Protein Energy Malnutrition Decreases Immunity and Increases Susceptibility to Influenza Infection in Mice

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Background. Protein energy malnutrition (PEM), a common cause of secondary immune deficiency in children, is associated with an increased risk of infections. Very few studies have addressed the relevance of PEM as a risk factor for influenza.

Methods. We investigated the influence of PEM on susceptibility to, and immune responses following, influenza virus infection using isocaloric diets providing either adequate protein (AP; 18%) or very low protein (VLP; 2%) in a mouse model.

Results. We found that mice maintained on the VLP diet, when compared to mice fed with the AP diet, exhibited more severe disease following influenza infection based on virus persistence, trafficking of inflammatory cell types to the lung tissue, and virus-induced mortality. Furthermore, groups of mice maintained on the VLP diet showed significantly lower virus-specific antibody response and a reduction in influenza nuclear protein-specific CD8+ T cells compared with mice fed on the AP diet. Importantly, switching diets for the group maintained on the VLP diet to the AP diet improved virus clearance, as well as protective immunity to viral challenge.

Conclusions. Our results highlight the impact of protein energy on immunity to influenza infection and suggest that balanced protein energy replenishment may be one strategy to boost immunity against influenza viral infections.

Keywords. Nutrition; protein energy; malnutrition; influenza; immunity.
defined as an imbalance between food intake (protein and energy) and the body’s requirement to ensure the most favorable growth [9, 10]. PEM is the most fatal form of malnutrition in developing countries, with more than 150 million children <5 years suffering worldwide [11]. There are 2 major forms of PEM—kwashiorkar and marasmus. Although inadequate protein in the diet, even with a adequate caloric intake, is the major cause of kwashiorkar, consumption of insufficient protein and calories are known to be responsible for marasmus [12]. Few studies, however, have linked these 2 major forms of PEM to higher incidence of influenza infection and mortality [13, 14].

In this study, we used a mouse model to address the impact of PEM on influenza A virus infection. We evaluated the effect of 2 isocaloric diets supplementing distinct levels of protein energy (18%, adequate protein [AP]; 2%, very low protein [VLP]) on infection of weanling mice with either a laboratory strain (A/PR/8/34 [A/PR8]) or a 2009 H1N1 pandemic influenza virus (A/Mexico/4108/2009 [A/Mex]). Our findings demonstrate the deleterious impact of PEM on influenza virus disease and subsequent immune responses and furthermore show that supplementing protein energy can restore immune function and improve the outcome of influenza infections in the mouse model.

MATERIALS AND METHODS

Mice and Diets

Four week-old female C57BL/6 mice (Jackson Laboratory, Maine) were randomly assigned to isocaloric diets (Harlan Laboratories, Indianapolis, Indiana) containing either 18% (AP diet; TD09530) or 2% (VLP diet; TD09532) protein. Establishing the isocaloric diets, containing comparable levels of micronutrients, required balancing the energy source with carbohydrate levels in the VLP diet. The composition and caloric content for the diets used in the study are shown in Supplementary Table 1. Mice were pair fed to confirm comparable feed consumption. For protein resupplementation experiments, after 3 weeks of feeding respective experimental diets (AP and VLP) to mice, we supplemented an additional VLP group of mice with the AP diet (hereafter referred to as supplemented protein [SP] diet) and, 3 weeks later, investigated their response to influenza A virus infection. All animal research conducted in the present study was approved by the CDC-IACUC and was conducted in an AAALAC-accredited facility.

Viruses and Infection

Influenza viruses A/PR8 and A/Mex were propagated in 10-day-old embryonated chicken eggs and stored at −80°C. The mice were deeply anesthetized with 2,2,2-tribromoethanol in tert-amyl alcohol before intranasal inoculation with virus (25, 50, or 100 mouse infectious dose [MID50]) diluted in phosphate-buffered saline (PBS). The 50% MID50 was determined as described elsewhere [16]. As per CDC-IACUC guidelines, any mouse that lost >25% of its preinfection body weight was humanely killed.

Lung Virus Titer Estimation

Lungs collected at days 3, 6, 9, and 12 postinfection were homogenized in PBS and titrated in eggs to determine virus infectivity (limit of detection, 101.5 EID50/mL). Allantoic fluid from the inoculated eggs was added to wells containing 0.5% turkey red blood cells (RBCs) in PBS. Virus titers were calculated by the Reed and Muench method and are expressed as the mean log10 EID50/mL ± SEM [16].

Hemagglutination Inhibition Assay

Serum samples were treated with receptor-destroying enzyme (RDE) and tested for reactivity to viruses by the standard hemagglutination inhibition (HI) assay with 0.5% turkey RBCs as described elsewhere [17]. Briefly, 25 µL of PBS was added to a 96 well plate containing 25 µL of RDE-treated serum samples. Eight hemagglutination units of virus was added and incubated at room temperature for 30 minutes. Finally, 50 µL of 0.5% turkey RBCs in PBS was added and incubated at room temperature for 30 minutes. The serial dilution of serum showing complete inhibition of hemagglutination was recorded as the HI titer.

Histopathology

On day 6 postinfection, 3 mice per group were killed, and lungs were collected in 10% neutral buffered formalin. After 72 hours, the samples were transferred to 70% ethanol, sectioned, and stained with hematoxylin and eosin [18].

Flow Cytometry

Single cell suspensions were prepared from mouse tissues following influenza infection. Spleen and lung tissue were digested by treatment with type-I collagenase (1:4 dilution) and made into a single cell suspension in MACS buffer (500 mL phosphate-buffered saline, 2.5 mL fetal bovine serum, 2 mL EDTA), using a cell strainer (VWR, California). For analysis of NK cell and neutrophil infiltration in lung tissue, Alexa Fluor 700-anti-CD45, Pacific Blue-CD11b, FITC-anti-Ly6G, PerCP-Cy5.5-anti-CD3, and APC-Cy7-anti-NK1.1 (BD Biosciences, California) antibodies were used. Virus-specific CD8+ T cells and intracellular interferon γ (IFN-γ), after in vitro stimulation of 10⁶ splenocytes/well with 0.1 MOI of virus for 3 days, were analyzed using pentameric complexes of the PE-H-2Db-influenza A (A/PR8) NP 366–374 ASNENMETM (Proimmune, UK), Alexa Fluor 700-anti-CD8, PE-Cy7-anti-CD4, and APC-Cy5.5-anti-IFN-γ following the manufacturer’s recommendation. Approximately 10⁵ cells were acquired and analyzed on an LSRII flow cytometer (BD Bioscience, California). Data were analyzed with FlowJo software (Treestar, Oregon).
ELISA
Following the manufacturer’s protocol, serum leptin concentrations were measured using a mouse enzyme-linked immunoasorbent assay (ELISA) kit (GenWay Biotech, California). Briefly, samples and standards were added to antibody-coated strips, followed by a biotinylated-labeled antibody, streptavidin-HRP conjugate and, finally, a substrate and stop solution. The plate was read using a BioTek Synergy plate reader (BioTek, Vermont). Measured values were then converted to concentrations (pg/mL) based on standard curves.

Statistical Analysis
Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, California). The Student t test was used to analyze differences among treatments for serum leptin concentrations, virus titer, and flow cytometry. A 2-way analysis of variance (ANOVA) was used, in conjunction with the Bonferroni post-test, on all virus-induced morbidity data; data were presented as mean ± SEM. The Mann–Whitney U test was used to determine significance among antibody (HI) titers. The Logrank (Mantel-Cox) test was used to compare percent survival among groups of mice. All differences were considered statistically significant when the P value was ≤0.05.

Results
Low Protein Diet Enhances Virus-Induced Mortality in Mice Infected With Either a Laboratory Strain or 2009 H1N1 Influenza Isolate
Because PEM is associated with an increased risk of infections in children [5, 19], we used weanling mice to examine the effects of diet-induced PEM on influenza virus infection. First, on beginning the feeding regimen with either the AP or VLP diet, we found that feed consumption was comparable between AP and VLP groups of mice (data not shown). Inability to gain body weight and lower levels of serum leptin, 2 markers of PEM [20–22], were assessed over 4 weeks of feeding custom diets. As shown in Figure 1A, although the AP diet group of mice gained body weight, the VLP group of mice failed to achieve similar growth. The mean serum leptin concentration was significantly lower in the VLP group of mice compared with mice maintained on the AP diet (Figure 1B).

Next, to determine the outcome of PEM on susceptibility to influenza virus infection, we infected mice maintained on either the AP or VLP diet with sublethal doses of influenza A viruses (A/PR8, 50 or 100 MID50; A/Mex, 100 MID50) and assessed for virus-induced mortality and morbidity. Following viral infection, the feed consumption, despite transient decline for both the AP and VLP groups, was comparable (data not shown). Also, compared with preinfection, the leptin levels did not vary after infection of mice with either virus (data not shown). Interestingly, as shown in Figure 1C (left and middle panels), the VLP group of mice, in response to infection with A/PR8, showed higher mortality when compared with mice maintained on the AP diet. Similarly, when compared with mice fed with the AP diet, the VLP group of mice showed higher mortality in response to infection with A/Mex virus (Figure 1C, right panel).

Low Protein Diet Leads to Impairment in Virus Clearance and Enhanced Inflammatory Cell Recruitment in Lungs of Influenza A Virus-Infected Mice
The consequence of PEM on virus-induced mortality following administration of a sublethal dose of influenza-A virus prompted us to investigate its effects on virus clearance and antiviral response in the lungs. To address this, we examined virus titer in lung tissue homogenates of mice maintained on AP or VLP diets. As shown in Figure 2, virus titer, for both viruses, in the AP and VLP groups of mice was comparable at days 3 and 6 postinfection. Furthermore, mice maintained on the AP diet demonstrated a decline in virus titer by day 9 postinfection and efficiently cleared virus by day 12 postinfection. However, mice on the VLP diet, when compared to those on the AP diet, showed significantly higher virus titer for A/Mex at day 9 postinfection, and for both viruses at day 12 postinfection.

To address the influence of PEM on inflammation in the lungs, we performed histological analysis of lung tissues harvested on day 6 postinfection from the AP and VLP groups of mice. As shown in Figure 3A, both AP and VLP groups of mice demonstrated inflammatory lesions following infection with either A/PR8 or A/Mex viruses compared with PBS-treated mice. To quantitatively assess the differences in lung inflammation, we measured infiltration of neutrophils, a key cell type known to orchestrate inflammation in influenza-infected hosts [23, 24] and found that groups of mice maintained on the VLP diet had a significantly higher percentage of neutrophils in response to infection with either influenza virus compared with mice fed the AP diet (Figure 3B; left panel). The defect in virus clearance in lungs of mice maintained on the VLP diet also prompted us to investigate changes in lung natural killer (NK) cells, an innate immune cell type known to secrete interferon γ (IFN-γ) key antiviral cytokine in effector tissues during the early stage of infection [25, 26]. We found a significant reduction in the percentage of NK cells in lungs of the VLP diet group of mice when compared with the AP diet group (Figure 3B, right panel).

Low Protein Diet Leads to Decrease in Virus-Specific Antibody and NP-Specific CD8+ T Cells in Influenza A Virus-Infected Mice
Because we observed higher mortality, as well as impairment in viral clearance, we investigated the effects of PEM on adaptive
A, B. Very low protein (VLP) diet leads to reduced growth and a decrease in serum leptin concentration. Mice maintained on the adequate protein (AP) and VLP diets were assessed daily for percent change in original body weight (A) and serum leptin concentration (B) at 5 weeks after beginning the feeding regimen. A, Results are shown from 1 of 2 independent experiments and consists of 5 mice per group. B, Serum leptin concentration is shown from 6 mice in each group. Error bars represent mean ± SEM. The differences in mean body weights between the VLP and AP groups were statistically significant as follows: \( P < .001 \) for day 9 and \( P < .0001 \) for days 10–38. C, Mice on the VLP diet show increased susceptibility to influenza infection. Mice maintained on the AP or VLP diets were infected with either A/PR8 or A/Mex influenza and assessed for virus-induced mortality (percent survival). Data represent results from 3 independent experiments.

Figure 1. A, B. Influenza A virus–infected mice maintained on a very low protein (VLP) diet show a defect in viral clearance in the lungs. Lung tissues harvested from mice on the adequate protein (AP) or VLP diets, on days 3, 6, 9, and 12 postinfection (A/PR8, 50 MID\textsubscript{50}; A/Mex, 100 MID\textsubscript{50}), were homogenized and assayed for virus titer as described in Materials and Methods. Data represent values from 2 independent experiments with each experiment consisting of \( n = 6 \) lung tissues per group at each time point. Values represent mean ± SEM.
immunity to influenza infection. As shown in Figure 4A, in response to either A/PR8 or A/Mex infection, serum HI titer in the VLP diet fed mice, was significantly less than the AP diet fed group of mice at 30 days postinfection. To assess the effects of PEM on virus-specific CD8+ T-cell responses, we examined the kinetics (days 8, 15, and 30 postinfection) of influenza NP-specific CD8+ T cells in the spleen of AP and VLP diet fed mice infected with A/PR8. First, flow cytometric analysis of splenocytes harvested from naive mice maintained on VLP diet revealed a reduction in the total number of splenocytes (leukocyte, B-cell, and T-cell subsets) compared with the AP diet-fed group (Supplementary Table 2). Upon A/PR8 infection, the total numbers of splenocytes were increased in AP diet group of mice on day 30 postinfection compared with naive mice (Supplementary Tables 2 and 3). In contrast, mice fed the VLP diet and infected with A/PR8 failed to show any increase in either the splenocyte numbers or the proportion of B and T cells (Supplementary Tables 2 and 3). Notably, evaluation of B-cell and T-cell subsets in A/PR8 infected mice showed comparable percentages of T-cell and T-cell subsets between the AP and VLP diet fed mice but a significant decrease in the percentage of B cells (Supplementary Table 3). Importantly, splenocytes harvested from the A/PR8-infected VLP group of mice, showed a lower percentage of influenza NP-specific CD8+ T cells at days 8, 15, and 30 postinfection, when compared with mice maintained on the AP diet (Figure 4B) despite comparable percentages of T-cell subsets (Supplementary Table 3).

**Supplementing Protein Energy-Malnourished Hosts with the Diet Containing Higher Protein Level Modulates PEM-Associated Immune Deficits and Decreases Incidence of Influenza Virus-Induced Mortality**

Nutritional supplementation can be an effective strategy, either as a supportive or adjunct approach, for the promotion of disease prevention [27, 28]. Provision of adequately
balanced nutrition may help with modulation of immunity against infections [29, 30]. To test this hypothesis in our experimental findings of PEM-induced immune deficits and enhanced influenza disease outcome, after 3 weeks of feeding respective experimental diets to mice, we supplemented an additional VLP group of mice with the AP diet (SP diet; Figure 5A) and, 3 weeks later, investigated their response to influenza A virus infection. Because infection with both A/PR8 and A/Mex led to increased morbidity, mortality, and changes in markers of innate and adaptive immune responses, only A/PR8 virus infection was used for the following protein supplementation experiments. As shown in Figure 5B, the SP group of mice began to gain body weight as soon as their diet was changed. More importantly, after infection, the SP group showed a significant reduction in morbidity and improved survival when compared with the VLP group of mice (Figures 5C and 5D). At day 9 postinfection, lung virus titer in the SP group was significantly reduced compared with the VLP group of mice and, in fact, was comparable to that observed in the AP diet-fed mice (Figure 6A). Moreover, analysis of lung homogenates harvested from the 3 groups of mice on day 6 postinfection for production of IFN-γ showed that the SP group of mice had significantly higher levels when compared with the VLP group of mice (Figure 6B).

Next, we investigated the effects of protein supplementation on adaptive immune responses to influenza virus infection. As shown in Figure 7A (left panel), we found that numbers of influenza virus NP-specific splenic CD8+ T cells harvested on days 8 and 15 postinfection and restimulated in vitro, were significantly higher in the SP group compared with mice fed with the VLP diet. Both CD4+ and CD8+ T-cell subsets harvested from the SP group of mice on days 8, 15, and 30 days postinfection and restimulated in vitro secreted significantly higher levels of IFN-γ compared with the VLP group of mice (Figure 7A; middle and right panels). Finally, the serum HI titer in the SP group of mice on day 30 postinfection was significantly higher compared to that of mice fed the VLP diet (Figure 7B) but was comparable to that observed for the mice fed the AP diet. Taken together, these data indicate that protein supplementation of mice that had initially received a protein-deficient diet, restored the ability to elicit appropriate adaptive immune responses.

Figure 4. Mice maintained on the very low protein (VLP) diet show a decrease in influenza-specific hemagglutination inhibition (HI) antibody titer and NP-specific CD8+ T cells. A, Serum samples harvested from mice fed the adequate protein (AP) and VLP diets, at 30 days postinfection (A/PR8, 50 MID50; A/Mex, 100 MID50), were analyzed for HI titer by HI assay as described in Materials and Methods. B, Splenocytes harvested at days 8, 15, 30 days postinfection (A/PR8, 50 MID50) were analyzed for influenza NP-specific CD8+ T cells using flow cytometry as described in Materials and Methods. B, Shown is the percent of NP-specific CD8+ T cells. Values in panel A are combined from 2 independent experiments. Values in panel B represent mean ± SEM, n ≥ 3 per group.
DISCUSSION

PEM, particularly in developing countries, represents one of the most common forms of childhood malnutrition [5, 31]. PEM increases susceptibility to multiple infectious diseases [10, 32]. In this article, we examined the consequences of PEM on influenza A virus infection in mice. We found that infection of mice manifesting signs of diet (VLP)-induced PEM, with either a laboratory strain or a 2009 H1N1 pandemic virus, resulted in higher rates of virus-induced morbidity and mortality compared with mice that received adequate nutrition. The VLP group of mice also showed impaired virus clearance, as well as an increase in inflammatory cells in the lungs. In addition, the VLP group of mice demonstrated a significant decrease in adaptive immune functions, including production of virus-specific HI antibodies, influenza (NP)-specific CD8+ T cells, and IFN-γ-producing T cells.

Importantly, these effects could be reversed by supplementing additional protein in the diet of the VLP group of mice, which resulted in significantly improved immunity to influenza virus challenge and enhanced host survival. Taken together, our results demonstrate multiple immune deficits associated with PEM and emphasize an immune stimulatory role for protein supplementation during influenza virus infection.

Although other studies have established the impact of PEM on immunity to microbial infections [5, 6, 20, 32], very few studies have addressed the relevance of malnutrition, PEM especially, as a risk factor for seasonal influenza [33–35]. Bellei et al [33] found that influenza vaccine-induced antibody responses were poor in elderly subjects that presented malnutrition. Using an energy restriction diet in the mouse model of influenza infection, Ritz et al [34] showed that mice maintained on an energy-restricted diet exhibit increased morbidity, and a reduction in NK cell numbers and function. In our study, similar to the
Figure 6. Switching from the very low protein (VLP) diet to the adequate protein (AP) diet enhances antiviral innate immune response and promotes virus clearance. Groups of mice fed with the AP, VLP, or supplemental protein (SP) diet regimen were either infected with influenza virus (A/PR8) or administered with phosphate-buffered saline (PBS), as described in Materials and Methods. Lung homogenates were assayed for virus titer on days 6 and 9 postinfection (A) and interferon γ (IFN-γ) on day 6 postinfection (B), as described in Materials and Methods. Data in panels A and B represent results from 2 independent experiments and consists of lung tissue harvested from 6 mice at each time point. Values represent mean ± SEM.

Figure 7. Switching from very low protein (VLP) diet to adequate protein (AP) diet modulates virus-specific adaptive immunity. Groups of mice fed with the AP, VLP, or supplemental protein (SP) diet were either infected with influenza virus (A/PR8) or administered with phosphate-buffered saline (PBS), as described in Materials and Methods. Percent NP-specific CD8+ T cells, percent interferon γ (IFN-γ)+CD4+ T cells, and percent IFN-γ+CD8+ T cells on days 8, 15, and 30 postinfection in the spleenocytes (A) and hemagglutination inhibition (HI) antibody titer on day 30 postinfection in the serum (B) were analyzed as described in Materials and Methods. A, Data represent results from 2 independent experiments with 3–6 mice per group. B, Each symbol represents an individual mouse from 2 independent experiments consisting of 8 mice per group, and the horizontal line indicates the mean value for the group.
experimental approach previously described for studying effects of PEM on LCMV infection [20], we used experimental diets to address the impact of PEM on influenza infection. Of note, the protein level in the AP diet used in our study is 18%, which is within the range recommended by the US Department of Agriculture and the US Department of Health and Human Services for children up to 3 years of age [36].

Previous studies have demonstrated an increased viral burden in clinical settings and experimental models of malnutrition. After initiating highly active antiretroviral therapy, Wang et al [37] found a higher baseline HIV load and an association with malnutrition. In a mouse model of PEM, Pena-Cruz et al [7] found that, compared with a 20% protein diet, mice fed a 2% protein energy diet had significantly increased virus titer in the lungs following Sendai virus infection. We found that although the group of mice fed with the AP diet had essentially cleared virus by day 12 postinfection, the group maintained on the VLP diet still exhibited substantial levels (approximately \(10^8\)EID\(_{50}\)/mL) of virus in the lungs. This delay in virus clearance in the lung tissue of the VLP group of mice may be responsible for the increase in neutrophil infiltration in our studies, as also suggested by other studies [38, 39].

Previous studies have established roles for several cell types and soluble mediators in bridging non-specific innate response with pathogen-specific adaptive immunity by priming antigen specific B and T lymphocytes [40–42]. In our studies, we found a significant reduction in both virus-specific antibody titer and percent NP-specific CD8\(^+\) T cells in the VLP group of mice infected with A/PR8 virus. Mutations in the peptide sequence corresponding to H-2D\(^b\) CD8\(^+\) T-cell epitope (amino acid sequence; A/PR8-ASNENMETM and A/Mex, ASNENVEIM) (J.P. Patel and S. Gangappa, et al unpublished observations) of the NP protein in A/Mex virus limited the application of commercially available influenza NP-specific pentamer for tracking NP-specific CD8\(^+\) T cells in A/Mex infected groups of mice. However, similar to our results, Chatraw et al [20] found a reduction in frequency of LCMV-specific CD8\(^+\) T cells and CD8\(^+\) IFN-\(\gamma\) +T cells in spleens from groups of mice fed with a low protein diet. It is possible that, in the face of diet-induced PEM, antigen processing and/or dendritic cell subsets responsible for priming antigen-specific T cells, could be defective in number, trafficking, or function [41, 43]. Alternatively, in our model, in the VLP group of mice, CD4\(^+\) T cells required for either facilitating virus-specific antibodies or cytotoxic CD8\(^+\) T cells [44, 45] could be impacted by altered proliferation, function, or survival. Although the CD4\(^+\) T cells primed in mice fed the VLP diet showed a relative decrease in IFN-\(\gamma\) production, our experiments to determine possible defects in subsets of dendritic cells (conventional DC and plasmacytoid DC) in the lymph nodes did not show any striking differences between the AP and VLP groups of mice (data not shown).

What are the challenges for improving immunity to influenza in a PEM population? Similar to micronutrient deficiencies [46, 47], PEM impacts overall growth and development of individuals [5]. As a result, a number of immune deficits, as evident from our studies, could ensue and pose a threat by limiting an individual’s ability to mount appropriate host responses to influenza infection important for efficient viral clearance and recovery. Therefore, studies to define the non-compromising limits for PEM (moderate vs severe PEM) and assessment of immune responses to influenza infection could aid in designing appropriate interventions for overcoming PEM-specific immune deficits. Our studies, using an experimental model of “severe” PEM and influenza infection, identify multiple virus-specific immune deficits, and, more importantly, underscore the benefit of a nutritional intervention strategy for dealing with influenza in certain malnourished populations. Studies focused on influenza infection in a mild to moderate range of PEM, as well as evaluations of vaccine-specific responses in dietary protein supplemented hosts, remain promising areas for future investigations.

**Supplementary Data**

**Supplementary materials** are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). 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 members of the Immunology and Pathogenesis Branch in the Influenza Division, Centers for Disease Control and Prevention, Vic Veguilla and Jessica Belser in particular, for providing reagents and constructive comments on this research. 

*Financial support.* A. T. was supported by Emerging and Infectious Diseases/APHIS fellowship.

*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.

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