Microbiology and Immunology

Microbiol Immunol 2012; **56**: 836–842
doi:10.1111/j.1348-0421.2012.00513.x

ORIGINAL ARTICLE

Prevalence, molecular characteristics and risk factors for cryptosporidiosis among Iranian immunocompromised patients

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ABSTRACT

Cryptosporidium spp. **is a major cause of diarrhea in developing countries, mainly affecting people with compromised immune systems in general and HIV-infected individuals with low CD4 + T-cell counts in particular. This infection is self-limiting in healthy persons; however, it can be severe, progressive and persistent in those who are immunocompromised. There are few published studies concerning cryptosporidiosis and *Cryptosporidium* genotypes in Iranian immunocompromised patients and none of them describe risk factors. This study was undertaken to identify prevalence, genotypes and risk factors for cryptosporidiosis in immunocompromised patients. Three fecal samples were obtained at two day intervals from each of the 183 patients and processed with modified Ziehl–Neelsen staining methods and 18S rRNA gene amplification and sequencing. The overall infection prevalence was 6%. *Cryptosporidium parvum* was identified in isolates from five HIV-infected patients, one patient who had undergone bone marrow transplantation and one with chronic lymphocytic leukemia. *Cryptosporidium hominis* was identified in isolates from two HIV-infected patients and two patients with acute lymphocytic leukemia. According to univariate analysis, the statistically significant factors were diarrhea (OR = 21.7, CI = 2.83–78.4, $P = 0.003$), CD4 + lymphocytes less than 100 cells/mm^3 (OR = 41.3, CI = 13.45–114.8, $P < 0.0001$), other microbial infections (OR = 7.1321.7, CI = 1.97–25.73, $P = 0.006$), weight loss (OR = 73.78, CI = 15.5–350, $P < 0.0001$), abdominal pain (OR = 10.29, CI = 2.81–37.74.4, $P = 0.001$), dehydration (OR = 72.1, CI = 17.6–341.5, $P < 0.0001$), vomiting (OR = 4.87, CI = 1.4–16.9, $P = 0.015$), nausea (OR = 9.4, CI = 2.38–37.2, $P < 0.001$), highly active antiretroviral therapy (OR = 0.089, CI = 0.01–0.8, $P = 0.015$) and diarrhea in household members (OR = 7.37, CI = 2.04–26.66, $P = 0.001$). After multivariate analysis and a backward deletion process, only < 100 CD4 + T-lymphocytes/mm^3 maintained a significant association with infection. The authors recommend that this infection should be suspected in patients with diarrhea, weight loss and dehydration in general and in diarrheal individuals with < 100 CD4 + T-lymphocytes/mm^3.**

Key words cryptosporidiosis, immunodeficiency, Iran, prevalence.

Since human infection with *Cryptosporidium* (*C*) was first documented in 1976 (1), this protozoon parasite has been recognized worldwide as a major cause of gastro-enteritis-like syndromes in humans (2). *Cryptosporidium*

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Received 19 April 2012; revised 24 July 2012; accepted 12 September 2012.

List of Abbreviations: ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; C, *Cryptosporidium;* CD, cluster of differentiation; CI, confidence interval; CLL, chronic lymphocytic leukemia; HAART, highly active antiretroviral therapy; NHL, non-Hodgkin's lymphoma; OR, odd's ratio; WHO, World Health Organization.

was identified as a "neglected pathogen" by the WHO in 2004 (3). The disease it causes ranges in seriousness from mild to severe and the signs and symptoms depend on the site of infection and nutritional and immune status of the host. In patients with intact immune systems, cryptosporidiosis is self limiting; however, infection in immunocompromised patients, particularly those infected by HIV and those who have developed AIDS, can be fatal (4, 5). There is no effective and specific medication for cryptosporidiosis. It is clear that an intact immune system is the main factor that limits this infection (6).

Evidence is also emerging that the clinical picture may vary with the infecting species. At least eight of the currently identified 20 *Cryptosporidium* species and seven of the more than 40 genotypes have been detected in humans; however, some of these may have been incidental findings (7). Those currently considered human pathogens include *C. hominis, C. parvum, C. meleagridis, C. felis, C. canis* and the *Cryptosporidium* rabbit genotype (8). *C. parvum and C. hominis* are the major species of *Cryptosporidium* that affect humans. However, unusual species and genotypes can induce infection in specific groups, including both immune-competent and immune-compromised populations (9).

It is now well known that people with compromised immune systems have a higher risk of *Cryptosporidium* infection and that carriage of this parasite is associated with diarrheal diseases in most cases.(9) Furthermore, the disease is much more severe and prolonged in patients with diarrhea than in otherwise healthy individuals. There is good evidence that risk of fecal carriage, severity of illness and development of unusual complications of cryptosporidiosis are directly related to T-cell immune deficiency, particularly decreased CD4 + lymphocyte counts (4).

Cryptosporidiosis can affect all segments of the gastrointestinal tract (10, 11). Since microscopic examination cannot accurately identify *Cryptosporidium* genotypes, molecular tools are essential for detecting and differentiating *Cryptosporidium Spp.* Such identification in turn informs our understanding of transmission routes and the health-related implications of various species and genotypes (8, 12).

A number of factors prompted us to carry out the present study. They included the increasing use of immunosuppressive agents in solid organ transplant recipients and cancer patients, the overwhelming number of HIV/AIDS patients in Iran (a United Nations Joint Project on HIV/AIDS/WHO report estimated the number of individuals living with HIV as 86,000 in 2007, which is approximately double that in 2001) (13), the limited knowledge about the prevalence of *Cryptosporidium* species in immunocompromised patients and the risk factors for infection in this group.

MATERIALS AND METHODS

Study subjects

This study was conducted at Ali-Asghar and Seyed-Alshohada hospitals, Isfahan city, Isfahan province, central Iran from November 2008 to March 2009. Both of these hospitals are major central referral centers to which many patients from other areas of Iran are referred. In all, 183 immunocompromised patients were included in this study. Eligibility criteria were immunosuppression due to HIV infection (with decreased white cell counts), hematological malignancies and use of immunosuppressive drugs after solid organ transplant or for treatment of chronic or intractable hematologic diseases. The ethics committee of Baqiyatallah University of Medical Sciences approved the study protocol. After informed written consent had been obtained, the study nurse administered a comprehensive questionnaire to each patient. This author-compiled checklist included items on patient variables including age, sex and weight; sociodemographic and intra-familial factors; location of dwelling; occupation; number of household members with diarrhea; zoonotic factors including exposure to pets and farm animals; and environmental factors including source of drinking water and exposure to lake, river or swimming pools. Clinical characteristics including diarrhea, weight loss, vomiting, abdominal pain and nausea, presence of concomitant microbial infections, antiretroviral use and laboratory characteristics including CD4 + T-cell counts were recorded. This checklist was filled out by a physician who confirmed patient's symptoms by physical examination and so on. Diarrhea was defined as three or more watery or loose stools in a 24-hour period. Diarrhea that persisted for more than two weeks was considered chronic; otherwise, it was classified as acute. Weight loss was considered significant when referred patients lost more than 10% of their baseline body weight during their hospitalization.

Specimen collection and processing

Three fecal samples were collected at two days intervals from each patient and placed in a disposable plastic cup. The samples were taken immediately to the laboratory and stored at -20°C until analysis. The fecal specimens were concentrated using a sucrose solution with a specific gravity of 1.200 at a centrifuge speed of $800 \times g$ for 10 mins. All samples were stained by the modified Ziehl-Neelsen method and examined under bright field microscopy. A

sample was considered *Cryptosporidium* positive if typical oocysts 4–6 μm in diameter were visible.

DNA extraction

Fecal samples were subjected to six cycles of freeze–thaw in liquid nitrogen and a 95°C water bath to rupture the oocysts. DNA was isolated from aliquots of frozen stool using the QIAamp DNA stool minikit (Qiagen, Gaithersburg, MD, USA) according to the manufacturer's instructions.

18S rRNA gene amplification and sequencing

A two-step nested PCR protocol was used to amplify the 18S rRNA gene (830 bp). The fragment of the 18S rRNA gene was amplified by PCR using the following primers: 5′-TTCTAGAGCTAATACATGCG-3′ and 5′-CCCATTTCCTTCGAAACAGGA-3′ for primary PCR and 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′ and 5′-AAGGAGTAAGGAACAACCTCCA-3′ for secondary PCR. For the primary PCR step, a PCR mixture containing 1 × PCR buffer, 3 mM $MgCl_2$, 0.2 mM each dNTP, 2.5 U Taq, 2.5 μL of BSA (0.1 g/10 mL) and 1 μM for each forward and reverse primer in a total of 50 μL reaction volume was used. A total of 35 cycles, each consisting of 94°C for 45 s, 59°C for 45 s, and 72°C for 1 min, were performed; an initial hot start at 94°C for 3 mins and a final extension step at 72°C for 7 mins were also included. For the secondary PCR step, the PCR mixture was identical except that a concentration of 1.5 mM $MgCl_2$ was used. A total of 40 cycles, each consisting of 94°C for 30 s, 58°C for 90 s, and 72°C for 2 mins, were performed; an initial hot start at 94°C for 3 mins and a final extension step at 72°C for 7 mins were also included. PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide staining. The PCR products were purified using the terminator V3.1 cycle sequencing kit (Applied Biosystems, Foster, CA, USA). Sequences were assembled using the SeqMan program (DNASTAR, USA).

Statistical analysis

The characteristics of study participants are presented as mean and percentage. As appropriate, Student's *t*-test was used to compare the means of continuous variables, whereas categorical variables were compared using Fisher's exact test or Pearson's X^2 test. A logistic model was used to assess any association between potential risk factors and *Cryptosporidium spp*. infection; $P < 0.02$ according to univariate analysis was considered significant and is presented with the OR. Wald's test was used to assess the significance of variable associations. Correlations between exposure and outcome that considered possible confounding variables were evaluated by multivariate analysis by means of a logistic regression model. Only variables with $P < 0.05$ on Wald's test were included in the multivariate model; a backward deletion process was used. Analyses were carried out using computer software SPSS ver.12 (SPSS, USA). For both univariate and multivariate analyses, associations were considered significant at $P < 0.05$.

RESULTS

We studied 183 immunocompromised patients. Their medical conditions were HIV infection in 47 (25.7%), ALL 43 (23.5%), AML 13 (7.1%), CLL 18 (9.8%), various solid cancers 22 (12%), NHL 11 (6%), post-bone marrow transplant 13 (7.1%) and post-renal transplant 16 (8.7%). One hundred and fifty one patients (82.5%) were male and 32 (17.5%) female. The majority of patients (72.7%) were over 30 years old, non-diarrheic (87%), had CD4 + T-cells counts > 100 cells/mm^3 (93.4%) and were urban dwellers (76%). We considered patients had *Cryptosporidium* infection if their fecal samples contained typical oocysts of 4–6 μm when examined after using a modified acid-fast staining technique. We identified oocysts of *Cryptosporidium* in the feces of 11 of the 183 patients (6%). Demographic, environmental and clinical characteristics of the studied patients are shown in Table 1. We identified two genotypes, *C.parvum and C.hominis*, by 18s rRNA gene amplification, sequencing and analysis.

We identified *C. parvum* in isolates from five HIV-infected patients, one patient who had undergone bone marrow transplantation and one with CLL and *C.hominis* in isolates from two HIV-infected patients and two patients with ALL (Table 2).

The age of *Cryptosporidium* infected patients ranged from 29 to 54 years, with a mean of 40.8 ± 0.5 years. Most patients were male (81.8%); of the two infected female patients one had HIV and the other had received a bone marrow transplant. We identified concurrent microbial infections in 5 of 11 patients, all of whom were HIV positive. The mean number of CD4 + T-lymphocytes (cells/mm^3) in *Cryptosporidium* infected individuals was 228.7 ± 1.8; only four HIV positive patients had <100 cells/mm^3 ($P < 0.0001$) (Table 2).

Results of univariate analysis are shown in Table 3. We found significant correlations between *Cryptosporidium* infection and CD4 + cell counts < 100 cells/mm^3 ($P < 0.0001$); diarrhea in household members ($P < 0.002$) and concomitant microbial infections ($P < 0.006$). In addition, the presence of diarrhea ($P < 0.003$), weight loss ($P < 0.0001$), abdominal pain ($P = 0.001$), dehydration ($P < 0.0001$), vomiting ($P < 0.015$) and nausea

Table 1. Demographic, environmental and clinical characteristics of the study population and *Cryptosporidium* infected patients

	Factor	Number (%)	*Cryptosporidium* infected (%)	*P*-value
Age (years)	<30	50 (27.3)	1 (2)	0.432
	≥30	133 (72.7)	10 (75)	
Sex	Male	151 (82.5)	9 (6)	0.465
	Female	32 (17.5)	2 (6.2)	
Diarrhea	Yes	24 (13)	10 (41.7)	<0.001*
	No	159 (87)	1 (0.6)	
Type of diarrhea	Acute	7 (29.2)	3 (42.8)	0.506
	Chronic	17 (70.8)	8 (47)	
CD4 + cell count (no/mm^3)	<100	12 (6.6)	4 (33)	<0.002
	≥100	171 (93.4)	7 (4)	
Other microbial infections	Yes	23 (12.6)	5 (21.7)	0.019
	No	160 (87.4)	6 (3.7)	
Weight loss	Yes	11 (6)	8 (73)	<0.0001
	No	172 (94)	3 (1.7)	
Abdominal pain	Yes	32 (17.6)	7 (21.9)	0.021
	No	159 (81.4)	4 (2.5)	
Dehydration	Yes	14 (7.6)	8 (57.2)	0.0002
	No	169 (92.4)	3 (1.8)	
HAART	Yes	25 (53.2)	1 (4)	<0.003
	No	22 (46.8)	7 (31.8)	
Fever	Yes	65 (36)	4 (6)	1
	No	118 (64)	7 (6)	
Vomiting	Yes	40 (22)	6 (15)	0.034
	No	143 (78)	5 (3)	
Nausea	Yes	46 (25)	8 (17)	0.026
	No	137 (75)	3 (2)	
Contact with pet or farm animals	Yes	38 (20.8)	2 (5.3)	0.560
	No	145 (79.2)	9 (6.2)	
Exposure to lake, river or swimming pool water	Yes	15 (2.7)	2 (13.3)	0.072
	No	168 (97.3)	9 (6.2)	
Type of drinking water	Tap water	157 (85.8)	7 (4.4)	0.055
	Well water	26 (14.6)	4 (15.4)	
Diarrhea in household members	Yes	40 (2.2)	7 (17.5)	0.042
	No	143 (97.8)	4 (2.8)	
Location of dwelling	Urban	139 (76)	8 (5.7)	0.058
	Rural	44 (24)	3 (6.8)	

*, *P*-values in bold are statistically significant.

($P = 0.001$) were significantly predictive of cryptosporidiosis (Table 3).

We found no significant association with age, sex, type of diarrhea, fever, contact with pet or farm animals, exposure to lake, river or swimming pool water, type of drinking water and location of dwelling (Table 3). For the multivariate analysis, we used cryptosporidiosis as the main outcome and the significant variables according to univariate analysis after assessment by the Wald test as explanatory variables. Patients with cryptosporidiosis had a higher risk of developing diarrhea, weight loss and abdominal pain. Most risk factors showing individually significant associations with cryptosporidiosis become non-significant when included in a multivariate model. Exclusion of these factors from the model one at a time did not affect its coefficients, as confirmed by the likelihood ratio test. The best fitting model was the variable 'diarrhea of household members' versus 'CD4 + cell count < 100 cells/mm^3)' (likelihood ratio test 34.52; 1 d.f.; $P < 0.0001$). Table 4 shows the model with two variables and Table 5 the final model with only one variable. Only 'CD4 + <100 cells/mm^3)' maintained a significant association with infection.

DISCUSSION

We found that *Cryptosporidium* infection was present in 14.9% of patients with AIDS/HIV, 4.6% with ALL,

Table 2. Genotypes of isolates and clinical and epidemiological features of patients with *Cryptosporidium* infection

Isolate code	Source	Age	Sex	Genotype	Immunological status	CD4 + cell count (no/mm^3)	Other microbial infections
P1	AAH	36	Male	*C. hominis*	HIV+	82	*Giardia lamblia, Candida albicans*
P2	AAH	49	Male	*C. parvum*	HIV+	44	*Cytomegalovirus*
P3	AAH	33	Male	*C. parvum*	HIV+	140	NDc
P4	AAH	50	Male	*C. parvum*	HIV+	179	*Entamoeba coli, Chilomastix mesnilli*
P5	AAH	43	Male	*C. hominis*	HIV+	88	*Blastocystis hominis*
P6	AAH	44	Female	*C. parvum*	BMT	490	None
P7	SAH	33	Female	*C. hominis*	ALL	321	None
P8	SAH	42	Male	*C. parvum*	CLL	655	None
P9	PTCs	29	Male	*C. hominis*	ALL	320	None
P10	PTCs	36	Male	*C. parvum*	HIV+	65	*Cytomegalovirus,* hepatitis B virus
P11	PTCs	54	Male	*C. parvum*	HIV+	132	ND

AAH, Ali-Asqhar hospital; BMT, bone marrow transplantation; ND, not determined; PTCs, private therapeutic centers; SAH, Seyed-Alshohada hospital.

Table 3. Univariate analysis of potential risk factors for *Cryptosporidium* infection among immunocompromised patients

Risk factor	Odds ratio	95% CI	χ^2	*P*-value
Age	0.25	0.03–2.01	1.96	0.145
Sex	0.95	0.19–4.62	0.004	0.605
Diarrhea	21.7	2.83–78.4	16.97	<0.003
Type of diarrhea	0.84	0.14–4.97	0.35	0.605
CD4 + cells <100 cells/mm	41.3	13.45–114.8	32.16	<0.0001
Other microbial infections	7.13	1.97–25.73	11.52	0.006
Weight loss	73.78	15.5–350	70.1	<0.0001
Abdominal pain	10.29	2.81–37.74	17.27	0.001
Dehydration	72.1	17.6–341.5	65.7	<0.0001
Fever	1.04	0.29–3.69	0.004	0.593
Vomiting	4.87	1.4–16.9	7.32	0.015
Nausea	9.4	2.38–37.2	14.1	0.001
HAART	0.089	0.01–0.8	6.41	<0.015
Contact with pet or farm animals	0.84	0.17–4.05	0.04	0.591
Exposure to lake, river or swimming pool water	2.72	0.53–13.92	1.55	0.224
Type of drinking water	3.89	1.05–14.4	4.71	0.053
Diarrhea in household members	7.37	2.04–26.66	11.9	0.001
Location of dwelling	0.83	0.21–3.29	0.06	0.518

*, *P*-values in bold are statistically significant.

Table 4. Multivariate analysis of the association between risk factors and *Cryptosporidium* infection: the model with two variables

Risk factor	Odds ratio	95% CI	Regression coefficient	SE	*P*-value
CD4 + cell count (no/mm^3)	20.4	2.8–65.3	2.3	0.6	0.0005
Diarrhea in household members	4.2	1.2–11.6	0.7	0.3	0.19

Table 5. Multivariate analysis of the association between risk factors and *Cryptosporidium* infection: final model

Risk factor	Odds ratio	95% CI	Regression coefficient	SE	*P*-value
CD4 + cell count (no/mm^3)	27.6	2.8–78.4	3.2	0.5	0.0000

5.5% with CLL and 7.7% of bone marrow transplant patients, with an overall prevalence of 6% in this sample of immunocompromised patients in Iran. There are few published studies concerning *Cryptosporidium* infection in Iranian immunocompromised patients. Nahrevanian *et al*. reported *Cryptosporidium* infection in 8.7% of AIDS patients and 2.3% of patients with hematological malignancies, with an overall 1.4% prevalence in immunocompromised patients attending 10 health centers in Iran (14). Zali *et al*. identified *Cryptosporidium* infection in 7% of HIV positive individuals in a study aimed at determining the prevalence of parasitic pathogens in this patient group (15). In another study reporting molecular characterization of *Cryptosporidium* isolated from humans and animals in Iran, Meamar *et al*. identified *Cryptosporidium* in 8 out of 15 isolates from AIDS patients, seven of which they identified as *C.parvum* and one as *C.hominis* (18).

Berenji *et al*. conducted a study in pediatric patients with lymphatic and hematological malignancies in Mashhad (center of Khorasan Razavi province, north-west Iran) hospitals and detected 22% *Cryptosporidium* infections

overall, with a prevalence of 19% in patients with ALL, 2% with AML and 1% with NHL (16). In a case-control study, Sharif *et al*. identified 5% *Cryptosporidium* infections overall, including in 3% of patients with ALL, 1% of those who had received bone marrow transplants and 1% with NHL (17). Using 18s rRNA gene amplification and sequencing, Meamar *et al*. evaluated the prevalence of *Cryptosporidium* genotypes in HIV-positive and -negative patients and identified that 88.9% of HIV infected individuals were infected with *C. parvum* and 11.9% with *C. hominis*, whereas in HIV negative patients 62.5% were infected with *C. parvum* and 37.5% with *C. hominis* (18). Thus, the reported prevalence of *Cryptosporidium* infection in Iranian immunocompromised patients ranges between 1.5% and 22% with a mean of 7%. It is well documented that, in the Middle East, *C. parvum* is the dominant species both in immunocompetent and immunocompromised individuals (15, 19, 20). In the present study, we found no sex difference in the frequency of cryptosporidiosis. However, patients older than 30 years had a higher risk of this infection. Similar age related increases in *Cryptosporidium* infection have previously been reported (21), but this may be because there are few immunocompromised patients younger than 30 years. In relation to the clinical features of *Cryptosporidium* infection, we found that diarrhea, weight loss, abdominal pain, dehydration, vomiting and nausea were significantly associated with *Cryptosporidium* infection. Manabe *et al*. and a review by Hunter *et al*. have also reported a high prevalence of these clinical symptoms (4, 22).

In some studies, *C. hominis* was associated with diarrhea, nausea, vomiting and general malaise, whereas *C. parvum* and other species were associated with diarrhea only (7). However, in the present study we found no differences between *Cryptosporidium* genotypes in severity of clinical manifestations, which is possibly because all study patients were immunosuppressed. Other microbial infections occurred more frequently in *Cryptosporidium* infected patients, particularly in those with HIV. Immunesuppression, especially when advanced, is a major risk factor for existence of co-pathogens in these individuals (4, 22). After backward deletion of significantly associated risk factors for cryptosporidiosis according to univariate analysis, only two factors remained significant: CD4 + T-cell counts < 100 cells/mm^3 and diarrhea in household members. Only CD4 + T-cell counts < 100 cells/mm^3 reached statistical significance in multivariate analysis as a predictor of the risk of cryptosporidiosis.

It is clear that CD4 + T-lymphocytes are necessary for resolution of cryptosporidiosis. The risk of *Cryptosporidiosis* in immunosuppressed patients correlates with CD4 + T-lymphocytes counts (23, 24). In the present study, the majority of infections occurred in HIV positive individuals (63.3%), of whom 57% had CD4 + T-lymphocytes counts < 100 cells/mm^3. The evidence indicates that *Cryptosporidium* does not pose a particular risk to cancer patients in general. The exception to this rule seems to be leukemia and other hematological malignancies (25, 26). The severe disease seen in bone marrow transplant patients usually appears to depend on and reflect the underlying diagnosis for which the transplant was performed (4).

The introduction and use of HAART for immune reconstitution has dramatically reduced the incidence of cryptosporidiosis in HIV/AIDS patients. However, HAART is still not widely available in many non-industrialized countries, where cryptosporidiosis remains an important emerging disease (2).

In conclusion, the results of this study indicate that the presence of *Cryptosporidium* may be high among HIV infected patients, patients with hematological malignancies (especially ALL and CLL) and in bone marrow transplant patients, living in Isfahan province, central Iran; however, evaluation of immunocompromised patients in other areas is required. In addition, cryptosporidiosis is more likely to be present in patients with particular signs and symptoms, such as diarrhea, weight loss, and dehydration. Moreover, we recommend that patients with CD4 + T-lymphocyte counts < 100 cells/mm^3 be assessed for cryptosporidiosis. Our overall recommendation is to consider cryptosporidiosis as a cause of diarrhea in HIV infected patients and patients with CD4 + T-lymphocyte counts < 100cells/mm^3. Additional precautions, including avoiding contact with diarrheal individuals among their household members, may help to prevent fecal-oral transmission.

ACKNOWLEDGMENTS

We would like to acknowledge all who collaborated in this study, especially the patients who provided specimens.

DISCLOSURE

The authors declare that they have no conflicts of interest related to this study.

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