Original Article

Heterophilic Interference of Rheumatoid Factor in TSH Immunometric Assay: A Cross-Sectional Observational Study

Shaikh N. Nayeemuddin, Akash Panigrahi[1], Rana Bhattacharjee[2], Subhankar Chowdhury[3]

Department of Endocrinology, ESIC Medical College and Hospital, Hyderabad, Telangana, [1]Department of General Medicine, Tata Main Hospital, Jamshedpur, Jharkhand, [2]Department of Endocrinology, Medical College, Kolkata, West Bengal, [3]Department of Endocrinology and Metabolism, Institute of Post Graduate Medical Education and Research, Kolkata, West Bengal, India

Abstract

Introduction: Considering the inherent vulnerability of immunoassays for heterophilic interference and the potential of Rheumatoid Factor (RF) to act as a heterophile-like antibody, we conducted this study to investigate if RF leads to any such heterophilic interference in seropositive rheumatoid arthritis (RA) patients. The study was done on the TSH assay as it is a noncompetitive, double antibody sandwich assay, which is known to be vulnerable to heterophilic interference. **Methods:** In this cross-sectional observational study, eighty-four consecutive newly diagnosed RF-positive RA patients underwent TSH, Free T4, and anti-TPO estimation using the chemiluminescence technique (CLIA) on Siemens Immulite 1000 platform. The samples were screened for TSH interference using four methods: 1) analysis on a different platform, 2) assessment of linearity using doubling dilutions, 3) polyethylene glycol (PEG) precipitation, and 4) addition of a commercial blocker. **Results:** Ten samples had a loss of linearity on serial dilution, indicating potential interference. After heterophile blocker treatment, five cases exhibited interference. One patient had diagnostic interpretation discordance on the second platform. No sample on PEG precipitation suggested the influence of antibodies. It is worth noting that even in cases where interference was suspected, the clinical interpretation was largely unaffected by the correction of TSH values based on mean dilution or measurement after heterophile blocker treatment. **Conclusion:** RF can cause heterophilic interference in TSH immunoassays used commercially. However, in most cases, this interference does not affect clinical decision-making.

Keywords: Dilution, heterophile antibody, immunoassay, interference, rheumatoid arthritis, rheumatoid factor, TSH

Introduction

Antibody-based methods or immunoassays are the most widely used laboratory techniques in hormone assays. These methods rely on antibodies derived from animal sources to quantify different analytes. The affinity and specificity of these antibodies permit the accurate measurement of analytes present in extremely low concentrations even in complex and protein-rich solutions such as human serum. However, immunoassays are inherently vulnerable to interference from heterophilic antibodies and endogenous antibodies that bind to these assay antibodies.

Interfering heterophile antibodies are low-affinity antibodies with broad specificities, commonly directed against the Fc fragment and mostly found in patients without known exposure to animals from which the assay antibodies are derived.[1] This heterophilic interference may lead to falsely low or high analyte levels in immunoassay systems, depending on the interference site and type of assay. Immunometric assays are particularly more vulnerable to such interference. There have been numerous case reports of heterophilic interference in thyroid function tests,[2-15] with Thyroid Stimulating Hormone (TSH) as the most common analyte affected. Although currently, manufacturers routinely add blocking agents to their assay formulations, not all heterophile interference can be blocked as suggested by some case reports.

Rheumatoid Factor (RF) is found among 5–10% of the general population and in approximately 70% of Rheumatoid

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Address for correspondence: Dr. Rana Bhattacharjee, Associate Professor, Department of Endocrinology, Medical College, 88, College Street, Kolkata - 700 073, West Bengal, India.
E-mail: dr.r.bhatta@gmail.com

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Arthritis (RA) patients.[16] There is a significant homology between Fc-domains in RF antibodies and Fc-domains in antibodies from several animal species, with some previous studies suggesting they have common immunological origins.[17,18] Consequently, RF has the potential to interfere with immunoassays by binding to animal antibodies.[16] RF-positive RA individuals are an especially vulnerable group for heterophilic-like interference with TSH owing to a high prevalence of RA and an increased proportion of autoimmune thyroid disease in RA.[19]

As far as we know there has been no study evaluating the heterophile interference of TSH assay among patients with RA. It is pivotal to know whether the current TSH immunoassays are affected by RF because if not recognized, heterophilic interference would lead to unnecessary over or undertreatment in a large number of patients. The commonly used methods to detect interference are 1. Analysis on different platforms, 2. Assessment of linearity on doubling dilutions, 3. Depletion of antibodies to remove heterophilic antibodies from samples, and 4. Addition of blockers to the sample to neutralize interfering antibodies.[20] Our study aimed to determine if RF causes heterophilic interference in TSH immunoassays.

Materials and Methods

This was a single-center cross-sectional observational study conducted in the department of endocrinology in a tertiary care center in the city of Kolkata, India. RA patients were diagnosed as per the American College of Rheumatology (ACR)/ European League Against Rheumatism (EULAR) criteria 2010. Eighty-four consecutive patients above 16 years of age with newly diagnosed RF positive (RF more than 20 IU/L by nephelometry method) RA were selected from Rheumatology OPD of the same institute. All participants provided written informed consent, and the study was conducted in accordance with the Declaration of Helsinki. The study was approved by the Institutional ethics committee.

Patients taking drugs known to cause thyroid abnormalities (lithium, interferon alpha, amiodarone, or biological anti-rheumatic agents), with a history of partial or total thyroidectomy, and with a history of neck radiotherapy were excluded from the study. Pregnant individuals were also excluded. This was a pilot study with 84 individuals since there was no prior research on the prevalence of heterophilic interference with RF in TSH immunoassays. Demographic and clinical data were collected from participants according to a protocol, and they underwent measurements of RF, TSH, Free Thyroxine (free T4), anti-thyroid peroxidase (anti-TPO), and four tests to screen for interference in TSH assay.

Serum TSH, free T4, and anti-TPO and all tests for screening interference in TSH assay except the second platform method were estimated by chemiluminescence technique (CLIA) using commercially available kits from Siemens Diagnostics (Germany) on Immulite1000 platform. The analytical sensitivity (as provided by the manufacturers) for TSH, free T4, and anti-TPO were 0.01 μIU/mL, 0.3 ng/dL, and 7 IU/L, respectively. The laboratory reference ranges for TSH, Free T4, and anti-TPO were 0.4–4 μIU/mL, 0.8–1.9 ng/dL, and <35 IU/L, respectively.

The TSH test (3rd generation) on the Immulite-1000 was a two-site chemiluminescent immunometric assay (sandwich assay) using solid-phase beads coated with a capture antibody (monoclonal murine anti-TSH) and a detection antibody (polyclonal goat anti-TSH conjugated with bovine alkaline phosphatase). The provider's intra-assay coefficient of variation for TSH was between 4.5 and 13.5%, and during the lab run, it ranged from 4.9 to 10.1%.

Dilution studies were performed manually using Siemens TSH diluent (provided by the manufacturer) and appropriate pipettes with dilutions of 1:2, 1:4, and 1:8 performed and measured in the same run as the undiluted sample. To assess linearity, the TSH concentration was back-calculated by multiplying it with the dilution factor, and the average of the three diluted samples was paired with the concentration in the undiluted sample for statistical analysis. Linearity was defined as a recovery of 80–120% of the expected value after dilution, and any sample that showed recovery outside this range was deemed to have nonlinear dilution and was suspected of interference.[21,22]

In the blocking method, "HAMA Blocking Reagent (85R-1001)" from Fitzgerald was used with a dilution of 1:500, as recommended by the manufacturer. After treatment, samples were incubated at room temperature for 1 hour before TSH measurement.[21] TSH values post-heterophile blocker treatment that fell between 80–120% of the untreated values were considered to be free from interference.[23,24]

For the depletion of antibodies, polyethylene glycol (PEG) precipitation was done with PEG 6000 (Laboratory version) at 25% concentration. PEG solution was prepared by adding 25 mg of PEG 6000 in 100 ml of distilled water, followed by a thorough vortex until a clear solution appeared. PEG treatment of the sample was done at 1:1 dilution (250 μL of the sample with 250 μL of PEG solution); followed by a thorough vortex for 20 min. This was followed by stabilization time for 30 min after which the samples were centrifugated at 1500 G for 15 min at room temperature. The supernatant was used for analysis. The percentage recovery of TSH after PEG precipitation was calculated and compared to the untreated TSH value, taking into account a dilution factor of 2. A recovery rate of less than 40% was considered indicative of interference due to antibodies.[25,26]

Assessment of TSH on a different platform was done for all the patients. Abbott i Architect was used as the second platform which was a chemiluminescent microparticle immunoassay. This test uses a solid phase of microparticles of anti-beta TSH mouse monoclonal antibody, and the detection antibody is a conjugate of anti-alpha TSH mouse monoclonal acridinium labeled with bovine stabilizers. The normal range

for TSH as per the Abbott i Architect platform was 0.35 to 4.95 μIU/mL.

Statistical analysis was conducted using IBM SPSS Statistics version 19. The distribution pattern of the data was determined using the Shapiro-Wilk normality test. Fisher's exact test was used to analyze nominal variables, while Mann-Whitney U-test was used for continuous variables. A *P* value less than 0.05 was considered statistically significant. Cohen's kappa values were also calculated to assess agreement between two nominal groups.

Ethical aspects

All participants provided written informed consent, and the study was conducted in accordance with the Declaration of Helsinki. The study was approved by the Institutional ethics committee. (institutional Ethical Committee approval number: IPGME&R/IEC/2019/429 Dated - 03.07.2019).

Results

The mean age of the study population was 42.6 (± 9.1) years, with the majority in the age group of 35 to 49 years. There was female predominance with 71/84 (84.5%) female and 13/84 (15.5%) male participants. All participants were positive for RF (>20 IU/L) with 52 (61.9%) having an RF more than three times the upper limit of the normal. The median RF level in the study population was 75.5 IU/ml (IQR = 45.9-138.75). We found that 53.6% (45/84) of the patients tested positive for anti-TPO antibodies. The median RF level among anti-TPO positive cases was 100 IU/mL (IQR = 48.5–149.45), which was numerically higher than anti-TPO negative cases, with a median of 63.7 IU/mL (IQR = 45.8–110). However, the difference was not statistically significant (Mann Whitney U test, $P = 0.074$).

As the normal TSH range on the second platform (Abbott i Architect) differed from the first platform (Immulite-1000), we compared those based on the difference in diagnostic interpretation. There was an interpretation discordance in only 1 out of 84 participants, whose TSH by the second platform was 3.31 (Euthyroid range), in comparison to 4.45 (Subclinical hypothyroid range) on the first one. Post-PEG precipitation TSH recovery was between 100% to 40% in all the samples [Figure 1]. No sample showed recovery of less than 40%, which was taken as a cutoff to suggest the influence of immunoglobulins or antibodies.

On dilution studies, seven samples had average TSH recovery above 120%, while three samples had TSH recovery below 80%; thus, 10 out of 84 (11.9%) samples were classified as suspected of interference based on criteria taken for loss of linearity, which refers to a deviation from a linear dose-response relationship [Figure 2]. The median RF level among samples showing loss of linearity was 133 IU/ml, which was numerically higher than samples with normal dilution study who had a median RF level of 71.9 IU/mL. Loss of linearity was seen in 17.31% of participants with RF levels greater than three times the upper limit of normal (ULN), compared to 3.13% in participants with RF levels less than three times ULN. However, both parameters did not reach statistical significance [Table 1]. The median TSH among participants with loss of linearity was 1.97 μIU/ml, which was similar to the median TSH of 2.25 μIU/mL observed in participants with normal dilution study [Table 1]. The median anti-TPO level in cases with interference on dilution was significantly higher than in cases without interference. Additionally, the loss of linearity on dilution was significantly higher in patients who tested positive for anti-TPO antibodies compared to anti-TPO negative patients. [Table 1].

On heterophile blocker treatment, four samples had TSH recovery of less than 80%, while one sample had a recovery of over 120%; thus, 5/84 were classified as suspected of interference [Figure 3]. The median RF level among participants with suspected interference on blocker treatment was 116 IU/mL numerically higher than participants with a normal result who had a median RF level of 73.6 IU/mL,

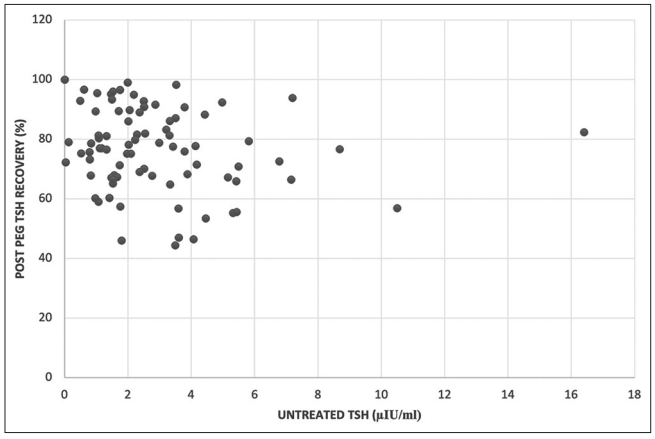

Figure 1: TSH recovery on PEG treatment. Scatter diagram with X-axis – Untreated TSH value of each sample, Y-axis – TSH recovery (in %) of the same sample on PEG treatment

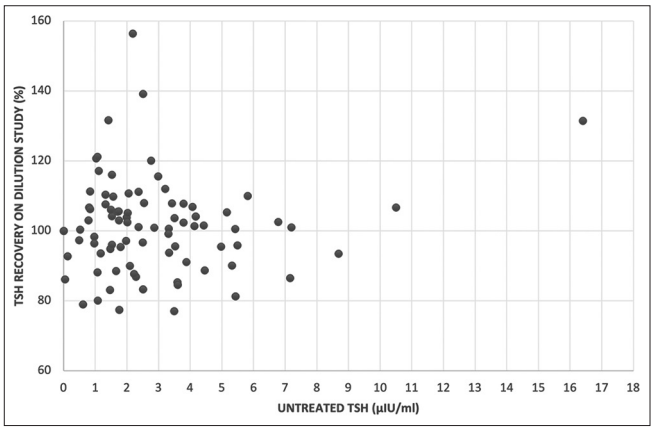

Figure 2: TSH recovery on dilution study. Scatter diagram with X-axis – Untreated TSH value of each sample, Y-axis – TSH recovery (in %) of the same sample on dilution study

Table 1: Comparison of loss of linearity on dilution study and interference post-heterophile blocker treatment in different subgroups

	Loss of linearity on dilution-absent	Loss of linearity on dilution-present	*P*	Post-HB treatment – interference negative	Post-HB treatment – interference positive	*P*
RF (IU/mL) (median)	71.9 (IQR=45.4 – 130)	133 (IQR=97.8 –150.8)	0.053	73.6 (IQR=45.8 – 138)	116 (IQR=79.45 – 223)	0.199
RF group-						
<3 times ULN	31/32 (96.88%)	1/32 (3.13%)	0.081	31/32 (96.885%)	1/32 (3.13%)	0.645
>3 times ULN	43/52 (82.69%)	9/52 (17.31%)		48/52 (92.31%)	4/52 (7.69%)	
TSH (µIU/mL) (median)	2.25 (IQR=1.42-3.8)	1.97 (IQR=1.06–2.94)	0.507	2.22 (IQR=1.3–3.76)	1.52 (IQR=1.07–2.92)	0.355
Hypothyroidism						
TSH <4	57/66 (86.4%)	9/66 (13.6%)	0.682	61/66 (92.42%)	5/66 (7.81%)	0.580
TSH >4	17/18 (94.4%)	1/18 (5.6%)		18/18 (100%)	0/18 (0%)	
Anti-TPO status						
Absent	38/39 (97.4%)	1/39 (2.6%)	0.017	36/39 (92.3%)	3/39 (7.7%)	0.659
Present	36/45 (80%)	9/45 (20%)		43/45 (95.6%)	2/45 (4.4%)	
Anti-TPO (IU/mL) (median)	32.35 (IQR=13.3–62.9)	54.05 (IQR=41.52–85.5)	0.027	38.3 (IQR=16.3–63.7)	30 (IQR=23.2–53.2)	0.676

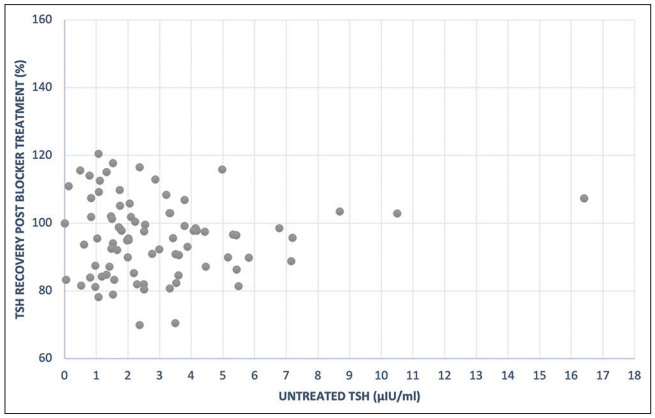

Figure 3: TSH recovery post blocker treatment. Scatter diagram with X-axis – Untreated TSH value of each sample, Y-axis – TSH recovery (in %) of the same sample on blocker treatment

though not statistically significant [Table 1]. In all these five cases with suspected interference on blocker treatment, the baseline as well as post-blocker TSH values were <4 µIU/mL [Figure 3]. The proportion of cases showing post-blocker interference was similar in patients with anti-TPO positivity compared to anti-TPO negative cases. Additionally, the levels of anti-TPO antibodies were similar in cases with or without blocker interference [Table 1].

The number of samples with anti-TPO positivity was numerically higher among cases showing interference by any of the four methods compared to those without interference (71.42% vs. 50%), but this difference did not reach statistical significance ($P = 0.24$).

Out of the five cases showing interference in the heterophile blocking study, only two had a loss of linearity in the dilution study. The rest of the three cases were showing interference only on blocking studies and were euthyroid. We measured the agreement of the results from two studies (dilution and blocker treatment) by Cohen's kappa, Kappa value came as k = 0.203 ($P = 0.045$), denoting a fair agreement between the two screening methods.

Discussion

We have used four different methods to detect interference in every participant regardless of baseline TSH values. Based on the criteria used, we found interference in 14 cases – 12 of them by a single method and 2 of them by two different methods. Interference was seen in 10 and 5 cases based on serial dilution studies and heterophile blocker treatment, respectively, while 1 case had diagnostic interpretation discordance when measured in the second platform. One person had TSH of 4.45 µIU/mL on Immulite-1000 and 3.31 µIU/mL on Abbott i Architect, suggesting subclinical hypothyroidism when measured by the former whereas TSH was within normal limits when measured by the latter. However, the other three screening tests for interference were negative for this individual. In clinical practice, differences in TSH levels from the same sample using two different assays (assays having antibodies from different animal sources) can be a simple way to suggest the presence of heterophilic interference.[9] But the sensitivity by this method can be low as demonstrated by Ismail *et al.* in 2002,[24] who only detected interference in 1 out of the 59 clinically suspicious results by assessment on a second platform. This lower detection rate by the second platform could be explained by the polyspecific nature of heterophilic antibodies.[18] Most case reports detecting interference by a second platform assessment had shown huge differences in results. These discrepancies were most probably due to interferences by Human anti-mouse antibodies (HAMA) or Human anti-animal antibodies (HAAA), which usually have stronger affinity and appear following exposure to animal antigens.

Hattori *et al*.[27,28] while using PEG precipitation for macro-TSH assessment, reported a mean post-PEG recovery value of approximately 40%. In another study, the mean post-PEG

recovered TSH was 47% among controls.[25] Based on these studies, we used post-PEG TSH recovery of less than 40% as an indicator of potential interference from high molecular weight proteins. In our study, PEG precipitation did not lead to the detection of interference in any of the 84 cases. As TSH is a glycoprotein like prolactin, macromolecules of TSH can be easily precipitated by PEG. Even though PEG precipitation can be helpful, it is a relatively nonspecific technique, and a proportion of the monomeric form is likely to be coprecipitated. The extent to which this occurs is both analyte and method dependent.[29]

Based on the serial dilution study, interference was suspected in 10 out of 84 cases. Only 2 of these 10 samples showed interference after treatment with a heterophile blocker. Ismail *et al*.[24] detected interference in 16 out of 59 clinically suspicious results using serial dilution studies, with 6 cases only being detected during dilution and not with heterophile blocker treatment. Serial dilutions may also reveal nonlinearity in other types of interference, such as macro-TSH, thyroid hormone autoantibodies, or the hook effect.[22,24]

The heterophile blocker treatment revealed interference in five cases. However, despite the suspected interference, all five cases showed TSH values within the normal range [Figure 3]. Only two out of five of those had shown interference by other methods. Ismail *et al*.[24] reported that 28 out of 59 suspected cases in their series exhibited interference, with 12 samples showing interference only with heterophilic blocking studies, 6 samples showing interference only with dilution studies, and 10 showing interference in both methods.

In our study, the two cases that showed interference in both dilution and blocker methods had baseline TSH of 3.49 and 1.01, and in both cases, even after correction using the mean of dilutions or post-heterophile blocker treated-TSH, the TSH values remained within the normal range, suggesting that the interference had minimal clinical implications in these samples.

In samples that exhibited interference in either dilution or blocking studies, the median RF levels were numerically higher in samples that exhibited interference compared to those that did not, but this difference was not statistically significant. Additionally, other studies of RF interference with multiplex cytokine assays have shown that the relationship between RF level and interference is unpredictable.[16,30,31] Importantly, corrected TSH (based on the mean of dilutions or post-HB treated TSH) did not change the clinical interpretation in most of the concerned cases. This indicates that RF is a weaker heterophile antibody causing a lesser degree of interference and having minimal effect on the interpretation of TSH assay results by modern immunoassay platforms.

In our study, we observed an association between the proportion of anti-TPO positivity and interference on dilution ($P = 0.017$), as well as numerically higher median anti-TPO levels in cases with suspected interference on dilution compared to cases without interference ($P = 0.027$). This could be due to an autoimmune association, as suggested by the numerically higher RF levels in anti-TPO-positive cases. However, the lack of statistical significance in the association between higher RF levels and anti-TPO positivity, as well as the lack of statistical association between interference on dilution and RF levels in our study, indicates that a larger study in the future may help us arrive at a conclusion in this regard.

The agreement between dilution and blocking methods was fair, as indicated by a kappa value of 0.203 ($P = 0.045$). If clinical significance and potential changes in management plans were used as measures of interference, neither serial dilution nor the heterophile blocking method detected clinically significant interference among seropositive RA participants, indicating a minimal degree of interference.

We suggest the use of all four approaches in suspected cases, with the sequence being influenced by the resources available to the individual laboratory. Blocker treatment is considered the most specific screening test for heterophilic interference in theory. However, the sensitivity of blocker treatment is uncertain due to the polyreactive nature of heterophilic antibodies. Availability and cost are also hurdles in the routine use of heterophile blockers in suspected cases in the laboratory. In this regard, dilution studies are better placed, as diluents are easily available and cheaper, and dilutions are performed routinely in laboratories for various other reasons, making it a preferable first-line screening method for heterophilic interference.

The interference from RF on TSH assays does not appear to have a significant impact on clinical decision-making in the majority of cases in our study. However, it is important to suspect and test for interference when there is a discrepancy between the clinical presentation and a lack of correlation with other biochemical or hormonal parameters. Effective communication between clinicians and laboratories is crucial to identify any interferences.

One of the key strengths of our study was the use of four methods to screen for interference in every sample. However, there are some limitations to this study that are worth noting. Firstly, this study should be considered a pilot as there is limited research on the prevalence of heterophile interference in RF-positive samples. Additionally, we used a manual method for dilution because automatic/onboard dilution was not available for the specific assay used in this study. Furthermore, due to the absence of a gold standard for detecting heterophile interference, we were unable to determine the sensitivity and specificity of each screening method. In addition, our study excluded cases that received treatment with biological agents, and therefore our results should not be extrapolated to individuals undergoing those treatments.

In conclusion, RF can cause heterophilic interference in commercial TSH immunoassays, which can be identified

through serial dilution or treatment with heterophile blocking agents. However, this interference does not significantly impact clinical decision-making in most situations. Further research using various immunoassay platforms is needed to gain a deeper understanding of this potential issue.

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Conflicts of interest

There are no conflicts of interest.

Acknowledgment

None.

Authors Contribution

Dr. SC conceived the study. Dr. SNN selected the patients, collected samples, conducted tests, and drafted the initial manuscript. Dr. AP also performed tests and conducted statistical analysis. Dr. RB supervised the project and finalized the documents. All authors contributed to the study execution, provided input for the write-up, and approved the final manuscript.

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