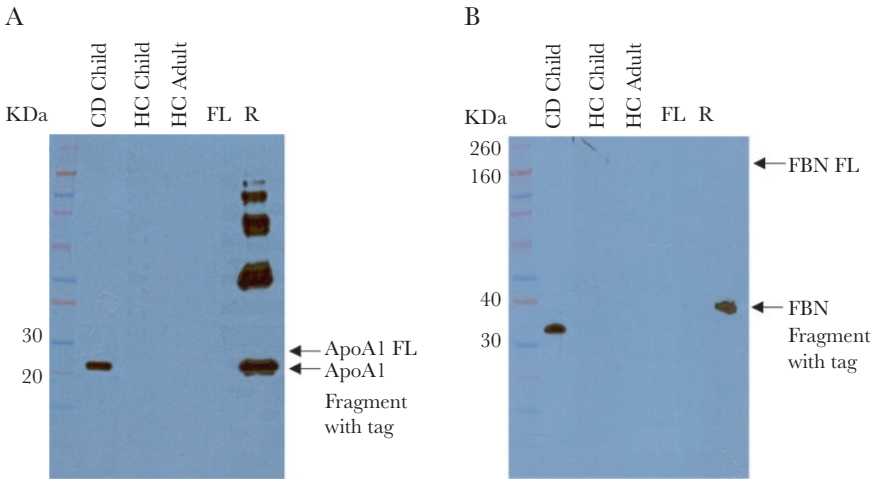

Figure 1. Immunoblot analysis of ApoA1 (24.7) and FBN (28.9) fragments in CD and HC. Each antiserum detects expected bands in a CD child but not in child or adult HCs. The specificity of the rabbit anti-ApoA1 (24.7) serum (A) and rabbit anti-FBN (28) (B) for the corresponding fragments is demonstrated by their inability to detect native full-length proteins, ApoA1 (~28.1 KDa) and FBN (~220 KDa), while reacting with 6xHis tag recombinants of the 24.7-KDa ApoA1 and 28.9-KDa FBN (migration at ~35 KDa) fragments, respectively. Abbreviations: ApoA1, apolipoprotein A-1; CD, Chagas disease; HC, healthy control; FBN, fibronectin; FL, full-length; R, recombinants.

corresponding fragments in infected mothers (Supplementary Figure 1) and did not show reactivity with any of the HC children and adults (not shown).

Using the immunoblot described above, ApoA1 and FBN fragments were undetected at S2 in all (30 of 30 samples; 100%) and 29 of 30 (96.6%) CD children, respectively. However, the only specimen (child of 9.9 years) that was FBN positive was very faint. Interestingly, ApoA1 and FBN fragments were undetected by immunoblot at S1 in 20 children (66.6%) and 16 children (53.3%), respectively (Figure 2), who remained seropositive at this point. In some children, the band intensity of fragments at S1 was reduced as compared with S0 (children of 4 months for ApoA1 and 10 months for FBN) (Figure 2).

Profiling Analysis of Serum Proteins

To confirm the immunoblot results and further emphasize the potential of ApoA1 24.7-KDa and FBN 28.9-KDa fragments as biomarkers of cure for CD, serum protein profiles were determined by mass spectrometry. Additionally, we searched for proteins corresponding to full-length ApoA1 in CD and HC children. Upon MS data analysis, proteins of around 28 KDa were detected at 38.5 minutes of the liquid chromatography gradient in HC samples. The peak 827.7288 m/z, which predicts

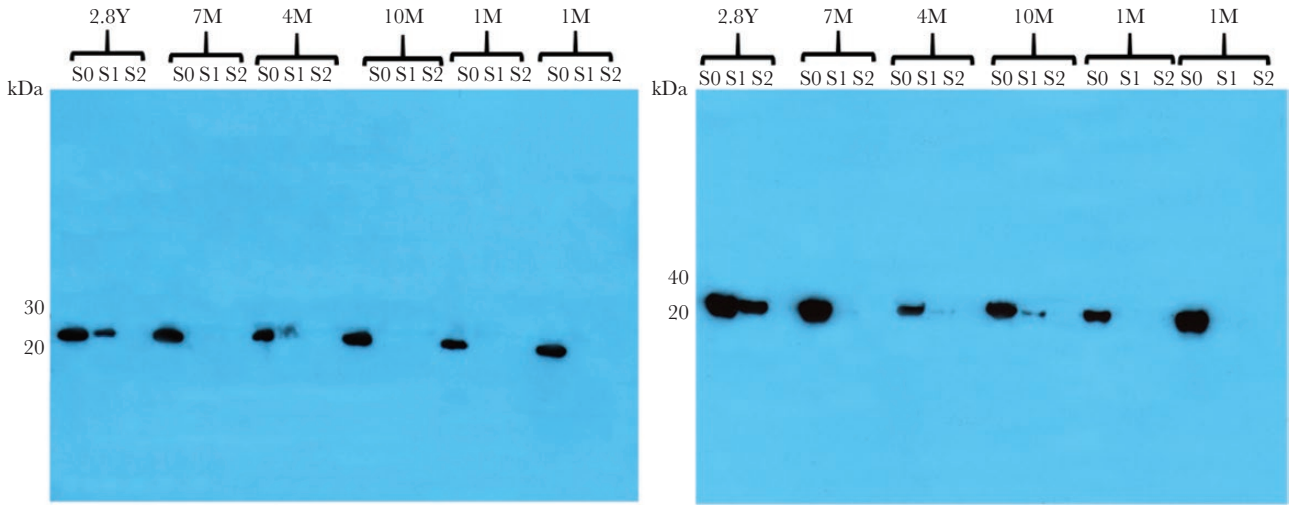

Figure 2. Immunoblot analysis of apolipoprotein A-1 (24.7) and fibronectin (28.9) fragments in serum samples from Chagas disease (CD) children at different time points. Serum from CD children age >1 year and younger were collected at diagnosis (S0), end of treatment (S1), and at seronegative conversion (S2). Immunoblot analysis was performed on 1 uL of serum using polyclonal antibodies against neo-peptides PALEDL and PFTDV from APOA1 24.7-KDa and FBN 28.9-KDa fragments, respectively.

a mass of 28 109 Da, was present in the extracted ion chromatogram of HCs and statistically upregulated in HCs compared with CD patients ($P = .03617$) (Figure 3A). A second MS acquisition for the precursor ion in HC confirmed that the protein corresponds to full-length ApoA1 (Figure 3B).

CD and HC serum fractions 1 and 3 were analyzed by MS and searched for peaks corresponding to the molecular weight of ApoA1 and FBN fragments. Peaks were present in CD samples but undetected in HC at the same retention times (Supplementary Figure 2), which strongly suggests that these fragments are upregulated in CD and correspond to the ApoA1 and FBN fragments that were identified in our previous studies [17, 18].

The same peaks were searched for in serum fractions of CD children at the different time points in the study. For this, the CD serum samples were pooled according to the immunoblot

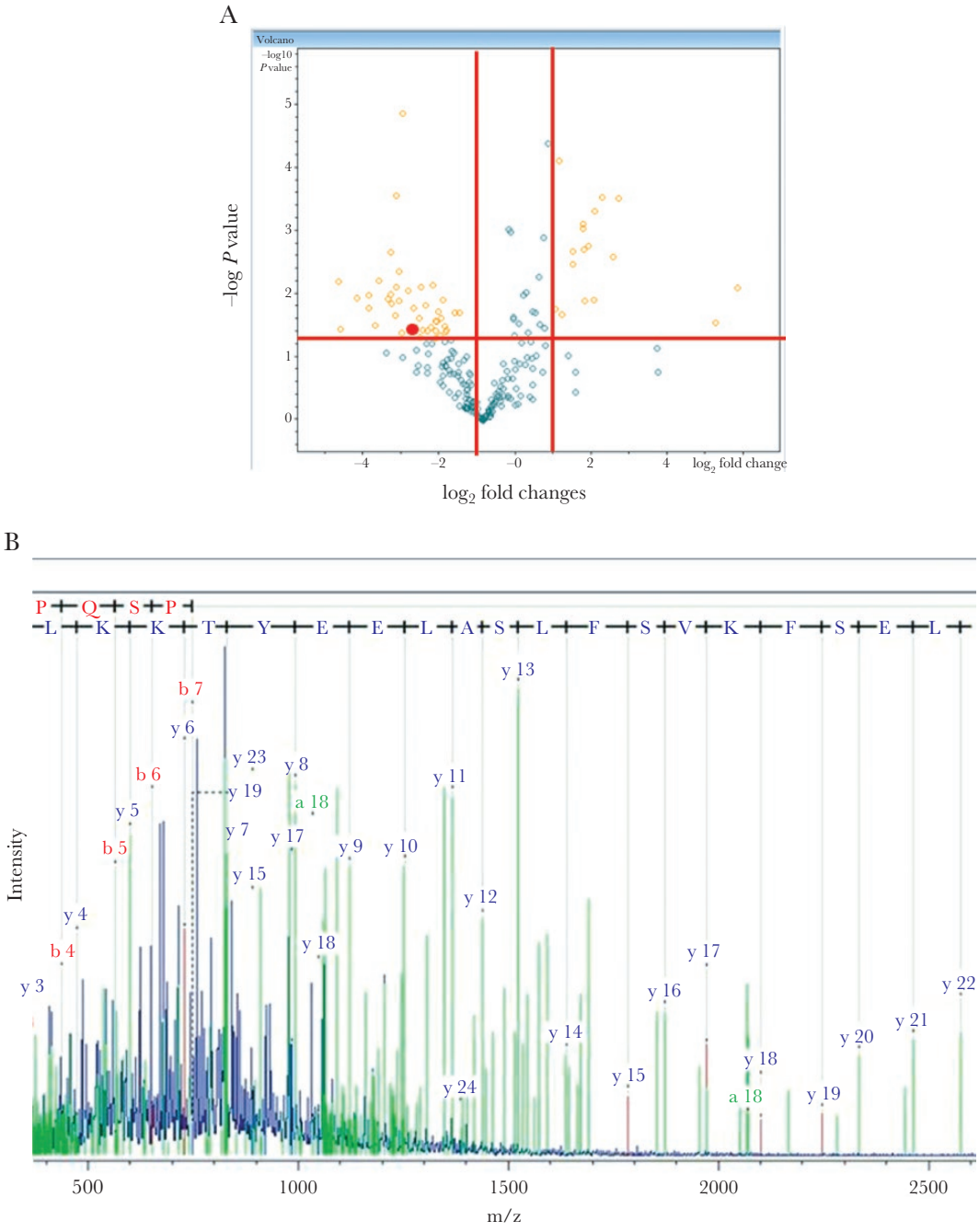

Figure 3. A, Volcano plot for all proteins identified in serum sasmples of Chagas disease (CD) and healthy control (HC) children. Mass of 28 109 Da, eluted at 38.5 minutes of the liquid chromatography gradient, (red point) is significatively upregulated in HC compared with CD. B, Tandem mass spectrometry spectra of 28 109 Da mass precursor peak and Biotools results that confirm the apolipoprotein A-1 identity.

results. As observed in Figure 4, the masses corresponding to the ApoA1 fragment (24 756 Da) and the FBN fragment (28 786 Da) were undetected at S2, whereas the mass corresponding to full-length ApoA1 (28 093 Da) was detected. As was observed by immunoblot, the detection of the ApoA1 24.7-KDa mass was reduced at S1 compared with its intensity at S0 (Figure 4A). In contrast, the intensity of full-length ApoA1 increases at S1 and S2 compared with its intensity at S0 (Figures 4 and 5). The fragments were undetected by MS in serum samples that were immunoblot negative at S1 (Figure 5).

DISCUSSION

Confirmation of parasitological cure during the chronic stage of Chagas disease is still challenging. Current diagnostic tools have limitations, can lead to inconsistent results, and cannot measure cure rates without decades of follow-up [29–31]. This lack of suitable tests of parasitological cure and treatment efficacy assessment is the biggest challenge in CD prognosis and drug research and development.

In our last 2 cohort studies [17, 18], we presented the first proteomic approach to identifying diagnostic biomarkers for Chagas disease using top-down proteomics. We demonstrated that highly sensitive and specific host biological markers were able to distinguish asymptomatic CD adult patients from HC in 2 different populations [17, 18]. ApoA1 fragments of 24.7, 13.6, and 9.1 KDa, as well as a 28.9-KDa FBN fragment, were upregulated in asymptomatic CD adults and returned to levels similar to those seen in HCs after 3 years of Nfx treatment. However, all adults were still seropositive at 3-year follow-up, and it was not

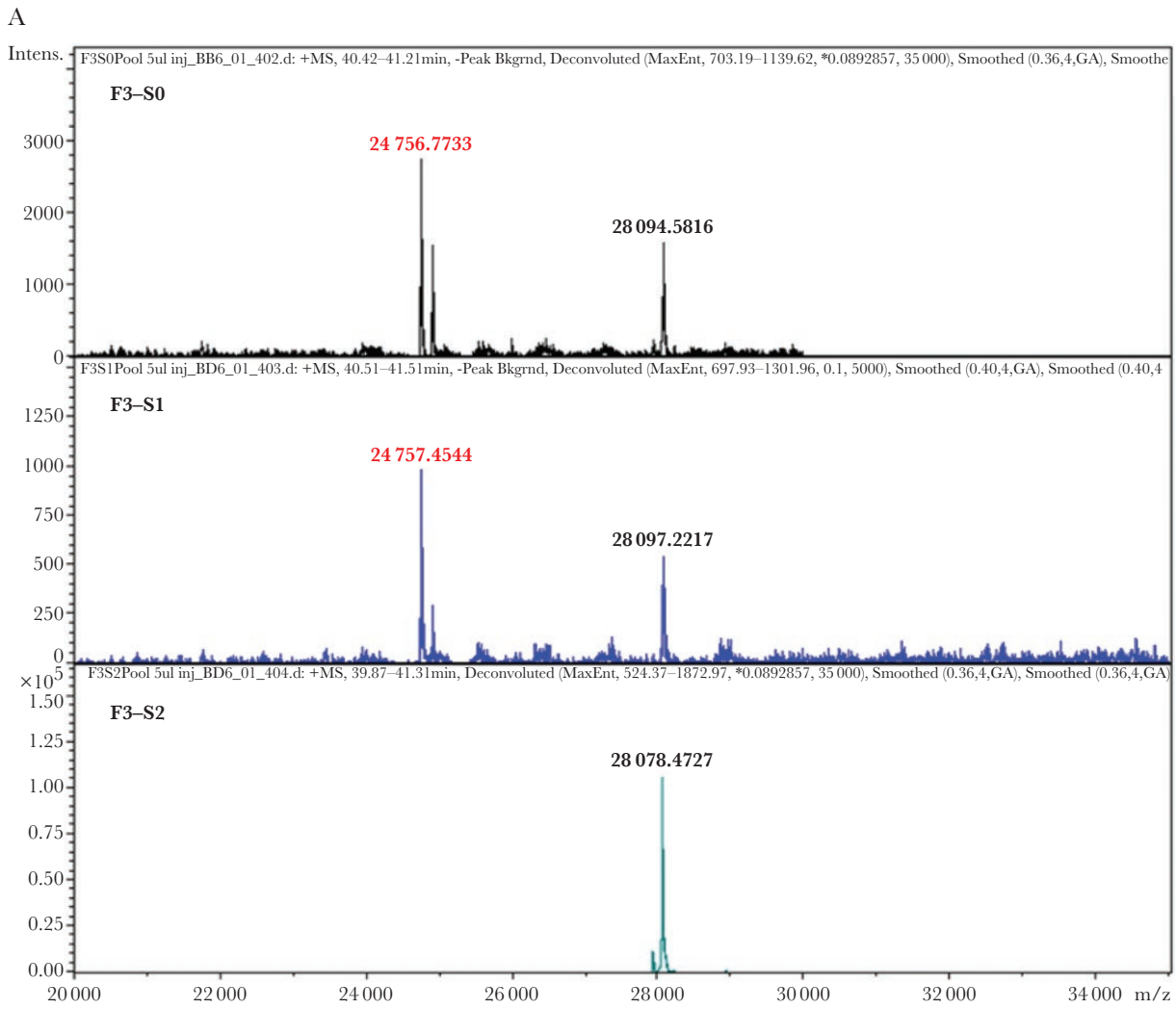

Figure 4. Representative mass spectrometry spectra of biomarkers 24.7 (A) and 28.9 (B) for samples from Chagas children patients treated with benznidazole. Proteins of 24 756 and 28 786 Da are present at diagnosis (S0), the intensity of 24 756 Da is reduced after treatment (S1), and both biomarkers are no longer detected at seroreversion (S2). A peak of 28 078 Da corresponding to apolipoprotein A-1 (~28.1 KDa) is detected at seroreversion.

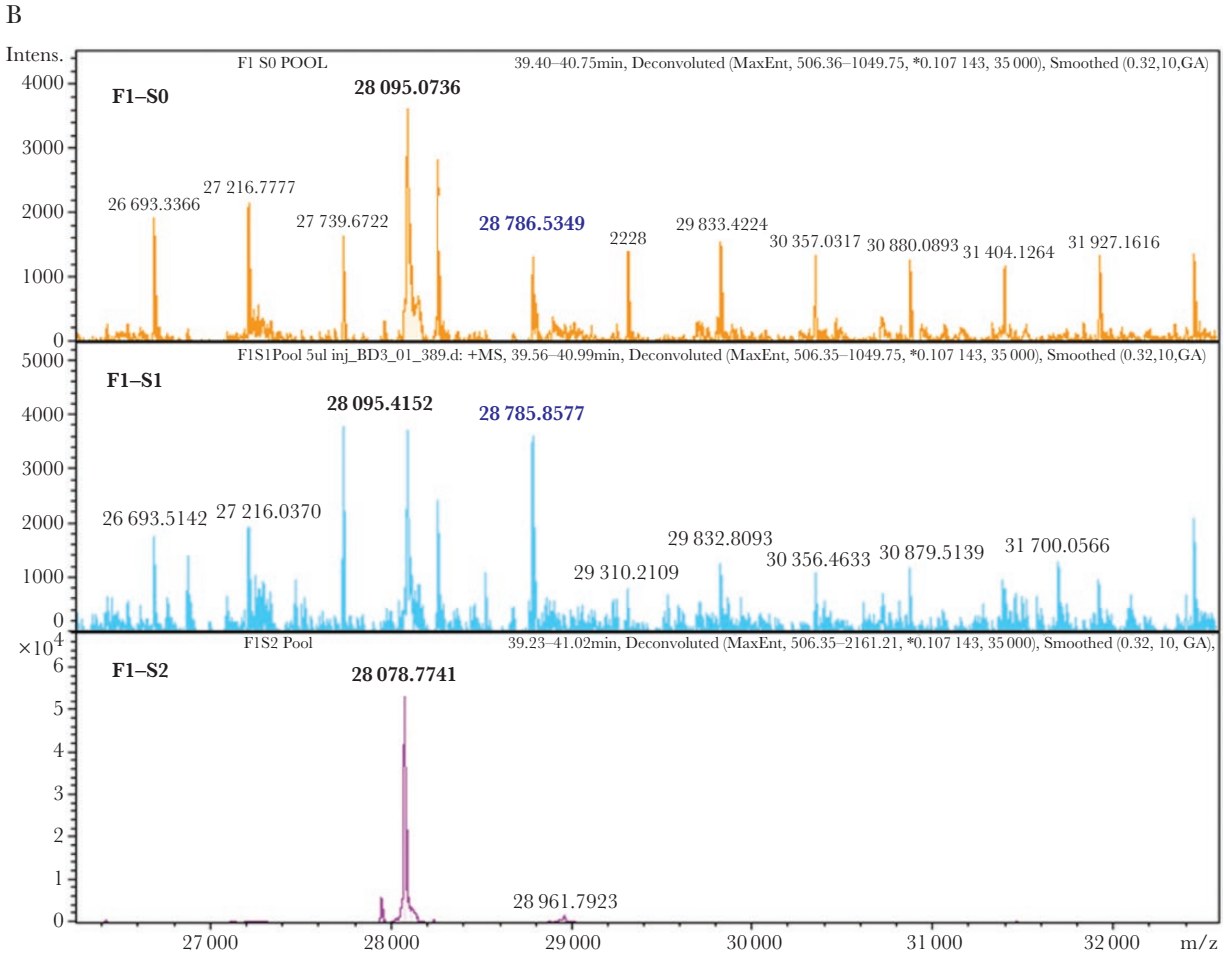

Figure 4. *Continued*

possible to compare our biomarker results with seroreversion, which is currently the gold standard of parasitological cure.

In the current study, taking advantage of the quicker serological conversion in children compared with adults, we collected serum samples of CD children at diagnosis, end of BZ treatment, and seroreversion. BZ treatment is well tolerated in children [28], and in the present study, all children were followed periodically after treatment until 2 serological tests were negative. This was the perfect scenario for comparing the presence or absence of previously identified biomarkers with seroreversion and validating their use as biomarkers of parasitological cure.

Using both immunoblot and MS, we observed that ApoA1 (24.7-KDa) and FBN (28.9-KDa) fragments were present in Argentinian CD children, but not in HCs. MS analysis also showed that full-length ApoA1 protein was statistically upregulated in HC children and its abundance increased in serum from CD children after treatment and at seroreversion. This pattern is consistent with those seen in our previous studies with Bolivian and Venezuelan samples [17, 19], which demonstrates the reproducibility of our approach across the South American population, an important factor considering the variety of infective *T. cruzi* strains in patients in these regions. This reproducibility was also achieved using different proteomics platforms, reinforcing the robustness of our data.

These fragments were absent in samples from CD children treated with BZ and considered cured as determined by seroreversion. To our knowledge, this is the first time that a correlation has been established between the presence/absence of a newly identified marker and seroconversion in Chagas disease. Potential CD biomarkers have been studied in CD children during the early chronic indeterminate stage. Soluble platelet selectin (sP-selectin) and soluble vascular cell adhesion molecule–1 (sVCAM-1) titers decreased in 66.7% and 41% of the children treated with BZ compared with controls [32]. Equally, interferon-γ profiles and M2 muscarinic receptor autoantibody (anti-M2R AAb) were decreased 6 months after BZ treatment. Anti-M2R AAb reactivity declined to between 29.7% and 88.1% of the initial level [33]. Specific cellular surface markers associated with a type 1–modulated cytokine pattern were elevated after BZ treatment in a different study [33]. However, none of these biomarkers has been further studied and/or shown correlation with seroconversion.

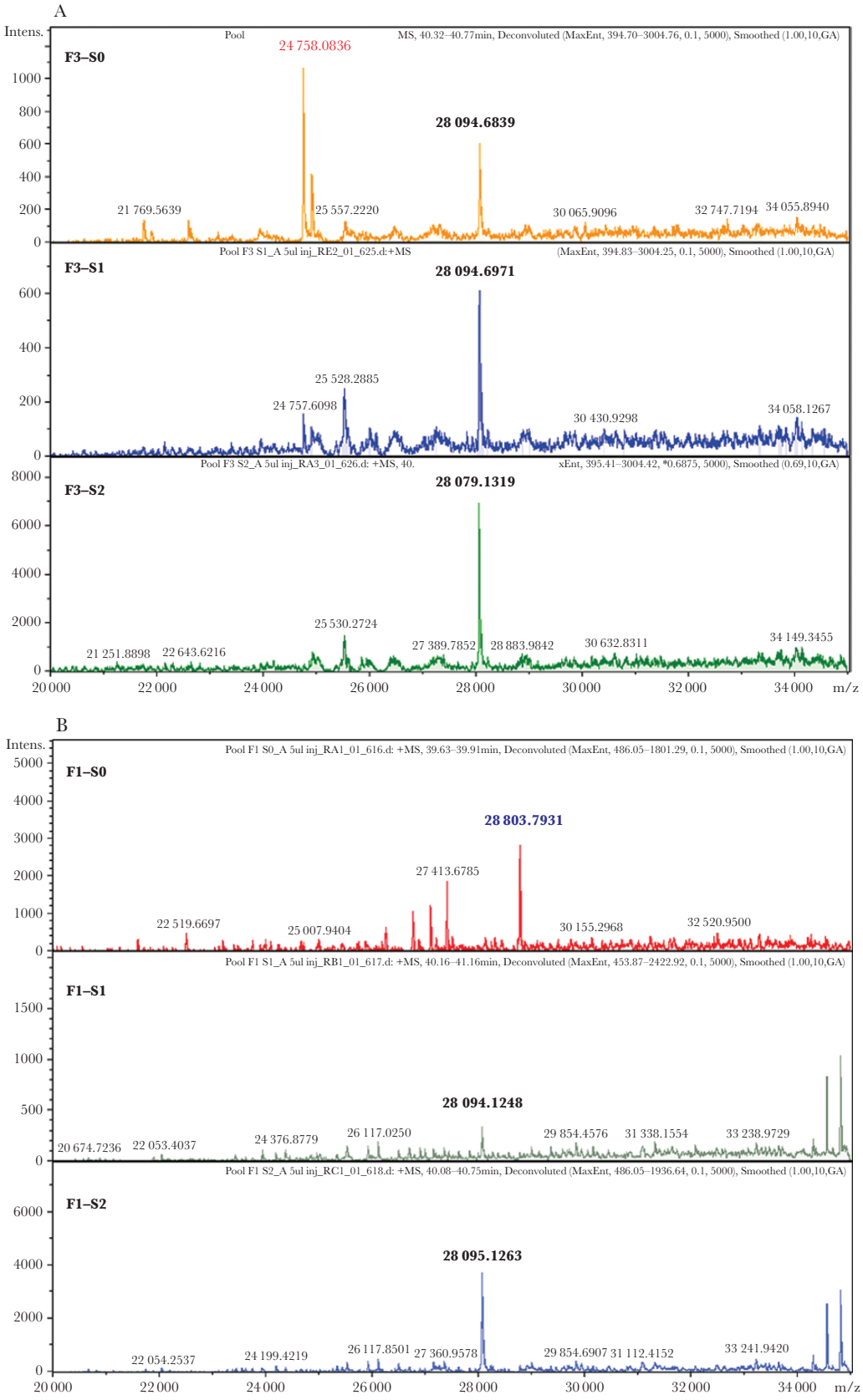

Figure 5. Mass spectrometry spectra of biomarkers 24.7 (A) and 28.9 (B) for children's samples that show negative immunoblot results at the end of benznidazole (BZ) treatment (S1). Proteins of 24 758 and 28 803 Da are present at diagnosis (S0) but are undetected at the end of BZ treatment (S1) and seroconversion (S2); instead, proteins corresponding to full-length apolipoprotein A-1 (~28.1 KDa) are detected at S1 and S2.

Interestingly, the ApoA1 (24.7-KDa) and FBN (28.9-KDa) fragments were absent at the end of BZ treatment in 66.6% and 53.3% of the total child population, respectively, even though the children remained seropositive at this time point. This suggests that these biomarkers could predict parasitological cure earlier than conventional tests or that some children might not require a full 60 days of BZ treatment. Additional studies are needed to continue the validation of these new biomarkers; in particular, the kinetics of the disappearance of the fragments following treatment needs to be further evaluated. Serum samples collected at different time points during treatment, as well as between treatment and seroreversion, should give us a better idea of their potential to determine cure earlier than conventional serology.

Moreover, a test or multiplex allowing the testing of more samples from adults and children at a higher throughput is needed before moving toward clinical use of ApoA1 and FBN fragments as biomarkers of parasitological cure for Chagas disease.

Overall, our data strongly suggest that ApoA1 and FBN fragments, together with ApoA1 full-length protein, are valid markers to be considered for discriminating infected from uninfected samples in clinical settings, and for the assessment of treatment efficacy in Chagas patients. Immunoblot results matched the detection of fragments by MS, which suggests that the immunoblot assay derived from our proteomics studies is good enough to evaluate CD patients and determine parasitological cure. This is a successful translation of proteomic-based studies into accessible tools for bench diagnosis.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Acknowledgments

The authors wish to thank Louise Burrows for the editing of this manuscript.

Author contributions. E.R.L., E.C., and M.N. contributed to the literature search, data analysis, and initial manuscript drafting. E.R.L., A.R., and M.N. contributed to data collection and figures. E.C., F.B., S.M., G.M., and J.A. contributed to sample collection. E.C., F.B., S.M., G.M., J.A., and M.N. contributed to study design. E.R.L., E.C., F.B., S.M., G.M., J.A., and M.N. contributed to data interpretation. F.B., S.M., G.M., and J.A. contributed to review of the manuscript. All authors approved the final version to be published and agreed to be accountable for all aspects of the manuscript.

Financial support. This study was done in collaboration with the Drugs for Neglected Diseases *initiative* (DND*i*) organization. For the work described in this paper, DND*i* received financial support from the Department for International Development/United Kingdom and Médecins Sans Frontières/Doctors Without Borders (MSF/DWB)/International. The National Reference Centre for Parasitology is supported by Public Health Agency of Canada/National Microbiology Laboratory grant WPG-6-39147 (005), the Foundation of the Montreal General Hospital, The Foundation of the McGill University Health Centre, and the Research Institute of the McGill University Health Centre.

Potential conflicts of interest. E.C. works for DND*i*. All authors declare no competing interests. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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