Human Cellular Immune Response Against 
*Giardia lamblia* 5 Years After Acute Giardiasis

Kurt Hanevik,1,3 Einar Kristoffersen,2,4 Staffan Bruserud,1 Emma Ringqvist,5 Steinar Sørnes,1 and Nina Langeland1

1Institute of Medicine and 2Gade Institute, University of Bergen, 3Centre for Tropical Infectious Diseases, Department of Medicine, and 4Department of Immunology and Transfusion Medicine, Haukeland University Hospital, Bergen, Norway; and 5Department of Cell and Molecular Biology, Uppsala University, Sweden

**Background.** Clinical and epidemiological studies have suggested the development of acquired immunity in individuals previously infected with *Giardia lamblia*. However, there are no data on the long-term cellular immunity and genotype cross-reactivity. An outbreak of assemblage B giardiasis in a nonendemic area made it possible to evaluate the long-term cellular mediated immunity and its specificity toward the 2 *Giardia* assemblages known to infect humans.

**Methods.** Peripheral blood mononuclear cells from 19 individuals infected with *Giardia* assemblage B 5 years previously and from 10 uninfected controls were cultured with antigens from assemblage A and B *Giardia* trophozoites for 6 days. Cell-mediated immunity was measured by a 3H-thymidine proliferation assay and flow cytometric analysis of activation markers HLA-DR, CD45RO, CD25, and CD26 in T-cell subsets.

**Results.** Proliferation responses were significantly elevated in the group previously exposed to *Giardia* for nearly all *Giardia* antigens tested. Individual responses toward *Giardia* trophozoite whole cell, cytosolic, and excretory-secretory antigens from both assemblages correlated well. Activation marker responses were mainly seen in CD4 T cells.

**Conclusions.** *G. lamblia* infection induces long-term, albeit variable, cellular immune responses that are not assemblage specific and that are largely driven by CD4 T-cell activation.
giardiasis-related intestinal mucosal injury [13]. Important roles have also been shown for mast cells and interleukin (IL) 6 [14], as well as for B-cell antibody production [15, 16]. An ex vivo study of human intestinal lymphocytes stimulated by Giardia trophozoites showed the importance of interferon (IFN)–γ, probably secreted by CD4 T cells [17].

Mice challenged with a second Giardia infection shed far fewer cysts than were shed during the primary infection [18]. In humans, adults are less affected than children, and specific antibodies against Giardia are found in populations in endemic areas [2, 19]. Residents in Creston, Canada, who were infected in a waterborne Giardia outbreak in 1985 were significantly less likely to be reinfected during a second outbreak 5 years later [20]. There are therefore strong indications that acquired immunity toward this noninvasive luminal-dwelling parasite is important for protection. Except for 1 case report 6 years after infection [21], there are no data on the long-term cellular immunity after acute giardiasis or on immunologic cross-reactivity between assemblages. A better understanding of the development and persistence of Giardia-specific immunity in humans and of the methods to assess these events are needed in efforts to develop a protective vaccine [22, 23].

A G. lamblia assemblage B waterborne outbreak [24] occurred in 2004 in Bergen, Norway, a nonendemic area [25]. The circumstances offered a rare opportunity to evaluate the presence of long-term cellular immunity from a single, well-documented infection. In the present study, peripheral blood mononuclear cells (PBMCs) from Giardia-infected individuals, 5 years after clearance, were tested for reactivity toward antigen fractions and purified antigen from the 2 human-infecting assemblages A and B. The results support a long-term cellular immune response toward Giardia that is not assemblage specific and that is largely driven by CD4 T-cell activation.

METHODS

Patients and Controls
A total of 19 healthy individuals with microscopy-confirmed G. lamblia infection during the Bergen outbreak 5 years previously [25] were recruited. None of these individuals had confirmed or suspected giardiasis at any time before or after their outbreak-related infection. All had been successfully treated with 1 or 2 courses of metronidazole. This group was termed the exposed group. Ten individuals without known previous Giardia infection were recruited as unexposed controls. All controls except one had not travelled in Giardia-endemic areas, and none had been exposed to contaminated water during the outbreak in Bergen. All were free from gastrointestinal symptoms. Exposed individuals and controls were screened by microscopic examination and 18S polymerase chain reaction [26] of feces to rule out ongoing giardiasis. All study participants were previously vaccinated against tuberculosis with BCG vaccine.

Antigens
Giardia antigens were obtained from cultures of Giardia reference strains WB-C6 (ATTC 50803, assemblage A) and GS/M (ATTC 50581, assemblage B) grown in TYDK medium (Diamond’s TYI-S-33 supplemented with bile according to the methods of Keister [27]). Trophozoites were collected, washed 4 times in cold phosphate-buffered saline (PBS), and then resuspended in 5 mL PBS and freeze-thawed twice before sonication (1 minute at 20 W) as described previously [5]. Membrane and cell fragments were removed by centrifugation for 10 minutes (×14 000 g). This gave the following antigen preparations: an excretory-secretory fraction, consisting of growth medium from the trophozoite culture at the time of harvesting the trophozoites (unused medium was used as a control); a whole-cell fraction, consisting of sonicated trophozoites before centrifugation; and a cytosolic fraction, consisting of sonicated trophozoites supernatant after centrifugation (washed and diluted TYDK medium was used as control).

Recombinant glutathione S-transferase (GST)–tagged α-1-giardin was expressed in and purified from Escherichia coli as described in Palm et al [16]. Mock-purified fraction from E. coli with empty GST-tag vector was used as a control. All antigen preparations were measured for their protein content by means of BCA protein assay kit (Pierce), diluted in X-vivo 15 medium to the relevant concentrations for lymphocyte stimulation assays, and kept frozen until use.

As antigen controls, we used Salmonella typhimurium lipopolysaccharide (LPS) (19516), Klebsiella pneumoniae LPS (L1519 [Sigma-Aldrich]), sterile filtered Candida albicans protein extract (403 skin prick test [Allergopharma]; 10 000 BU/mL), and tuberculin purified protein derivate (PPD) (Statens Serum Institut).

PBMC Acquisition and Culture
We isolated PBMCs by density-gradient separation from peripheral blood samples drawn in BD Vacutainer Na-citrate CPT tubes (Becton Dickinson). After PBMCs were harvested, they were washed twice in PBS with 1% bovine serum albumin before cells were dissolved in X-vivo 15 serum-free culture medium supplemented with 1-glutamin, gentamicin, and phenol red (BioWhittaker). The freshly isolated PBMCs (10⁵ cells/well; 200 μL medium per well) were cultured in 96-well U-bottom microtiter plates in X-vivo 15 medium. Cells were cultured in the presence of Giardia antigens, control antigens, or medium alone for 6 days at 37°C in a humidified atmosphere of 5% CO₂. All cultures were prepared in triplicate.

Proliferation Assay
Cellular proliferative responses were measured by the incorporation of ³H thymidine in newly synthesized DNA, using a conventional assay [28]. After 5 days of culture, the cells were pulsed with 37 kBq/well of ³H thymidine (Amersham International) and harvested 18 hours later onto glass-fiber pads. The amount of DNA-incorporated radioactivity was determined...
by liquid scintillation counting in a β-counter. Proliferation was
determined as counts per minute (cpm). As positive prolifera-
tion controls, we used pokeweed mitogen (L8777 [Sigma-
Aldrich]), anti-CD3 (1.5 mg/mL mouse immunoglobulin E
moab; CLB-T3/4.E [Central Laboratory, Netherlands Red
Cross Blood Transfusion Services [NRCBTS])], and anti-
CD28 (2 mg/mL mouse immunoglobulin G1 moab; CLB-
CD28/1 [Central Laboratory, NRCBTS]) at final dilutions of
1:500 for anti-CD3 and 1:250 for anti-CD28.

The results were expressed as stimulation indices (SIs), in
which responses in stimulated cells are divided by the response
for corresponding unstimulated cells. The median (SD) un-
stimulated cell responses were low, at 526 (577) cpm and 450
(355) cpm in the exposed and unexposed groups, respectively,
indicating no significant difference between groups (P = .64).

Flow Cytometric Analysis
The late activation markers HLA-DR and CD25, the recall an-
tigen activation marker CD26, and the memory cell marker
CD45RO have been described in flow cytometric analysis of
specific cell-mediated immunity toward viral and bacterial an-
tigens [29–32]. To characterize further the long-term cellular
immune response to Giardia, we examined the presence of these
activation markers in the CD4 and CD8 T-cell subsets of cul-
tures stimulated with 10 μg/mL Giardia A and B cytosolic
fractions, 1 μg/mL S. typhi LPS, and 10 μg/mL tuberculin PPD.

The triplicate cell cultures were pooled, mixed, and washed
once in PBS with 1% fetal calf serum. Cell suspensions (50 μL)
were then stained for 30 minutes in the dark with combinations
of 5 fluorescent dyes to identify T-cell subsets: CD3-ECD
(Beckman Coulter), CD8a-FITC and CD4-PerCP/Cy5.5 (BioL-
egend), and the activation markers CD45RO-PE and HLADR-
PE/Cy7 or CD26-PE and CD25-PE/Cy7 (BioLegend). Relevant
isotype controls were obtained from the same manufacturer and
used at the same concentrations. After staining, cell suspensions
were washed once and resuspended in PBS-paraformaldehyde
solution (1%) and analyzed the same day, using a Beckman
Coulter Cytomics FC 500 MPL flow cytometer equipped with an
argon diode laser for 5-color detection. In a typical acquisition,
7 × 10^4 lymphocytes (range, 2.3 × 10^4 to 1.7 × 10^5 lympho-
cytes) were collected. The collected data were analyzed with
FlowJo 7.6 software (Tree Star).

Statistical Analysis
Stimulation indices obtained by the proliferation and flow cy-
tometric analyses were compared between the groups, using the
Mann–Whitney U test. A P value of <.05 was used to define
statistical significance.

Ethical Considerations
The study was approved by the Regional Committee for Ethics
in Medical Research and the Norwegian Social Society Data
Services in Bergen. Written informed consent was obtained from
the participants.

RESULTS
Baseline characteristics of the 19 exposed individuals infected
with Giardia 5 years previously did not differ significantly from
those of the 10 unexposed controls regarding age, sex, and
baseline routine peripheral blood leukocyte, monocyte, or
lymphocyte cell counts (Table 1).

Proliferation Assay
PBMCs were stimulated with Giardia trophozoite sonicate
fractions, an excretory-secretory fraction, or a purified antigen
and measured for proliferation responses by 3H-tymidine DNA
incorporation (Table 2). For the whole cell–antigen fractions,
the responses were generally stronger for stimulation with as-
semble A, compared with assemble B. Both assemble antigen
antigens gave significantly stronger responses in the exposed
group, compared with the unexposed control group.

Responses toward the cytosolic fractions were tested in
4 different protein concentrations (ie, 62.5 μg/mL, 25 μg/mL,
10 µg/mL, and 4 µg/mL), and the titration curves were similar for both assemblages (data are shown for concentrations of 10 µg/mL). In some individuals, the response to the stronger concentration of the cytosolic fractions were weaker than the response to the more diluted antigen, indicating the possible presence of toxic or inhibitory components in both assemblages that limited the response at the higher concentrations.

The excretory-secretory fractions were tested in 3 different concentrations (ie, 1250 µg/mL, 250 µg/mL, and 50 µg/mL) and gave a striking difference between assemblages. The assemblage B–derived excretory-secretory components gave responses in the exposed group that were significantly stronger than those in the unexposed group at all 3 concentrations tested, while there were no differences between groups at any concentrations for assemblage A antigens (data are shown for concentrations of 250 µg/mL).

Although at a group level responses were significantly weaker in the control group compared with the exposed group, variations between individuals were large, with a strong response in one control individual and weaker or no response in some of the exposed individuals. However, as demonstrated in Figure 1, in each individual the responses to the various Giardia antigen fractions correlated well.

The recombinant α-1-giardin protein elicited relatively weak responses that did not differentiate between groups. The responses to various positive and negative antigen and culture medium controls were not different between the exposed and unexposed groups.

### Table 2. Proliferation Responses in 3H-Thymidine Assay After Stimulation With Different Giardia Antigens From Assemblage A or B, by Study Group

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Giardiasis 5 years previously (n = 19)</th>
<th>No known previous Giardia infection (n = 10)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Giardia A whole cell 10 µg/mL</td>
<td>22.3 (23.1)</td>
<td>10.9 (18.1)</td>
<td>.035</td>
</tr>
<tr>
<td>Giardia B whole cell 10 µg/mL</td>
<td>16.9 (19.3)</td>
<td>8.1 (11.4)</td>
<td>.027</td>
</tr>
<tr>
<td>Giardia A exsecretory 250 µg/mL</td>
<td>9.4 (8.0)</td>
<td>10.6 (5.6)</td>
<td>.946</td>
</tr>
<tr>
<td>Giardia B exsecretory 250 µg/mL</td>
<td>6.3 (6.2)</td>
<td>2.8 (8.0)</td>
<td>.008</td>
</tr>
<tr>
<td>TYDK medium 250 µg/mL</td>
<td>1.0 (0.3)</td>
<td>0.9 (0.2)</td>
<td>.512</td>
</tr>
<tr>
<td>Giardia A cytosolic fraction 10 µg/mL</td>
<td>12.8 (13.0)</td>
<td>3.5 (6.2)</td>
<td>.006</td>
</tr>
<tr>
<td>Giardia B cytosolic fraction 10 µg/mL</td>
<td>12.2 (13.9)</td>
<td>3.5 (4.7)</td>
<td>.001</td>
</tr>
<tr>
<td>TYDK cytosolic fraction control</td>
<td>1.0 (0.2)</td>
<td>1.1 (0.3)</td>
<td>.759</td>
</tr>
<tr>
<td>α-1-giardin 10 µg/mL</td>
<td>6.6 (3.9)</td>
<td>5.3 (1.9)</td>
<td>.357</td>
</tr>
<tr>
<td>E. coli control 10 µg/mL</td>
<td>7.9 (5.6)</td>
<td>6.5 (6.2)</td>
<td>.803</td>
</tr>
<tr>
<td>K. pneumoniae LPS 1 µg/mL</td>
<td>3.4 (2.5)</td>
<td>4.3 (1.6)</td>
<td>.588</td>
</tr>
<tr>
<td>C. albicans 10 µg/mL</td>
<td>7.1 (9.1)</td>
<td>11.0 (9.4)</td>
<td>.228</td>
</tr>
<tr>
<td>S. typhimurium LPS 1 µg/mL</td>
<td>21.5 (18.6)</td>
<td>17.7 (10.5)</td>
<td>.498</td>
</tr>
<tr>
<td>Tuberculin (PPD) 10 µg/mL</td>
<td>26.3 (34.1)</td>
<td>27.7 (27.1)</td>
<td>.512</td>
</tr>
<tr>
<td>αCD3αCD28 (pos ctr)</td>
<td>70.6 (35.4)</td>
<td>60.1 (39.7)</td>
<td>.925</td>
</tr>
<tr>
<td>Pokeweed mitogen (pos ctr) 5 µg/mL</td>
<td>65.4 (43.1)</td>
<td>90.9 (35.3)</td>
<td>.339</td>
</tr>
</tbody>
</table>

Data are median stimulation index (SD).

Abbreviations: αCD3αCD28; anti-CD3 and anti-CD28 antibodies C. albicans, Candida albicans; E. coli, Escherichia coli; K. pneumoniae, Klebsiella pneumoniae; LPS, lipopolysaccharide, pos ctr, positive control; PPD, purified protein derivative; S. typhimurium, Salmonella typhimurium.

* By the Mann–Whitney U test.

### Flow Cytometric Characterization

T-cell activation by Giardia antigens from the 2 human-infecting assemblages A and B were measured by flow cytometry, using cell surface markers (Figure 2). Bacterial LPS was used as an unspecific positive control and tuberculin as a positive control for the specific immune response against a purified protein antigen. Analyses of the percentage of activated lymphocytes by use of the combined markers CD45RO and HLA-DR and the combined markers CD25+ and CD26bright were the most accurate to differentiate between the exposed and unexposed groups. Analysis of the marker CD25 alone showed the same trend as the combined markers but with less strong differences between the groups, while CD45RO positivity alone did not differ between groups (data not shown).

By analyzing the presence of cells expressing CD25+CD26bright in the whole lymphocyte population after stimulation with Giardia antigens, we could see a significant difference between the exposed and unexposed groups for both assemblage A (P = .001) and B (P = .021) (Supplementary Table 1). Analysis of the whole lymphocyte population was not possible in a satisfactory way for the CD45RO and HLA-DR combination because of autofluorescence and interfering monocyte population.

Results from specific activation of the CD4 and CD8 T-cell populations are given for CD25CD26bright cells (Supplementary Table 1) and for HLA-DR+CD45RO+ cells (Supplementary Table 2). With both activation marker sets, the 10 µg/mL cytosolic fraction of assemblage A gave strong responses.
comparable to LPS and PPD positive controls and showed significant differences in CD4 cell activation between the exposed and unexposed groups ($P < .024$). The responses to the cytosolic fraction of assemblage B responses were generally weaker and did not seem to rise above the unspecific/cross-reacting background. Responses in the exposed group in CD8 and CD4 cells are illustrated in Figure 3.

**DISCUSSION**

In this study, we measured the strength and specificity of cell-mediated immunity toward various *Giardia* assemblage A and B antigens in individuals exposed to assemblage B infection 5 years previously. Also, we characterized the lymphocyte responses with activation markers to differentiate between CD4 and CD8 responses.

**Cytosolic and Excretory-Secretory Antigens**

Although the 2 human-infecting *Giardia* assemblages A and B have significant genomic differences, both showed a capacity to activate immune cells from individuals previously infected with assemblage B. The proliferation responses toward both assemblage antigens were variable but mostly correlated well in each individual (Figure 2). One unexposed control had strong responses against all *Giardia* antigens and may have had a previous undiagnosed *Giardia* assemblage A or B infection. This may also be true for some of the *Giardia* B–exposed individuals and other controls in this study and may account for some of the variability and cross-reactivity observed.

Ringqvist et al [33] identified 3 *Giardia* proteins (ie, arginine deiminase, ornithine carbamoyl transferase, and enolase) that were secreted in contact with human Caco-2 cells and that were previously shown to be immunodominant antigens for the humoral immune response in giardiasis [16]. These proteins and elongation factor 1-α [34] are to some extent also present in the excretory-secretory fraction of separately grown *Giardia* trophozoites. The difference observed between assemblages for the excretory-secretory fraction may indicate that there exists assemblage B–specific excretory-secretory protein(s) that can induce cellular immunity.

The recombinant *Giardia* cytoskeleton protein α-1-giardin, shown elsewhere to be an antigen for *Giardia*-specific humoral immunity [16], showed weak and nondifferentiating responses that were not different from the unspecific reactivity of the corresponding *E. coli* vector. It therefore does not seem to be inducing long-term cellular immunity, but it is possible that the *E. coli* expression has resulted in a misfolding that does not elicit proper immune responses.

![Figure 1](https://example.com/giardia.png)  
**Figure 1.** Correlation of stimulation indices in exposed individuals (●) and controls (○) in a proliferation assay for *Giardia* assemblage A and B cytosolic antigens. SI, stimulation index.
A study of Giardia cysts in Bergen sewage specimens before the outbreak showed assemblage A to be most prevalent [35]. Both exposed and unexposed participants may therefore have a similar background prevalence of assemblage A infection. One should also note that interassemblage variation can be large, especially in assemblage B [10], so that strain variations may be as important as assemblage-specific variations for the outcomes in this study.

On a similar note, Giardia parasites express only 1 of many variant surface proteins at any given time, and these are known to elicit a strong humoral host immune response [36]. With the assemblage-unspecific responses observed and the relatively crude antigens used in this study, the antigen components responsible for these responses are likely to be more preserved between assemblages. A previous study that used Giardia-specific T-cell hybridomas indicated that T-cell–stimulating antigens are expressed among different G. lamblia strains [37]. Further characterization and identification of the immunodominant proteins in trophozoite fractions could lead to several potential candidates for an assemblage-unspecific mucosal vaccine.

**Activation Markers**

On the basis of the proliferation assay of PBMCs, we expected to see significant differences between the exposed and unexposed groups with stimuli of both Giardia assemblages also in activation markers in the T-cell subpopulations. However, assemblage B antigen gave less than half the response compared with that of the LPS and PPD controls, which was too weak to mirror any previous Giardia exposure. Assemblage A antigen, on the other hand, elicited a close to maximal response, and a significant difference between exposed and nonexposed individuals was demonstrated, suggesting a detectable 5-year CD4 cell memory. Possibly, the assemblage B responses could have been picked up by other T-cell activation markers, it could contain immunomodulating components altering the response, or it may have induced proliferation in other cell populations than T cells. Thus, the difference between assemblages may indicate a qualitative difference between immune responses to different Giardia strains, or it could be an in vitro difference in which only assemblage A antigen elicits a sufficiently strong response to detect previous exposure, irrespective of the assemblage of the previous exposure. This poses an interesting focus for further studies.

When analyzing all lymphocytes for activated CD25<sup>+</sup>CD26<sup>bright</sup> cells, we found a significant difference between the 2 groups for both assemblages, although findings were still much more marked for assemblage A. CD26 is known to be induced by stimuli that favor development of a Th1 response, and CD26<sup>bright</sup> surface expression is also correlated with production of Th1-type cytokines, such as IFN-γ [38]. The strong and significant CD26<sup>bright</sup> CD4 T-cell responses induced by Giardia assemblage A antigens in the exposed group therefore are in line with the importance of CD4 T cells seen in previous research in mice [11, 12, 18] and humans [17, 21]. Because of its simplicity in analysis and ability to
The CD25/CD26 activation marker combination is promising for flow cytometric analysis of cell-mediated immunity toward Giardia and other pathogens, but it needs validation in future studies. The higher LPS-induced expression of markers HLA-DR and CD45RO in the CD8 T cells in the exposed group was unexpected and may be coincidental (Supplementary Table 2). However, it is tempting to speculate that previously described tight junction disruption and increased permeability during giardiasis have left the group previously exposed to Giardia with a stronger in vitro reaction toward LPS. It is also interesting to note that this group difference is only found in the CD8 subpopulation, which has been shown to be responsible for Giardia-induced villous atrophy and impaired disaccharidase activity in mice [13].

Cautionary Remarks

Used culture medium and sonicate of parasites are crude antigen mixes containing a large number of possible antigens. Cross-reactivity is therefore hard to avoid. When comparing assemblages, one should also know that assemblage A parasites grow faster than assemblage B parasites in culture, and we also noted this to occur in the preparation of Giardia antigen, in which assemblage A yielded protein concentrations that were 2–3-times higher than those for assemblage B cultures harvested at equal time points. Although protein concentrations were corrected for in the assays, assemblage growth rate differences may have contributed to variations in antigen composition, including in the excretory-secretory fraction.

Ebert [17] concluded that whole Giardia trophozoites induced mainly a mitogenic response in human intestinal lymphocyte cultures, and the presence of mitogenic components in antigen fractions in this study may be responsible for some of the background reactivity observed. Contamination of the TYDK culture medium with bacterial or viral antigens can also not totally be excluded, even if standard sterile techniques were implemented. The cross-reactivity observed in some of the assays may also be due to host immunity toward other pathogens or environmental antigens.

In conclusion, the data presented here show that G. lamblia infection may induce persistent cellular immunity lasting at least 5 years, with large interindividual variation and with cross-reactivity between the 2 human-infecting assemblages. The long-term Giardia antigen recognition mainly occurs in CD4 T cells. In efforts to develop a vaccine, further studies to better characterize the Giardia-specific, but assemblage- and strain-unspecific cellular immunity–inducing components of the Giardia parasite are warranted.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://www.oxfordjournals.org/our_journals/jid/).

Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.
Notes

Acknowledgments. We thank the staff at the hospital laboratory, the study participants, and especially Marita Wallevik, Trygve Hausken, Knut Arne Wensaas, Lucy Robertson, and Kristin Paulsen, for their cooperation, advice, and assistance in this project.

Laboratory work and data collection were done by K. H., E. R., and S. S. All authors participated in the planning, analysis, writing, and finalization of the paper and approved the final manuscript submitted.

Financial support. This work was supported by the Western Norway Regional Health Authority and the Institute of Medicine, University of Bergen. Data were analyzed and evaluated independently by the authors, without any interference from the funding institution.

Potential conflicts of interest. All authors: No reported conflicts.

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.

References