Impact of Antecedent Infections on the Antibodies against Gangliosides and Ganglioside Complexes in Guillain-Barré Syndrome: A Correlative Study

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Abstract

Background and Aims: Guillain-Barré Syndrome (GBS), an immune-mediated neuropathy, is characterized by antibodies against gangliosides/ganglioside complexes (GSCs) of peripheral nerves. Antecedent infections have been reported to induce antibodies that cross-react with the host gangliosides and thereby have a pivotal role in conferring an increased risk for developing GBS. Data pertaining to the impact of various antecedent infections, particularly those prevalent in tropical countries like India on the ganglioside/GSC antibodies is sparse. We aimed at exploring the association between six antecedent infections and the profile of ganglioside/GSC antibodies in GBS. Methods: Patients with GBS (n = 150) and healthy controls (n = 50) were examined for the serum profile of antibodies against GM1, GM2, GD1a, GD1b, GT1b, and GQ1b and their GSCs by ELISA. These antibodies were correlated with immunoreactivities against Campylobacter jejuni, Japanese encephalitis (JE), dengue, influenza, zika, and chikungunya infections. Results: The frequencies of antibodies against six single gangliosides (P < 0.001) and their GSCs (P = 0.039) were significantly higher in patients as compared to controls. Except for GT1b-antibody which was more frequent in axonal GBS, none of the other ganglioside/GSC antibodies correlated with the electrophysiological subtypes of GBS. Antecedent JE infection was significantly associated with increased frequency of antibodies against GD1a, GD1b, GT1b, and GQ1b. Antibodies against GSCs were not influenced by the antecedent infections. Interpretation: This study for the first time shows an association between antecedent JE infection and ganglioside antibodies in GBS. This finding reinforces the determining role of antecedent infections on ganglioside antibody responses and the subsequent immunological processes in GBS.

Keywords: Antecedent infections, autoantibodies, ganglioside complex, gangliosides, Guillain-Barré syndrome, Japanese encephalitis virus

Introduction

Guillain-Barré Syndrome (GBS), an immune-mediated neuropathy, is the commonest cause of neuromuscular paralysis. Antecedent infection with Campylobacter jejuni has been identified as the predominant risk determinant of GBS. In addition, a number of other infectious pathogens, such as Mycoplasma pneumoniae, Epstein Barr Virus (EBV), Dengue virus, Chikungunya virus, Hemophilus influenzae, Cytomegalovirus (CMV), Zika virus, and the severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) have been associated with increased risk of GBS.[1-5] However, there exist some variations in the patterns of association between these pathogens and the risk of developing GBS.[1-5] Contrary to the earlier studies, in our recent study, the Chikungunya virus was the most common infectious trigger of GBS, followed by C. jejuni infection.[6] It is noteworthy that not all individuals infected with these pathogens develop GBS. For example, only 1 in 1,000 individuals with C. jejuni infection develops GBS. This suggests that infectious triggers alone are not sufficient to drive the underlying pathogenetic processes in GBS.

The infectious pathogens potentially interact with the host immune cells and immune molecules and lead to the development of GBS.[7] One of the most widely recognized mechanisms through which C. jejuni causes GBS is ‘molecular mimicry’, i.e., cross-reaction between antibodies raised against pathogens and the gangliosides of peripheral nerves.[8,9] Antibodies against gangliosides and ganglioside complexes (GSCs) have been...
reported in GBS patients across various populations and they have been suggested to be the major drivers of the pathogenic processes as well as the severity of GBS. However, the repertoire of autoantibodies targeting the peripheral nerves is rather heterogeneous in GBS. Notably, the patterns of distribution of the ganglioside- and GSC- antibodies are not uniform across different populations. The precise factors that contribute to the variations in the frequencies of these antibodies in GBS are not well understood.

There is a growing recognition that the type of these antibodies, as well as the magnitude of their production in GBS, may depend on the burden of infectious pathogens in a population. Further, specific genes in these pathogens may also play a role in determining the induction of various ganglioside antibodies.

A positive correlation between preceding infections and the profile of ganglioside antibodies has been reported by previous studies in the French, Japanese, and Chinese populations. These studies focussed mainly on the association between C. jejuni, and to some extent on M. pneumoniae and CMV, and the induction of ganglioside antibodies. However, these studies are limited and they do not provide adequate insights into the causal relationship between the spectrum of preceding infections and antibodies against the individual gangliosides/ GSCs in GBS. As such the associations between all the major risk pathogens that are prevalent across various geographical territories and the ganglioside/GSC antibodies have not been tested. In tropical countries, arboviral infections such as chikungunya, dengue, Japanese Encephalitis (JE), etc. are more prevalent and they have also been linked to the risk of developing GBS. We have reported the association between JE, dengue, and chikungunya virus infections and the risk of GBS in the Indian population. However, the impact of such preceding arboviral infections on the ganglioside- and GSC-antibody profile is not known. To address these knowledge gaps, this study was aimed at exploring the association between six infections and antibodies against gangliosides and GSCs in patients with GBS.

**Subjects and Methods**

**Study participants**

The present study was conducted on patients with GBS admitted to the emergency services of a single neurology unit of the National Institute of Mental Health & Neurosciences (NIMHANS), Bangalore, India. Adults (age ≥ 18 years) fulfilling the National Institute of Neurological Disorders and Stroke (NINDS) diagnostic criteria were enrolled for the study. Patients who received treatment with intravenous immunoglobulin and/or underwent plasmapheresis prior to the study entry were excluded. Nerve conduction studies were carried out in all patients using standard protocols, and subtyping was done based on the criteria recommended by Rajabally et al. Healthy community controls were recruited from the blood donation camps organized by the Department of Transfusion Medicine and Haematology, NIMHANS. The patients and controls were matched for age, gender, and ethnicity. The study was approved by the Institute Ethics Committee [No. NIMH/DO/Ethics Sub-committee (BS&NS) 5th Meeting/2017, dated 13.6.2017]. All the study participants provided written informed consent prior to their participation in the study.

**Collection of blood samples**

Ten ml. of peripheral blood was drawn from the median cubital vein under aseptic conditions, of which 5 ml. into sterile Becton Dickinson (BD) serum vacutainers and the remaining 5 ml. into EDTA vacutainers. The serum and the EDTA tubes were centrifuged at 3000 rpm for 12 minutes to separate the serum and plasma, respectively. The serum and plasma samples were aliquoted and stored at -80°C until the immunoassays were performed and they underwent only one freeze and thaw cycle.

**Profiling of antibodies against gangliosides and ganglioside complexes**

A manual and validated Enzyme-Linked Immune Sorbent Assay (ELISA) was employed to determine antibodies against single gangliosides and GSCs in the sera of patients (n = 150) and controls (n = 50). The selection of ganglioside antigens was based on (i) homology in molecular architecture with the lipo-polysaccharides (LPS) in C. jejuni, and (ii) their abundance in peripheral nerves. Assessment of antibodies against six gangliosides (GM1, GM2, GD1a, GD1b, GT1b, GQ1b) and 15 GSCs (GM1 + GM2, GM1 + GD1a, GM1 + GD1b, GM1 + GT1b, GM1 + GQ1b, GM2 + GD1a, GM2 + GD1b, GM2 + GT1b, GM2 + GQ1b, GD1a + GD1b, GD1a + GT1b, GD1a + GQ1b, GD1b + GT1b, GD1b + GQ1b, and GT1b + GQ1b) were performed. The detection of anti-ganglioside and anti-GSC antibodies was accomplished by horseradish peroxidase (HRP) labelled anti-human antibody, which was visualized by a color-shifting substrate reagent and was read spectrophotometrically. A commercial ganglioside autoantibody detection kit (Bühlmann Laboratories AG, Schönenbuch, Switzerland) was utilized for standardizing the manually developed assay. Positive controls from the kit were used for assay validation and quality assurance.

To assay antibodies against single gangliosides, each individual ganglioside was dissolved in ethanol (0.2 μg/50 μl) and was added to the wells of the ELISA plate, while for antibodies against GSCs, two (0.1 μg each) gangliosides were mixed in a microwell and left for approximately 30 minutes. The plates were kept at 37°C for several minutes for drying and complete evaporation of ethanol. Thereafter, 50 μl of the blocking solution [1% Bovine Serum Albumin (BSA) in Phosphate-Buffered Saline (PBS)] was added to each well and was allowed to stand for 30 minutes at room temperature. The blocking solution was removed from the microwells, and the serum sample diluted (1:40) with 1% BSA in PBS was added to each well (50 μl/well) and was left to stand for 90 minutes at room temperature. The plate was washed three times with 300 μl of 0.1% BSA in PBS. Following this, HRP-conjugated secondary antibody diluted with 1% BSA in PBS was added to the wells (50 μl/well) and the plate was left for 90 minutes.
at room temperature. The plate was washed again with 0.1% BSA in PBS three times. Subsequently, 100 µL of OPD substrate solution (ortho-phenylenediamine dihydrochloride dissolved in 0.1M citrate-phosphate buffer) was added and the plate was left at room temperature for 2 minutes. The color reaction was stopped by the addition of 50 µL of 8N H₂SO₄. The optical densities (ODs) of the reactions were read with an ELISA plate reader at 490 nm, and the OD values were corrected by subtracting the OD of a well that was not coated with gangliosides (blank control) to obtain the ganglioside autoantibody reactivity.

The OD value 0.1 was used for defining the threshold level of seroreactivity. An OD ≥0.1 was considered seropositive for ganglioside antibodies and the OD <0.1 was considered seronegative. Seroreactivity to GSCs (e.g., GSC X + Y comprising gangliosides X and Y) was considered as ‘anti-X + Y autoantibody positive’ when the OD of anti-X + Y autoantibody was higher by 0.2 than the OD of anti-X or anti-Y autoantibody. When a serum sample showed both anti-X and anti-Y autoantibody reactivities, the serum was considered anti-X + Y autoantibody-positive only when the OD value of the anti-X + Y autoantibody was higher compared to the sum of the anti-X and anti-Y autoantibodies.

**Detection of antecedent infection**

The IgM antibody capture (MAC) micro-ELISA kit was used to detect *C. jejuni* antibodies in the sera (MyBioSource, San Diego, California, USA). Serum IgM antibodies to JE virus, dengue virus, and chikungunya virus were detected using the ELISA kits manufactured by the National Institute of Virology (NIV, Pune, India) and these findings have been recently published.[9] Besides this, in the current study, we examined seroreactivities to influenza and zika viruses in the patients with GBS (n = 150) and control subjects (n = 150). For the detection of the influenza virus, throat/nasal swabs samples were collected from the study participants. The QIAamp Viral RNA Mini Kit (Qiagen) was used for the extraction and purification of viral RNA from throat/nasal swabs. Molecular detection of influenza virus RNA was carried out by real time RT-PCR using a Center for Disease Control and Prevention (CDC, USA) standardized protocol. For the detection of the Zika virus, total RNA was extracted from the plasma samples using QIAamp® Viral RNA Mini kit. A real-time PCR assay standardized by the CDC, USA, was used for the qualitative detection of zika virus.

**Statistical analysis**

Statistical analyses were performed using SPSS-27 for Windows (SPSS Inc., Chicago, Illinois, USA). *P* <0.05 was considered to be statistically significant. Gaussian distribution of the variables. The profile of antibodies against the single gangliosides and GSCs was compared between the patient and control groups using the Chi-square test. Further, study participants were stratified into two groups based on the presence or absence of ganglioside/GSC antibodies. The associations of ganglioside/GSC antibodies with electrophysiological subtypes as well as immunoreactivity to infectious pathogens were tested using the Chi-square or Fisher’s exact test. Benjamini-Hochberg correction at α% was applied to control the false discovery rate (FDR).

**Results**

**Clinical and demographic profile of the study participants**

The cohort comprised 97 men (64.7%) and 53 women (35.3%) with GBS. In the control group, there were 30 men (60.0%) and 20 women (40.0%). The median age at the time of study entry was 37 years (IQR = 27 to 47 years) and 36.5 years (IQR = 30 to 43 years) in the patient and control groups respectively. Thus, the patient and control groups were matched for age and also for gender (*P* = 0.55 and 0.87, respectively). The median duration of GBS was 6 days (IQR = 4 to 10 days). Antecedent infections reported by patients included fever (n = 18, 12%), acute gastroenteritis (n = 18, 12%), and respiratory infection (n = 9, 6%). The Hughes disability scale (HDS) score at the time of study entry was 1, 2, 3, 4, and 5 in 1 (0.7%), 14 (9.3%), 44 (29.3%), 90 (60.0%) and 1 (0.7%) patient, respectively. Twelve patients (8%) eventually developed respiratory muscle weakness and required mechanical ventilation. Based on the criteria of Rajabally et al.,[21] there were 67 (44.7%), 43 (28.7%), 33 (22.0%), 5 (3.3%), and 2 (1.3%) patients with axonal, primary demyelinating, equivocal, inexcitable and normal electrophysiology, respectively.

**Autoantibodies against single gangliosides**

The frequency of autoantibodies against all the studied gangliosides was significantly higher among patients with GBS than in the controls (*P* < 0.001). GM1 autoantibody was the most common (80%), whilst GQ1b autoantibody was the least common (53.3%) among patients with GBS [Table 1].

**Antibodies against GSCs**

In the present cohort, 43 patients (28.7%) had autoantibody positivity for any one of the tested GSCs. Autoantibodies against GSCs consisting of GM1 as one of the components were the most common [Figure 1]. None of the control subjects had autoantibodies against GSCs.
Correlation between ganglioside/GSC antibodies and electrophysiological subtypes

The IgM autoantibodies against single gangliosides were compared between the two major electrophysiological subtypes of GBS viz. demyelinating (n = 43) and axonal (n = 67) [Table 2]. A significantly higher frequency of GT1b autoantibody was noted in the axonal (n = 54, 80.6%) as compared to demyelinating (n = 27, 62.8%) subtype of GBS (P = 0.039). However, there were no differences in the frequencies of GSC autoantibodies between the axonal and demyelinating subtypes of GBS in the present cohort.

Correlation between ganglioside/GSC autoantibodies and infection immunoreactivities

The data pertaining to the antecedent infections were taken from our recently published article. Zika virus RNA was detected neither among patients nor in healthy controls. Two patients and none of the healthy controls tested positive for influenza virus RNA. The association of IgM immunoreactivity against the six tested pathogens namely C. jejuni, JE virus, dengue virus, chikungunya virus, influenza virus, and zika virus and antibodies against gangliosides and GSCs were examined. Except for preceding JE virus infection, none of the other pathogens showed a statistically significant association with antibodies against the single gangliosides. GBS patients with evidence of preceding JE virus infection exhibited significantly higher frequency of autoantibodies against GD1a (P = 0.001), GD1b (P < 0.001), GT1b (P = 0.008), and GQ1b (P = 0.008) gangliosides [Table 3]. Notably, none of the preceding infections showed any association with antibodies against GSCs.

Discussion

It has long been understood that GBS is a post-infection autoimmune disease of the peripheral nervous system. Nevertheless, the immunological process underlying GBS pathogenesis still is an enigma in a substantial number of patients. Antecedent infections and ‘molecular mimicry’ between antibodies against infectious pathogens and the host gangliosides are recognized as the key underlying mechanism of GBS. Efforts have been made to delineate the interactions between antecedent infections and antibodies against gangliosides, but the previous studies were limited only to a few pathogens. Besides, there exists a lack of clear understanding regarding the association between antecedent infections and antibodies against gangliosides as well as GSCs.

In the present study, the prevalence of antibodies against the six tested gangliosides was found to be in the order of GM1>GM2>GT1b>GD1a>GQ1b. GM1 autoantibody was the commonest and was observed in majority (80%) of the patients. Similar to the present study, GM1 antibody was reported to be common in GBS patients in several other populations, including Korean, Spanish, and Chinese. It is important to note that the presence of GM1 antibody has been associated with the severity and prognosis of GBS. Autoantibody against GM1 from patients with GBS was observed to impede voltage-gated calcium channels (Ca$^{2+}$v). This leads to neuromuscular weakness due to Ca$^{2+}$ channel dysfunction in the motor nerve-endings in patients with GBS. It is interesting to note that 16% of the healthy controls in the present study had antibodies to at least one of the gangliosides. Previous studies have reported the presence of antibodies

### Table 1: Profile of antibodies against single gangliosides in Guillain-Barré syndrome

| Ganglioside Autoantibodies | GBS (n=150) | Controls (n=50) | Statistics ($\chi^2$) | P    |
|---------------------------|-------------|----------------|-----------------------|------|
| GM1 autoantibody          | 120 (80.0)  | 3 (6.0)        | 86.72                 | <0.001|
| GM2 autoantibody          | 117 (78.0)  | 2 (4.0)        | 85.21                 | <0.001|
| GD1a autoantibody         | 105 (70.0)  | 0              | 73.68                 | <0.001|
| GD1b autoantibody         | 107 (71.3)  | 1 (2.0)        | 72.57                 | <0.001|
| GT1b autoantibody         | 110 (73.3)  | 2 (4.0)        | 73.16                 | <0.001|
| GQ1b autoantibody         | 80 (53.3)   | 1 (2.0)        | 41.00                 | <0.001|
| Any ganglioside autoantibody | 142 (94.7)  | 8 (16.0)       | 123.76                | <0.001|

*GBS*: Guillain-Barré syndrome. *Numbers in parentheses represent percentages

### Table 2: Comparison of ganglioside antibody profile between demyelinating and axonal subtypes of Guillain-Barré syndrome

| Ganglioside Autoantibodies | Demyelinating GBS (n=43) | Axonal GBS (n=67) | Statistics ($\chi^2$) | P    |
|---------------------------|--------------------------|-------------------|-----------------------|------|
| GM1                       | 32 (74.4)                | 57 (85.1)         | 1.92                  | 0.165|
| GM2                       | 32 (74.4)                | 54 (80.6)         | 0.586                 | 0.444|
| GD1a                      | 28 (65.1)                | 50 (74.6)         | 1.44                  | 0.284|
| GD1b                      | 31 (72.1)                | 48 (71.6)         | 0.006                 | 0.959|
| GT1b                      | 27 (62.8)                | 54 (80.6)         | 4.27                  | 0.039|
| GQ1b                      | 27 (62.8)                | 33 (49.2)         | 1.93                  | 0.164|
| Positivity for any ganglioside autoantibody | 42 (97.7) | 62 (92.5) | 1.34                 | 0.247|

*GBS*: Guillain-Barré syndrome. *Numbers in parentheses represent percentages

### Table 3: Correlation of Japanese encephalitis IgM immunoreactivity and ganglioside antibodies in Guillain-Barré syndrome

| Ganglioside Autoantibodies | JE IgM immunoreactivity | Statistics ($\chi^2$) | P    |
|---------------------------|-------------------------|-----------------------|------|
|                          | Positive (n=60)         | Negative (n=90)       |      |
| GD1a                      | 51 (85.0)               | 54 (60.0)             | 10.71 | 0.001|
| GD1b                      | 55 (91.7)               | 52 (57.8)             | 20.21 | <0.001|
| GT1b                      | 51 (85.0)               | 59 (65.6)             | 6.69  | 0.008|
| GQ1b                      | 40 (66.7)               | 40 (44.4)             | 7.14  | 0.008|

*JE*: Japanese Encephalitis. *Numbers in parentheses represent percentages
against gangliosides in up to 15% of healthy individuals, who were considered to be ‘healthy controls’, but their prevalence in the general population is not known.\[11\,\text{[27]}

In the present study, GSC autoantibodies were detected in 28.7% of GBS patients (43/150), but in none of the control subjects. Autoantibody against the GM1 + GM2 complex was the most common anti-GSC antibody (n = 12, 8%) among patients. Similarly, in an earlier study from Italy, GSC autoantibodies were reported in 27% (17/63) of patients with GBS.\[28\] However, in an Italian GBS cohort, the most frequent anti-GSC antibody was against the GD1a + GD1b complex.\[29\] In a UK cohort of GBS, the frequency of anti-GSC antibodies was reported to be 21.7% (39/180).\[30\] Contrary to these studies, the prevalence of anti-GSC antibodies was found to be only 17% (39/234) in a Japanese cohort.\[31\] Thus, the distribution pattern of GSC autoantibodies varies across different ethnic groups. There exists a dearth of understanding on the individual as well as ethnic differences in anti-ganglioside and anti-GSC antibodies. It may be hypothesized that microbial exposures, being apparently unique to each individual and also to each ethnic group, may have some influence on the individual or ethnic differences in the autoantibody profiles. Besides, the profile of different ganglioside autoantibodies in GBS was reported to vary depending upon the technique used.\[32\,\text{[33]}\] Various studies have employed different types of immunoassays in profiling antiganglioside autoantibodies. Therefore, more precise information is required to explain the methodical and ethnic differences in anti-ganglioside and anti-GSC antibodies in GBS patients.

The antibodies against the individual gangliosides/GSCs were reported to be associated with the subtypes of GBS. In the present study, only the GT1b autoantibody was more frequent in the axonal subtype. The earlier studies demonstrated an association between the axonal form of GBS and GM1 and GD1a antibodies.\[16\,\text{[25]}\] In a large multi-centric study, GSC antibodies were associated with the axonal form of GBS.\[33\] However, in our study anti-GSC antibodies were not found to be associated with the electrophysiological subtypes of GBS.

The most salient finding in the current study was the association of antecedent JE virus infection with antibodies against GD1a, GD1b, GT1b, and GQ1b gangliosides in GBS patients. This is the first study showing an association between antecedent JE infection and ganglioside antibodies in GBS patients. Most of the previous studies focused on the association of C. jejuni, M. pneumoniae, CMV, and Epstein-Barr Virus. The current study focused on the association between the arboviral infections that are endemic to India and ganglioside antibodies. Of the studied pathogens, an association was observed between antecedent JE infection and ganglioside antibodies. It is noteworthy that no association between antecedent C. jejuni infection and antibodies against gangliosides was observed in the current study. Several studies reported an association between antecedent C. jejuni infection and ganglioside antibodies. Elevated titers of antibodies against GM1, GD1a, GD1b, and GQ1b were reported in GBS patients with antecedent C. jejuni infection in Japan.\[15\] Antibodies against GM1 and GD1a were reported to be associated with GBS developing after C. jejuni infection in the French population.\[16\] Other studies demonstrated an association between C. jejuni infection and GM1 antibodies.\[34\,\text{[36]}\] Interestingly, in a previous study, antibodies to C. jejuni were reported more frequently in GBS than controls (17.2% vs 7%) and antibodies against gangliosides such as GM1 and GD1b were present in 20% of patients with C. jejuni antibodies, while 9.6% of patients without C. jejuni antibodies also had anti-GM1 or anti-GD1b antibodies.\[37\] Thus, the findings on the association between antecedent C. jejuni infection and gangliosides antibodies are not consistent across studies. In addition, GM2 antibody was reported to be associated with CMV-associated GBS and GM1 antibody with M. pneumonia-associated GBS.\[36\] Notably, in the current study, antecedent infections were not found to be associated with any of the anti-GSC antibodies. In contrast, in an earlier cohort of GBS, an association between antecedent gastrointestinal infection with C. jejuni and antibodies against GSC was noted.\[30\] Studies on the association between antecedent infections and GSC-antibodies are albeit limited.

**Conclusion**

Infectious pathogens are the major risk determinants of GBS. The spectrum of microbial risk determinants of GBS is expanding, the two recently added viruses such as Zika and SARS-CoV-2 are examples of such an expansion. The functional interactions between infectious triggers and gangliosides/GSCs influence the risk, severity as well as prognosis of GBS. Given the determining role of pathogens on the risk of developing GBS, it is essential to identify their interacting immune partners and the subsequent pathophysiological trajectories. GT1b antibody was more frequent in the axonal variant of GBS in the current study. The association of antecedent JE infection with GD1a, GD1b, GT1b, and GQ1b antibodies in the present cohort of GBS provides additional insights into the role of antecedent infections in the immunobiology of GBS. This adds to the existing knowledge that besides C. jejuni, CMV, and M. pneumoniae, other pathogens also have the potential to influence the production of ganglioside antibodies. This may imply that the profile of infections antedating the onset of GBS in the tropics may differ from those in temperate regions. Further research is warranted in ethnically diverse populations with a larger number of gangliosides and GSC antigens and a wider spectrum of antecedent pathogens to obtain better insights into the functional interactions between infections and antibody responses.

**Declaration of patient consent**

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.
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Conflicts of interest
There are no conflicts of interest.

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