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Oral mucositis and microbial status in acute lymphoblastic leukemia subjects undergoing high-dose chemotherapy

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Aim: To assess oral microbial status in patients with acute lymphoblastic leukemia (ALL) undergoing high-dose chemotherapy and to unravel possible associations between nosocomial pathogens and the establishment of chemotherapy-induced oral mucositis (CIOM). **Methods:** Oral mucosa, saliva, and peripheral blood samples were collected from 46 ALL subjects one day prior to chemotherapy (D0) and 2 weeks after treatment initiation (D14). Clinical intraoral inspection was performed by a single practitioner, with mucositis classification performed according to the WHO oral toxicity scale. Blood components were quantified by automatic flow cytometry, while oral *Staphylococcus aureus* and *Pseudomonas aeruginosa* were detected by Polymerase Chain Reaction with species-specific primers. Associations among bacteria and clinical findings were determined by Fisher's Exact test, longitudinal bacterial changes by paired Macnemar, and correlations among blood parameters and mucositis status or bacteria via Mann-Whitney. **Results:** *S. aureus* displayed higher detection rates at D14 ($p < 0.05$) and was positively associated with mucositis, adoption of a non-solid diet (all $p < 0.001$), nausea and fever (all $p < 0.05$). Conversely, *P. aeruginosa* did not correlate to CIOM clinical parameters. At the systemic standpoint, lower hemoglobin levels associated with CIOM and fever events (all $p < 0.01$). **Conclusion:** The study evidences *S. aureus* as a potential pathogen in ALL-CIOM, reaffirming microbial control as an important preventive measure during high-dose immunosuppressive therapy. The weight of non-white-blood-cell parameters should be validated as novel CIOM biomarkers in prospective research.

Keywords: Oral mucositis. Acute Lymphoblastic Leukemia. Antineoplastic agents. Polymerase chain reaction. Bacteria.

Introduction

Acute lymphoblastic leukemia (ALL) is the most prevalent cancer in childhood and the second most common leukemia variant in adults[1,2]. For decades, the administration of chemotherapeutic agents (chemotherapy) has been the gold standard treatment approach for ALL, offering high success rates that may vary according to patient age, disease subtype, clinical profile and country of origin[2-4]. Although immunosuppressive therapy represents a pillar strategy in cancer, it frequently engenders a serious side-effect known as mucositis, with prevalence ranging from 30% to 98% of patients[5-8]. Indeed, oral mucositis (OM) is considered the most debilitating isolated condition during antineoplastic therapy[9], bearing significant impact on the patient's quality of life, treatment adherence, and prognosis[10,11]. It is especially significant in cases requiring hematopoietic stem cell transplantation, where high-dose therapeutic regimens are more commonly required[11]. In this reality, a significant portion of leukemia patients is expected to develop at least some degree of OM in the first weeks of hospitalization[8] and, therefore, caregivers must be prepared to be involved responsibly.

Despite its undeniable relevance for adherence and prognosis of cancer treatment, the pathobiological mechanisms that govern OM have not been fully disclosed, often leading caregivers to commit to multi-agent therapy in the hope of increasing favorable outcome[12]. OM is currently regarded as a cascade result of biologically complex host-related events[10,13] with proinflammatory mediators harboring important predisposing effects[10,11,14]. At the cellular level, chemical toxicity to epithelial cells is believed to be the prevailing factor[12], leading to cellular alterations, apoptosis and dose-dependent enlargement of the intercellular space[15]. On the other hand, over the past two decades, research on the role of the oral microbiome in OM has yielded contrasting results. Although the reduction of anaerobic Gram-negatives and fungi by broad-spectrum antimicrobials has yielded positive impact on radiotherapy-induced oral mucositis (RIOM)[16,17], the taxonomic identity of bacteria at the species level has not yet reached clear consensus. More recently, oral dysbiosis has been highlighted as a key factor in the establishment and duration of both chemotherapy-induced oral mucositis (CIOM) and RIOM, with the potential involvement of periodontopathogen groups revealed by molecular-based methodologies[18,19]. Moreover, upregulation of chemotherapy-induced mucosal inflammatory factors by specific bacterial species was observed in an in vitro model of CIOM, suggesting a mechanism of drug and microbe collusion[15]. Nevertheless, the relevance of unconventional and non-oral bacterial consortia in the onset and severity of CIOM remains unattended[7], reaffirming the importance of complementary disease association initiatives[20].

Considering the existing evidence and the prolonged period of hospitalization recommended by ALL treatment guidelines, the involvement of nosocomial pathogens in CIOM should not be ruled out. In this sense, we evaluated 14-day longitudinal changes in OM status, peripheral blood components, and presence of the opportunistic pathogens *S. aureus* and *P. aeruginosa* in ALL patients undergoing a high-dose canonical chemotherapy regimen, to shed additional light into the biological factors governing CIOM.

Materials and Methods

Ethical considerations: The study was approved by the Human Research Ethics Committee of the Amazonas State University (UEA, Manaus, Amazonas, Brazil, CAAE number 46295215.0.0000.5016) in compliance with the 196/96 Resolution of the National Health Council of the Brazilian Health Ministry and the 7th version of the Declaration of Helsinki (2013). Written informed consent was obtained from all the participants, parents, or legal guardians, prior to sample collection.

Patient selection: The study population consisted of acute lymphoblastic leukemia (ALL) patients regularly attended at the Hematology and Hemotherapy Hospital Foundation (FHEMOAM, Manaus, Amazonas, Brazil). Diagnosis was made by clinical and laboratorial analysis, including complete blood count, myelogram and immunophenotyping by flow cytometry, according to the guidelines of the Brazilian Cooperative Group for Treatment of Childhood ALL (GBTLI ALL-99)[12]. Volunteers were recruited from patients referred for chemotherapy, regardless of gender and age. Individuals previously submitted to antibiotic therapy in the 3-month period prior to sample collection, as well as those with systemic comorbidities, were not included. Samples were collected within the "remission induction phase" of the GBTLI ALL-99 protocol, in which low and high risk subjects were treated with a unified drug cocktail. The chemotherapy regimen consisted of a combination of prednisone (40 mg/m^2/day), vincristine (1.5 mg/m^2/week), L-asparaginase (5000 UI/m^2/day), and daunorubicin (25 mg/m^2/week) coupled with intrathecally delivered methotrexate, cytarabine and dexamethasone with doses adjusted by age group[21].

Whole blood collection and cell flow cytometry analysis: Peripheral blood samples were collected by venipuncture as part of the routine follow-up of ALL treatment. Samples were collected using 5.0 mL EDTA vacuum tubes at two distinct time points: 1 day prior to chemotherapy (D0) and 14 days after chemotherapy initiation (D14). Hemoglobin levels and platelet, leukocyte and neutrophil counts were quantified in a flow cell hematology analyzer (ADVIA 2120i, Siemens).

Intraoral examination and specimen collection: Clinical examination was performed by a single practitioner to ensure maximum consistency. Classification of mucositis was performed according to the World Health Organization (WHO) oral toxicity scale system: Grade I (mild: oral soreness, erythema), Grade II (moderate: oral erythema, ulcers, solid diet tolerated), Grade III (severe: oral ulcers, liquid diet only), Grade IV (life-threatening: oral feeding impossible). Oral pain was considered positive when the patient reported spontaneous oral discomfort or pain when speaking or swallowing. For maximum representation of the oral microbiota, samples were composed of a pool of saliva and mucosa swab samples, collected on D0 and D14. Samples were obtained by intraoral saliva accumulation for 1 minute, followed by displacement into 25 mL collection tubes containing Tris-EDTA buffer (TE) pH 8.0 as nucleic acid preserver. Mucosal swab samples were obtained by scratching the buccal mucosa, hard palate, buccal vestibule and mouth floor with a sterile cotton swab, followed by placement of the swab tip in the collection tubes.

Reference bacteria growth: *Staphylococcus aureus* and *Pseudomonas aeruginosa* reference strains were gently provided by the Oswaldo Cruz Foundation (Fiocruz, Manaus, Brazil). Bacterial cells were initially reactivated by streaking on trypticase soy agar plates at 37°C under aerobic conditions. After initial bacterial growth, one colony per species was transferred to a tube containing trypticase soy broth, and incubated in an orbital shaker at 37°C at 150 rpm for 24 hours, aiming for optimal cell growth.

PCR amplification of bacterial DNA: Samples and reference bacteria were vortexed for 1 min, sedimented by centrifugation at 10,000 x g, and the supernatant was discarded. DNA extraction was conducted with PureLink® Genomic DNA Mini Kit according to the manufacturer's instructions, with addition of lysozyme and proteinase K to ensure lysis of Gram-negative and Gram-positive bacteria. Genomic DNA was quantified by Qubit 3.0 colorimetric assay (Invitrogen, Carlsbad, USA), and nucleic acid quality was assessed by the A260/A280 ratio, with accepted values ranging from 1.8 to 2.0. The polymerase chain reaction (PCR) was performed in 20 µL total volume in Veriti® thermocycler (ThermoFisher, Waltham, USA) with species-specific oligonucleotide primers (Table 1)[22,23], according to the following conditions: 1 x PCR buffer, 1.5 mM $MgCl_2$ (1,5 mM), 0.2 mM dNTPs, 0.2 µM each primer, 1.0 U Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, USA) and 5.0 ng sample DNA. The thermocycling profile for *P. aeruginosa* was: initial denaturation at a 94°C for 2 min, followed by 25 cycles of DNA denaturation at 94°C for 1 min, primer annealing at 60°C for 40 sec, DNA extension at 72°C for 30 sec, and a final extension step at 72°C for 2 min. The thermocycling profile for *S. aureus* was: initial denaturation at a 94°C for 2 min, followed by 25 cycles of DNA denaturation at 94°C for 1 min, primer annealing at 55°C for 30 sec, DNA extension at 72°C for 30 sec, and a final extension step at 72°C for 2 min. PCR amplicons from reference bacteria (positive controls) and clinical samples were subjected to agarose gel electrophoresis. For this purpose, 3.0 µL of the amplified DNA was loaded into a 1.5 % agarose gel prepared with 1x TBE buffer (0.002 M EDTA, 0.089 M Tris, 0.089 M Boric acid) and Gelred stain (Biotium). Samples were electrophoretically shifted under 700 mA for 60 min, using 1 Kb Plus DNA Ladder (Invitrogen) as the amplicon size reference. The DNA was then visualized under UV for validation of PCR efficiency and specificity, and digitally registered via transilluminator (L-PIX, Loccus Biotecnologia).

Statistical analysis: The Shappiro-Wilk test was used as preliminary tool to verify data distribution. Associations among oral bacteria and clinical findings were assessed by Fisher's Exact test, or by the Chi-square test with Yates correction when applicable. Associations among blood parameters and mucositis or oral bacteria were evaluated with the Mann-Whitney test. Differences in bacteria detection frequencies between timeframes were assessed using paired-sample McNemar test.

Results

Overall, 46 ALL patients, 27 males (58.7%) and 19 females (41.3%) ranging from 2 to 59 years-old (median 7.5 years-old) were analyzed. Patient clinical, hematological

and dietary features are summarized in Table 2. In terms of oral health status, 19 subjects (41.3%) developed at least some degree of OM after 14 days of chemotherapy initiation, with the majority of cases represented by grade 1. PCR detection of bacteria revealed *P. aeruginosa* in 5/46 (10.9%) and 9/46 (19.6%) subjects, and *S. aureus* in 11/46 (23.9%) and 20/46 (43.6%) subjects (D0 and D14 respectively), with a paired sample Mcnemar test indicating significantly increased detection rates at D14 for *S. aureus* (p = 0.035), but not for *P. aeruginosa* (p = 0.344). Moreover, *S. aureus* was significantly associated with mucositis, fever, nausea, and adoption of semi-solid or liquid diet, whereas *P. aeruginosa* did not correlate with clinical or dietary parameters (Table 3). At the systemic standpoint, the application of the Mann-Whitney non-parametric test among blood component levels (hemoglobin, platelets, neutrophils and leucocytes), OM status and bacterial detection unveiled significant associations between lower hemoglobin levels (at D0 and D14) and OM onset, as well as between lower hemoglobin levels (at D14) and *P. aeruginosa,* as described in Figure 1.

Table 1. Species-specific oligonucleotide PCR primers used in the study.

Target species	gene	Oligonucleotide sequence	Amplicon size
Pseudomonas aeruginosa	*oprL*	Forward 5'–ATGGAAATGCTGAAATTCGGC–3'	504 bp
		Reverse 5'–CTTCTTCAGCTCGACGCGACG–3'	
Staphylococcus aureus	*nuc*	Foward 5'–GCGATTGATGGTGATACGGTT–3'	447 bp
		Reverse 5'–AGCCAAGCCTTGACGAACTAAAGC–3'	

Table 2. Clinical, dietary, and blood parameters of 46 ALL subjects submitted to high-dose chemotherapy regimen.

Clinical features (n)	D0	D14
Systemic features		
Fever	17 (37.0%)	16 (34.8%)
Nausea	12 (26.1%)	12 (26.1%)
Diarrhea	8 (17.4%)	3 (6.5%)
Oral mucositis		
Grade 0 (no mucositis)	46 (100%)	27 (58.7%)
Grade 1 (mild)	0	12 (26.1%)
Grade 2 (moderate)	0	3 (6.6%)
Grade 3 (severe)	0	2 (4.3%)
Grade 4 (life-threatening)	0	2 (4.3%)

Continue

Continuation		
Diet type and administration (n)		
Free diet, oral	46 (100%)	27 (58.7%)
Semi-solid, oral	0	14 (30.4%)
Liquid, oral	0	5 (10.9%)
Liquid, nasogastric tube	0	0
Liquid, parenteral	0	0
Blood parameters (median, IQR)	**D0**	**D14**
Leukocyte (cells/mL)	10,965 (19,617)	9,003 (19,022)
Neutrophil (cells/mL)	4,008 (7,494)	3,143 (7,393)
Platelets (per mL)	85,156 (67,593)	84,451 (64,509)
Hemoglobin (g/dL)	8.55 (3.48)	7.70 (3.41)

Table 3. PCR detection of *P. aeruginosa* and *S. aureus* in 46 acute lymphoblastic leukemia patients 14 days after high-dose chemotherapy initiation (D14), according to clinical and dietary parameters.

Parameter	Parameter status/type	Positive for *P. aeruginosa*	Positive for *S. aureus*
Mucositis	observed	26.3% (5/19) †	78.9% (15/19) † **
	not observed	14.8% (4/27)	18.5% (5/27)
Oral pain	observed	40.0% (2/5) †	80.0% (4/5) †
	not observed	17.1% (7/41)	22.0% (9/41)
Fever	observed	31.3% (5/16) †	68.8% (11/16) † *
	not observed	13.3% (4/30)	30.0% (9/30)
Nausea	observed	16.7% (2/12) †	75.0% (9/12) † *
	not observed	58.8% (20/34)	29.4% (10/34)
Diet	type I	14.8% (4/27) †	18.5% (5/27) † **
	type II	26.3% (5/19)	78.9% (15/19)

† Chi-square Test with Yates correction; † Fisher Exact Test (* $p < 0.05$; ** $p < 0.001$). Diet type I = free consistency; Diet type II = liquid or semi-solid

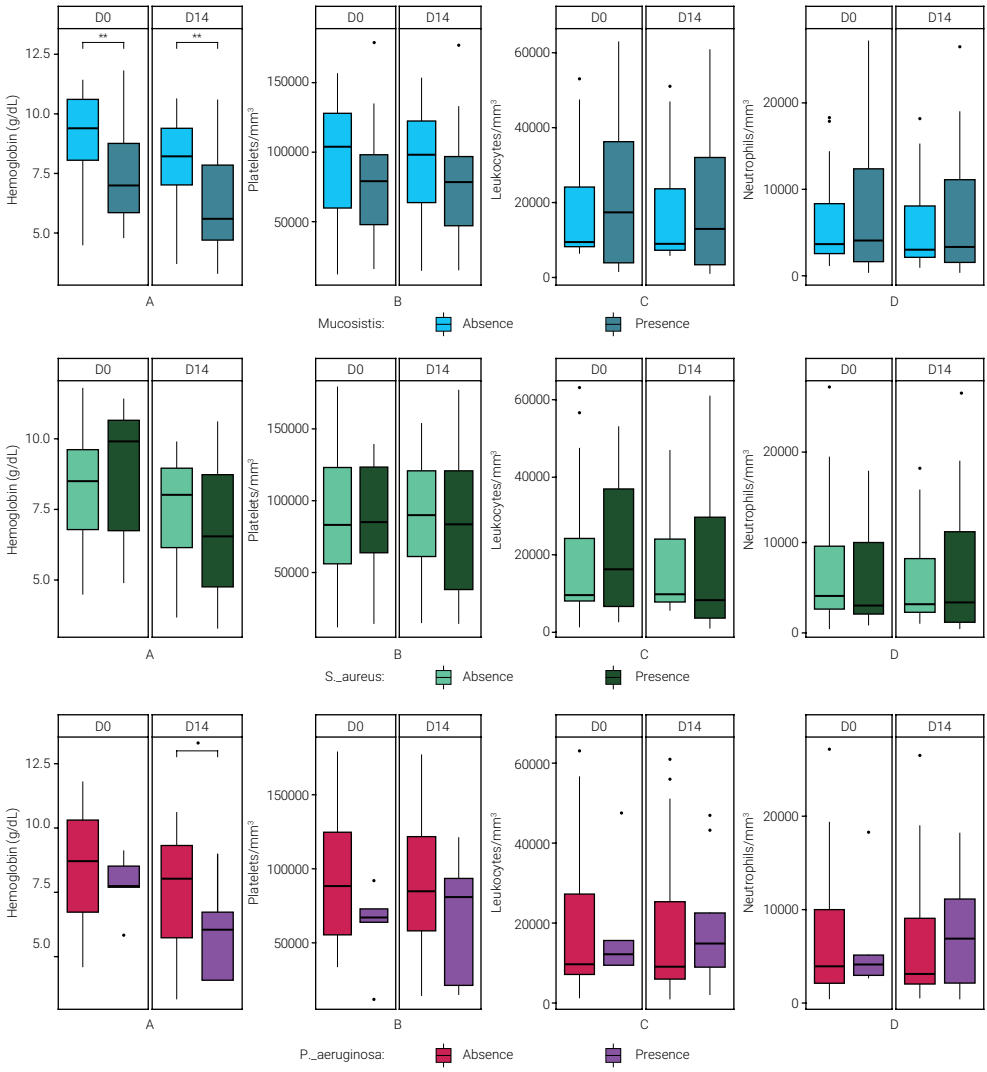

Figure 1. Hemoglobin (A) levels and platelet (B), leucocyte (C) and neutrophil (D) counts according to presence of mucositis and oral bacteria in 46 acute lymphoblastic leukemia patients, at baseline (D0) and 14 days after high-dose chemotherapy initiation (D14). Mann-Whitney test (* $p < 0.05$, ** $p < 0.01$).

Discussion

This study was conducted to assess the influence of high dose immunosuppressive chemotherapy on oral mucositis and bacterial status, and to investigate potential relationships among the detected bacteria, oral health and systemic parameters. We chose a 14-day timeframe based on a previous report, according to which OM clinical signs peak at around 7 and 14 days after chemotherapy installment, with spontaneous resolution around a week after[10]. Overall, the prevalence of chemotherapy-induced OM fell within the expected 30%-75% range reported by earlier studies[24,25], but lower than those disclosed by recent research on various cancer types[5,7,8]. In respect to OM severity, most cases belonged to the mild grade 1 OM WHO type, with few occurrences of grade 2, and very rare cases of grades 3 and 4.

As the severity-based subcategorization resulted in excess of data stratification, OM was considered binomially, with statistical analysis performed regardless of clinical levels, also considering the fact that bacterial abundances are generally comparable among different OM grades[20].

The weight imposed by oral bacteria on OM establishment and progression has been a long-lasting concern in hematology, and the preventive effect of oral hygiene on cancer patients brings incisive clues to a pathological role in CIOM[8]. Although the advent of 16S rDNA-based methodologies has revealed higher relative abundances of Gram-negative anaerobic bacilli and periodontopathic bacteria in CIOM and head-and-neck cancer RIOM[18,20], the influence of non-indigenous oral species on OM rests undetermined. In this study, *S. aureus* and *P. aeruginosa* were chosen as target species due to their well-established virulence arsenal and nosocomial transmission patterns. Truthfully, both species have been described as opportunistic pathogens of the skin, open wounds, lungs and the bloodstream[26,27], with *S. aureus* contributing to higher cancer mortality rates[28], and *P. aeruginosa* to antibiotic-resistance related deaths in acute leukemia[29], leading to the assumption of a potential role in OM.

Overall, our results revealed *S. aureus* as a putative pathogen in CIOM, presenting significant correlation with mucositis, nausea, and adoption of non-solid diet (all $p < 0.05$). In accordance, *S. aureus* lays among the most prevalent genera in saliva, buccal mucosa and lateral tongue of radio and chemotheray-treated subjects[20,30] and has been previously detected in intra and extra-oral complications, including apical periodontitis and panstomatitis[30-33]. Here, oral microbiome disruption promoted by chemotherapeutic agents[34] could be the prevailing determinant for *S. aureus* higher proliferation rates at D14, but prospective research based on broad microbial detection techniques should be conducted to validate such hypothesis. Furthermore, the association between blood stream detection of *S. aureus* and severe cases of CIOM has already been pointed out previously[33], partially accrediting our observations and, at the same time, suggesting the oral-to-bloodstream route as a potential pathway to bacteremia. On the other hand, although an increase in the number of subjects harboring *P. aeruginosa* was detected at D14 when compared to D0, we could not disclose relevant associations between this species and the establishment of OM, bestowing the results of a 16S rDNA-based study on CIOM in various cancer types[34]. Taken together, the results, along with the evident ubiquity of *S. aureus* in chemo and radiotherapy-treated cancer patients[20,30], stress the need for thorough oral microbial characterization in ALL prior to chemotherapy installment as CIOM risk-assessment measure. We also believe that judicious administration of antimicrobials is an important preventive step towards mitigation of OM, as previously illustrated by the positive outcomes obtained with broad-spectrum antibiotics such as tobramycin[16,17]. This holds greater importance for neutropenic subjects, whom are susceptible to bacteremia by *S. aureus*, thus prone to life-threatening risk[30]. It is worth noting that, even though our results indicate *S. aureus* as a putative pathogen in CIOM, dysbiosis could still be playing a relevant role[18,27] and, in this reality, efforts should be taken in assessing the ecological interactions that, together, could boost the cytotoxic effects elicited by chemotherapy[34].

Finally, the results also revealed a positive correlation between *S. aureus* and fever in ALL (D14) ($p < 0.05$). Nonetheless, since the presence of *S. aureus* was not directly assessed in peripheral blood, a systemic culprit could not be defined. In addition, as lower peripheral hemoglobin and platelet levels (both at D0 and D14) related to pyrexia (at D14) (Mann-Whitney, $p < 0.01$ and $p < 0.05$, respectively) (figure 1), the results point to underlying host-related mechanisms that may deserve further consideration. At the systemic standpoint, our results revealed that curtailed hemoglobin levels (at D0 and D14) are predictive of OM onset at D14 (Mann Whitney, $p < 0.01$) (figure 1) and are generally in line with the recent observations obtained in severe OM associated with head and neck cancer radiotherapy[35], suggesting non-white-blood-cell (non-WBC) parameters as promising variables for future investigations. On the other hand, we did not detect significant associations among neutrophil and leukocyte counts with systemic or intraoral clinical parameters, partially contrasting the findings of the same study. Regarding the association of peripheral blood components and bacteria, an inverse correlation was observed between hemoglobin levels and PCR detection of *P. aeruginosa* in saliva and mucosal samples. All other blood parameters had no relevant influence on either *P. aeruginosa* or *S. aureus* detection rates.

In conclusion, this study suggests *S. aureus* as a putative pathogen in ALL-CIOM and emphasizes the importance of preventive measures during the early stages of high-dose immunosuppressive therapy, including the adoption of higher oral hygiene standards and intrahospital professional oral assistance. Finally, the predictive power of non-WBC peripheral blood components in ALL-CIOM should be appraised in prospective research, aiming for more precise diagnosis and risk-directed case management.

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Conflicts of interest

The authors declare that there are no conflicts of interest in this work.

Author contribution

ACN Duarte: Study conception, sample collection, laboratory analysis;

AN Barbosa: Laboratory analysis;

CPB Saito: Stylistic manuscript revision;

EV de Paula: Study Design, manuscript revision;

D Saito: Study Design, manuscript drafting;

All authors have revised and approved the final version of the manuscript.

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