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Original Article

Comparative analysis of antibodies and lymphocytes in pulmonary tuberculosis patients with negative and positive cultures

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Abstract

Introduction: The sputum smear or the culture are the definitive diagnosis of pulmonary tuberculosis. Only a fraction of clinical patients are culture-confirmed.

Methodology: A total of 24 clinical cases (40 ± 14 years old) with positive smear and negative co-morbidity were studied. Cases were selected from 600 patients who attended the pneumology service over two years. A sputum sample was cultured in Löwenstein-Jensen medium with consequent amplification of the rrnA V2 promoter, the differentiation region 4, and the IS6110 insertion sequence of *Mycobacterium tuberculosis*. After the culture result, the patients were divided into negative (n = 14) or positive (n = 10) culture groups. In addition, 30 samples from healthy donors (45 ± 10 years) were studied. The numbers of CD4, CD8 and CD19 lymphocytes were determined by flow cytometry. Levels of IgA and IgG to *M. tuberculosis* were measured by ELISA.

Results: IgG and IgA levels were detected in patients with positive culture, while only IgA was found in patients with negative cultures. The lymphocyte populations in the two groups were similar. The presence of a pleural apical cap was found more frequently in patients with negative- (57%) than with positive cultures (10%).

Conclusions: The isotype profile in patients with positive cultures was both IgA and IgG positive, while in patients with negative culture, only IgA was found. The results will contribute to improve the diagnostic algorithm and appropriate treatment of patients with clinical tuberculosis. Further studies are needed to determine if this profile is predictive of the outcome of isolation.

Key words: Tuberculosis; diagnosis; culture.

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Introduction

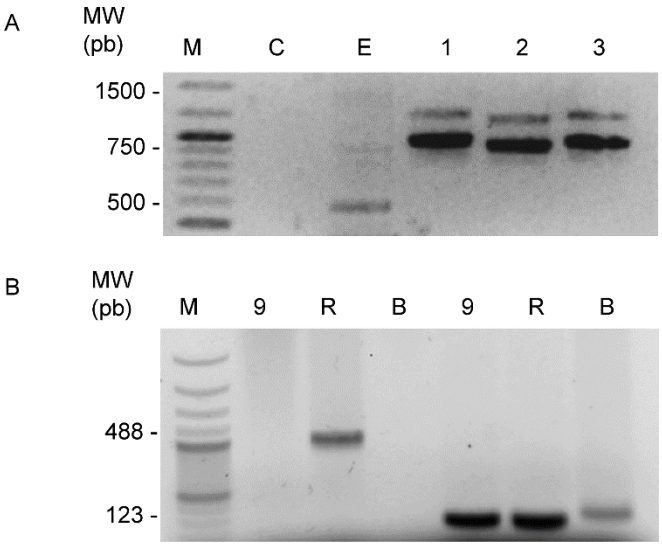
Tuberculosis is a chronic bacterial infection caused by the *Mycobacterium tuberculosis* bacillus. The main clinical entity is the pulmonary form, but other forms may be present such as ganglion, bone, miliary, or combinations of them. Although one of three people has been documented to be infected by the bacteria, only 5 to 10% of the carriers develop active tuberculosis [1]. Nowadays, the identification of the bacillus by microscopic examination of sputum smear or by culture is the definitive diagnosis, but the direct diagnosis presents certain challenges. Around 30-50% of tuberculosis patients are negative in the microscopy examination, and culture requires a long time for the growth of the bacillus (4 to 8 weeks), which may lead to delayed diagnosis. In addition, the successful isolation of the pathogen requires that the best specimen be properly collected, promptly transported and carefully processed. As a result, the sensitivity of culture ranges from 80% to 93% [2]. However, routinely, only 20% of patients with clinical tuberculosis are culture-confirmed, because it is not encouraging to receive a negative result after waiting several weeks. Automated liquid culture systems are faster and have a 10% greater yield than solid media, but such systems are expensive and prone to contamination [3]. Bacteriologic confirmation of childhood tuberculosis is typically successful in fewer than 40% of cases [4].

A number of alternative diagnostics have been standardized because there is a great need for rapid point-of-care tests that can be readily used at all levels

of the health system and in the community [5]. The tuberculin skin test is the recommended diagnostic test for latent tuberculosis, but it requires standardized application and interpretation, and a positive result depends on an adequate immune response. The usefulness of the IFN-gamma release assays in the diagnosis of active tuberculosis remains questionable [6]. Nucleic acid amplification tests [3] and PCR amplification [7] are the most promising developments in tuberculosis diagnostics. However, uses of such tests are restrictive because the assays require dedicated and expensive equipment. The serological test based on the detection of circulating antibodies against *M. tuberculosis*-specific antigens could represent a useful complement to microscopic examination for screening active tuberculosis. Although an antibody response to *M. tuberculosis* antigens occurs, there is great individual variability in the number and type of serologically reactive antibodies, making this diagnostic tool too unreliable [8]. Indeed, challenges for the development of effective serologic tests include the need to discriminate active disease from latent infection and to avoid cross-reactivity with *M. bovis* BCG or mycobacteria other than *M. tuberculosis.*

Diagnosis of tuberculosis in the early stage of the disease is extremely important, as is rapid detection and identification of mycobacteria from clinical samples. Host biomarkers are needed to diagnose tuberculosis, monitor treatment, and assess outcomes. Indeed, host biomarkers could potentially be used to predict the success or failure of culture, and thereby save time in differential diagnosis or beginning treatment. Thus, the aim of this work was to compare the immunological profile in tuberculosis patients with negative- and positive culture to identify possibly useful differences as an aid in diagnosis.

Figure 1. Amplification of PCR products to confirm the isolation of *Mycobacterium tuberculosis*.



Panel A shows products characteristic of the genus *Mycobacterium* (934 and 1031 pb), as well as a molecular weight marker (M), the mix without DNA (C) and a DNA control of *Escherichia coli* (E). Lanes 1, 2 and 3 show samples from three different patients. Panel B shows the product characteristic of *Mycobacterium bovis* (488 pb) in line T and the IS6110 insertion sequence (123 pb) characteristic of *Mycobacterium tuberculosis* in samples from three different patients (lines 1, 2 and 3). The molecular weight marker (M) and the mix control (C) are also shown.

Methodology

Populations of study

Twenty-four adult patients (42 ± 14 years of age, range 18 to 61 years) with active pulmonary tuberculosis from a group of patients (n = 600) were selected over two years from the Department of Pneumology at the General Hospital of Mexico, Ministry of Health. Patients entered the service because they had cough, asthenia, adynamia, fever and weight loss at the time of the study. All had a complete medical history accessible for review. Clinical tuberculosis was confirmed according to the *Norma Oficial Mexicana* (Mexican guidelines NOM-006-SSA2-2013 for the control and prevention of tuberculosis) and stained smear samples were positive for acid-fast bacilli. The number of patients was rigorously selected to include only immunocompetent patients who did not have a history of HIV, type 2 diabetes mellitus, or other co-morbidities. Haemolysed or insufficient samples were excluded. Samples of patients diagnosed with nontuberculous mycobacteria [9] were also excluded. Additionally, 30 samples from healthy donors (45 ± 10 years) with no personal or family history of tuberculosis were studied. All healthy donors were from an urban environment (Mexico City); their socioeconomic status was medium, without overcrowding, with cement housing, roof, floor and walls made of materials suitable for construction. In Mexico, vaccination with Bacille Calmette-Guerin (BCG) has been carried out routinely (except in clinically justified exceptions) in the first hours of life since 1951, so all the participants in this study were vaccinated with BCG. Each patient and healthy donor provided two biological samples, one whole blood to determine cell populations by cytometry and another serum to determine immunoglobulin isotypes by ELISA. The bioethical and clinical criteria of inclusion of the Ethics and Research Committee of the Institute for Epidemiological Diagnostic and Reference, Ministry of Health, were fulfilled. The informed consent of the patient was obtained at the General Hospital of Mexico.

Bacilli culture and classification of patients

A total of 200 µL of a sputum sample was used to isolate the bacilli in Löwenstein-Jensen culture medium in a Biosecurity Level Laboratory III. Isolation was confirmed by the amplification of the IS6110 insertion sequence, the rrnA V2 gene promoter and the *Mycobacterium tuberculosis* differentiation region 4 to rule out nontuberculous mycobacteria (Figure 1) [10]. Based on the results of culture, the patients were divided into two groups, one with negative cultures (n = 14) and the other with positive cultures (n = 10).

Flow cytometry to analyze the lymphocyte populations

CD4, CD8 and CD19 lymphocyte cell markers were analyzed in whole blood. For the analysis of each marker, 100 µL of blood was used, added to 20 µL of specific conjugates for CD4, CD8 and CD19 (Beckman Coulter, Brea CA, USA). The reaction was run for 30 minutes at room temperature, then 100 µL of labeled Immunotrol beads (Beckman Coulter, Brea CA, USA) were added. The samples were prepared in the Immunoprep kit (Beckman Coulter, Brea CA, USA), adding 600 µL of buffer for lysis, 265 µL of buffer for washing and 100 µL of buffer to fix the cells. Lymphocyte populations were analyzed using a flow cytometer (EPICS XL MCL, Beckman Coulter, Brea CA, USA) with the program assigned for the tuberculosis protocol (Panel Report COULTER EPICS).

ELISA for antibodies in serum samples

The specific antibodies were determined in the serum samples by a standard ELISA [11] with some modifications. Briefly, high-binding microtiter plates (Corning-Costar, Bloomington, MN, USA) were coated with 100 µL /well of a 10 µg/mL solution of a soluble extract of *Mycobacterium tuberculosis* (strain ATCC 25618 / H37Rv), diluted in 0.015 M phosphate-buffered saline, pH 7.2 (PBS) then incubated overnight at 4ºC. After washing with 0.05% tween 20 in PBS, the plate wells were blocked with 1% bovine serum albumin (BSA) in PBS during 30 min at 37ºC. Serum samples were tested in duplicates as for routine diagnosis using 100 µL/well and incubated for 1 h at 37ºC. A peroxidase-IgG-goat conjugate to human IgA, IgM or IgG isotypes (Jackson Research, West Grove, PA, USA) was added 1:1000 in BSA and incubated 1 h at 37ºC. Finally, 100 µL/well of 0.05 mg/mL o-phenylenediamine (Sigma-Aldrich, St. Louis, MO, USA) in PBS buffer, pH 7.2 was added during 30 minutes. The enzyme reaction was stopped with 50 µL/well of 4 N H_2SO_4. Absorbance values were read at 490 nm. The cutoff was the average absorbance plus two times the standard deviation obtained with the 30 samples from healthy donors.

PCR for the analysis of strains

A multiplex PCR was carried out to confirm the genus of *Mycobacterium* isolated from the sputum cultures from patients with clinical tuberculosis. Two sets of primers were used [10]. The first set amplified variant 2 at the rrnA operon sequence 5′-TCGATGATCACCGAGAACGTGTTC-3′, named Raq 1 and 5′-CACTGGTGCCTCCCGTAGG-3′, named Raq 8. The second set amplified a product of 1031 pb at region 4 of differentiation (5′-ACATGTACGAGAGACGGCATGAG-3′, named Y277-32F and 5′-AATCCAACACGCAGCAACCGA-3′, named Y277-32R). To confirm the species, a simple PCR was carried out using the primers IS6110HPF (5′-CCTGCGAGCGTAGGCGTCGG-3′) and IS6110HPR (5′-CTCGCTCAGCGCCGCTTCGG-3′) that amplified a product of 123 pb for the IS6110 repeated sequence in the genome of *Mycobacterium tuberculosis* [12]. To rule out *M. bovis*, the primers MTBF (5′-CGGGTATGCTGTTAGGCGACG-3′) and MTBR (5′-CCACCACAAGACATGCATG-3′) were used to amplify a 488 pb product.

Data processing and statistical analysis

Data on clinical characteristics were analyzed by the Mid-p exact test, 2 tails (OpenEpi: Open Source Epidemiologic Statistics for Public Health, Versión 3.01. www.OpenEpi.com). Data on number of lymphocytes and results of IgG isotyping was analyzed by the Kruskal-Wallis test using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA). Additional contrast tests were performed with Dunn's Multiple Comparison Test. Data in figures are shown as mean ± standard deviation.

Results

Clinical and socio-economic characteristics of the population

Table 1 shows the socio-economic and clinical characteristics of the patients with tuberculosis. The socio-economic backgrounds of patients with negative or positive cultures were similar. The group of patients comprised 24 adults (40.4 ± 13.7 years old), including 22 men and two women. The educational levels were divided into elementary (n = 10) and high school (n = 14). Most patients resided in urban areas (83.3%). Overcrowding at home was 50% in culture negative - and 57% in culture-positive patients.

The clinical characteristics of the patients with positive or negative cultures were also similar but, unexpectedly, a difference was found in the presence of a pleural apical cap in 57% of patients with negative cultures (57%) compared to 10% of those with positive culture (p = 0.02603; Mid-p exact, 2 tails. IC = 1 272 - 295.3). In general, patients started to have signs 8.5 ± 8.3 months before they came to medical consultation. All patients had BCG vaccination and positive sputum smear microscopy, but the PPD test (PPD) was positive in 30% of the culture positive patients and in 42.9% of the negatives. Other clinical characteristics as grains of millet, caverns, pleural spill and calcification, were below 42% in both groups. Most of patients presented with cough with phlegm (100%), asthenia/adynamia (100%), fever (91.6%), low weight (87.5%) and fatigue/weakness (100%). Only 8.4% had hemoptysis. In general, there were no familial antecedents of medical importance, although six cases (26.6%) recorded cohabitation with tuberculosis patients. In addition, seven cases (29.16%) mentioned drug addiction and 12 (50%) reported smoking.

Figure 2. Lymphocyte populations in serum samples from patients with active tuberculosis. The number of lymphocytes was determined by flow cytometry using antibody against CD4 and CD8, CD19 markers. The figure shows the data obtained with the samples from healthy donors (closed bars) and with the patients with negative (open bars) and positive (grey bars) cultures. Statistical differences within the groups are shown with an asterisk (p < 0.0001; Kruskal-Wallis). Contrast tests were performed by means of Dunn's Multiple Comparison Test).

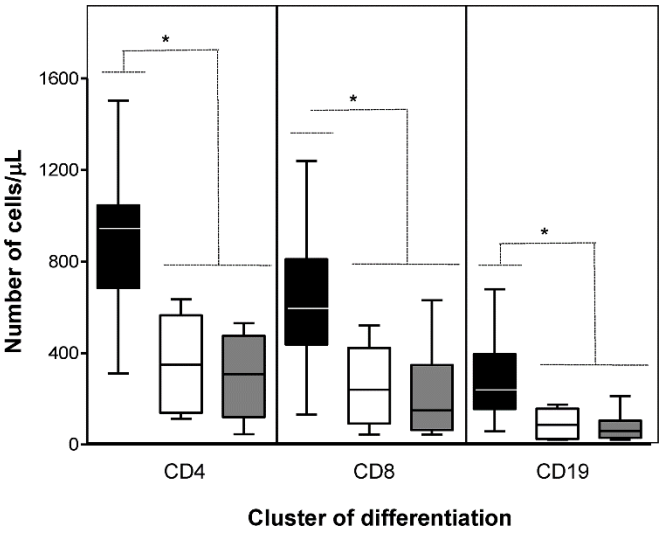


Table 1. Socio-economic and clinical characteristics of the patients with tuberculosis.

Item	Culture outcome[1]		Total
	Positive	Negative	
Number of patients	10	14	24
Socio-economic characteristics			
Number of women and men	1 and 9	1 and 13	2 and 22
Age of patients (in years)	39.0 ± 11	41.5 ± 16	40.4 ± 13.7
Maximum school grade (elementary or high school)	6 and 4	4 and 10	10 and 14
Residence of patients (rural or urban)	1 and 9	3 and 11	4 and 20
Overcrowding (three or more people per room)	5	8	13
Clinical characteristics			
Apical pleural[2]	1	8	9
Beginning of clinical signs (range 1 to 36 months)	9.4 ± 11.4	7.9 ± 5.6	8.5 ± 8.3
Bacille Calmette-Guerin vaccination	10	14	24
Sputum smear microscopy	10	14	24
Positive result to purified protein derivative test	3	6	9
Grains of millet	2	3	5
Caverns	2	4	8
Pleural spill	1	1	2
Calcifications	6	6	12
Treatment response	8	11	19
Cough with phlegm	10	14	24
Asthenia/adynamia	10	14	24
Fever	9	13	22
Low weight	9	12	21
Fatigue/weakness	10	14	24
Hemoptysis	2	0	2
Cohabitation with other tuberculosis patients	3	3	6
Drug addiction	3	4	7
Smoking addiction	6	6	12

[1] Number of patients; [2] A statistically significant difference found by Mid-p exact 2 tails (p = 0.02603; IC = 1.272 - 295.3).

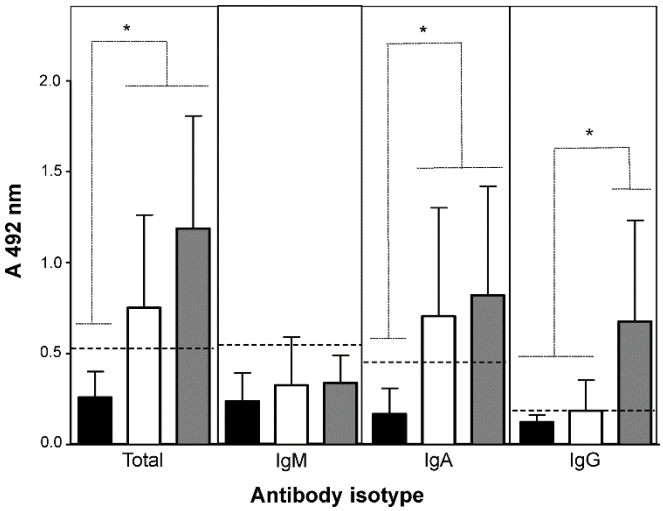
Lymphocyte populations

Figure 2 shows the determination of lymphocyte numbers with the markers CD4, CD8 and CD19. In general, populations of lymphocytes were reduced in patients with TB compared to healthy donors ($p < 0.0001$; Kruskal-Wallis). In the group of patients with negative cultures, the CD$ population was reduced by 54%, CD8 by 60% and CD19 by 64%, while in patients with positive cultures, lymphocyte populations decreased by 70, 62 and 74%, respectively.

Antibody determination

Total immunoglobulins and IgM, IgA and IgG isotypes were determined by ELISA. Figure 3 shows the levels of antibodies in the serum samples of patients with tuberculosis. The greatest value of diagnostic sensitivity (Table 2) was for IgG determination in patients with positive cultures (80%). In the case of patients with negative cultures, the peak of sensitivity was 50% with IgA. The rate of diagnostic specificity was 88.5 to 96.2%. Particularly, the positivity rate in patients with negative cultures was only 57.1% for total immunoglobulins, 50% for IgA and 42.9% for IgG. For patients with positive cultures, the positivity rate was 80% for both total immunoglobulins and IgG, while for IgA it was 70%. In the case of IgM, positivity rates were 14.3% for patients with negative cultures and 20% with positive cultures. Concordance in the determination of IgG and IgA was 85.7 % in patients with negative cultures and 70% with positive cultures. No concordance was found in the determination of IgM. In serum samples from healthy donors, the frequency of positivity was 3.8% to both total immunoglobulins and IgA, while for IgM and IgG it was 11.5%.

Figure 3. Levels of antibodies in serum samples of patients with tuberculosis against a soluble extract of *Mycobacterium tuberculosis*. The reactivity of IgM, IgA, and IgG isotypes as well as the total immunoglobulins was determined. The figure shows the average absorbance of the data obtained with samples from healthy donors (closed bars) and with patients with negative (open bars) and positive (grey bars) cultures. Statistical differences within the groups are shown with an asterisk ($p < 0.0001$; Kruskal-Wallis). Contrast tests were performed with Dunn's Multiple Comparison Test. The horizontal dotted line is the cut-off value.



Discussion

Nowadays, a number of techniques can confirm the microbiological diagnosis of tuberculosis, including the molecular tests which detect the DNA in tuberculosis bacteria in less than 2 hours, such as the GeneXpert test. The cost and the need for continuous equipment maintenance are disadvantages in their use. Mycobacterial culture takes more than four weeks and is often negative. Successful isolation of the pathogen is associated with correct collection, transport and adequate processing of the specimen. However, little is known about the possible influence of host factors such as immunocompetence on the potential detection of the bacterium. The data reported here suggest that the antibody profile of patients with negative cultures features IgA, while patients with positive cultures are positive for both IgA and IgG.

We found that clinical and socio-economic data of the patients were similar to what has been reported previously [13]. Recently, Cudahy and Shenoi [1] made an excellent review of the diagnosis of pulmonary tuberculosis, emphasizing that there is no perfect universal symptom screen and that symptom combinations may differ depending on the patient population´s immune status. However, we did find a significant difference in the frequency of apical pleural cap between culture negative and positive groups. The apical pleural cap refers to the formation of a hyaline

Table 2. Serological evaluation of antibodies to *Mycobacterium tuberculosis.*

Culture outcome	N[1]	Sensitivity (%)				Specificity (%)			
		Ig´s[2]	IgM	IgA	IgG	Ig´s	IgM	IgA	IgG
Positive	10	80.0	20.0	70.0	80.0	96.2	88.5	96.2	88.5
Negative	14	57.1	14.3	50.0	42.9				
Total	24	66.7	16.7	58.3	58.3	96.2	88.5	96.2	88.5

[1] Number of serum samples tested; [2] Total IgA, IgG and IgM isotypes detected using an anti-human polyvalent immunoglobulins conjugate.

plaque in the visceral pleura, which is caused by an intrapulmonary fibrosis. The etiologic origin of the apical pleural is controversial, since it is frequently found in people older than 45 years. Our data suggest that they might go unnoticed if all patients with tuberculosis are considered together, not separated as culture-negative and -positive. There was a clear association between the pleural fibrosis and the negative result for isolation of the bacteria, suggesting that the apical pleura may be a sequel to active tuberculosis. Interestingly, the patients in this study had long disease history (around 12 months), so the time in which the pleura fibrosis is developed after the infection with *Mycobacterium* cannot be determined accurately.

This is the first study that compares the immunological profile of tuberculosis patients with negative and positive cultures. The data suggest that the IgA could be used as a marker to predict the outcome of the culture, which would be helpful in the early identification of patients with tuberculosis. Our data suggest that patients with positive cultures show both IgA and IgG positive profiles. It has been reported that patients with active disease show a strong IgG response but poor IgA and IgM responses [8,14,15]. Although some studies show better values of diagnostic sensitivity for IgA than for IgG [16-18], we found a clear correlation between active disease and IgA and IgG.

In patients with negative cultures, the immunological profile is different, showing only IgA, which suggests a protective role that could influence the culture outcome. It has been reported that IgA antibodies are associated with the absence of disease, in addition to a significant immunomodulatory effect on cell-mediated immunity [19]. Recently, the anti-heparin-binding hemagglutinin (HBHA) IgA was reported as a potential biomarker of protective immunity, since healthy controls had significantly higher IgA and lower IgM antibodies compared to both untreated tuberculosis patients and contacts. HBHA is a surface protein involved in epithelial attachment and extrapulmonary dissemination of *Mycobacterium tuberculosis* [20]. Conde et al., [16] also reported that specific IgA was positive in 54% of healthy close contacts of pulmonary tuberculosis patients but only 8% of healthy controls without such contact or a prior personal history of tuberculosis. In experimental tuberculosis, a monoclonal IgA anti-a-crystallin given intranasally impaired short-term protection against aerosol lung infection and granuloma formation in BALB/c mice [21]. The same antibody was also protective in an intratracheal model of pulmonary infection [22].

Conclusions

We identified the isotype profile in patients with positive cultures as both IgA and IgG positive, while in patients with negative cultures, it was only IgA. These results are important to improve the diagnostic algorithm of patients with clinical suspicion of tuberculosis. Further studies are needed to determine whether this profile is predictive of the outcome of isolation of the bacteria from patient samples.

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Authors' contributions

FRCS conceived, designed the work, performed laboratory and data analysis and prepared the manuscript. AHS and RCS selected the patients with clinical tuberculosis and participated in the data analysis, AEAD, HGG, MIH, HH, RTR performed the laboratory work, participated in the data analysis and participated in the preparation of the manuscript. JLRA conceived, designed the work, performed data analysis, prepared the manuscript and is the guarantor of the present paper. All authors reviewed the manuscript and approved it for submission.

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