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Etiology of Pulmonary Infections in Human Immunodeficiency Virus–infected Inpatients Using Sputum Multiplex Real-time Polymerase Chain Reaction

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Background. There are limited data on the etiology of respiratory infections in human immunodeficiency virus (HIV)–infected patients in resource-limited settings.

Methods. We performed quantitative multiplex real-time polymerase chain reaction (PCR) for *Pneumocystis jirovecii* and common bacterial and viral respiratory pathogens on sputum samples (spontaneous or induced) from a prospective cohort study of HIV-infected inpatients with World Health Organization danger signs and cough. Mycobacterial culture was done on 2 sputum samples, blood cultures, and relevant extrapulmonary samples.

Results. We enrolled 284 participants from 2 secondary-level hospitals in Cape Town, South Africa: median CD4 count was 97 cells/μL, 64% were women, and 38% were on antiretroviral therapy. One hundred forty-eight had culture-positive tuberculosis, 100 had community-acquired pneumonia (CAP), 26 had *P. jirovecii* pneumonia (PJP), and 64 had other diagnoses. Probable bacterial infection (>10^5 copies/mL) was detected in 133 participants; the prevalence was highest in those with CAP (52%). *Haemophilus influenzae* and *Streptococcus pneumoniae* were the commonest bacterial pathogens detected; atypical bacteria were uncommon. Viruses were detected in 203 participants; the prevalence was highest in those with PJP (85%). Human metapneumovirus was the commonest virus detected. Multiple coinfections were commonly detected.

Conclusions. Sputum multiplex PCR could become a useful diagnostic tool for bacterial respiratory infections in HIV-infected inpatients, but its value is limited as quantitative cutoffs have only been established for a few bacterial pathogens and validation has not been done in this patient population. We found a high prevalence of respiratory viruses, but it is unclear whether these viruses were causing infection as there are no accepted quantitative PCR cutoffs for diagnosing respiratory viral infections.

Keywords. HIV; bacterial pneumonia; *Pneumocystis jirovecii* pneumonia; tuberculosis; respiratory viral infections.

Pulmonary infections remain the commonest human immunodeficiency virus (HIV)–associated complication in the antiretroviral therapy (ART) era [1]. A systematic review of hospitalizations in HIV-infected patients in the ART era reported that tuberculosis (TB), community-acquired pneumonia (CAP), and *Pneumocystis jirovecii* pneumonia (PJP) collectively accounted for 57% of inpatient deaths in adults globally [2]. Tuberculosis is the commonest cause of HIV-associated hospitalization worldwide, especially in low- and middle-income countries [3]. The view that PJP is uncommon in HIV-infected adults in sub-Saharan Africa has been challenged by a systematic review, which reported a prevalence of 22.4% among inpatients [4]. The risk of mortality in inpatients is increased by delayed diagnosis of TB, PJP, and CAP, but differentiating between them is difficult as they have overlapping clinical presentations.

The World Health Organization (WHO) algorithm for the diagnosis of TB in HIV-infected seriously ill patients recommends broad-spectrum antibiotics for all patients and that treatment for PJP should be considered without giving guidance on which seriously ill patients should be empirically treated for PJP [5]. Considerable progress has been made to improve the rapid diagnosis of TB in inpatients with the development of point-of-care urine lipoarabinomannan and Xpert MTB/RIF assays, both of which correctly identified >90% of participants with culture-confirmed TB in a prospective cohort study of seriously ill HIV-infected inpatients [6]. The etiology of HIV-associated CAP is thought to be similar to that in HIV-uninfected adults, but there are limited data on the role of atypical bacteria [1]. Bacterial cultures of sputum and blood are often negative in CAP due to the high proportion of patients who are on antibiotics. Tuberculosis in HIV-infected patients often presents acutely and mimics CAP [7, 8]. There is limited

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evidence on the role of viruses in HIV-associated pulmonary infections [1]. PJP is usually diagnosed clinically in low- and middle-income countries where facilities for bronchoscopy, sophisticated chest imaging, and microbiologic identification of *P. jirovecii* are limited. However, a systematic review found poor diagnostic value of chest radiography, symptoms, signs, and basic laboratory tests for PJP [9]. Finally, differential diagnosis is further complicated by coinfections, which are common, especially in patients with PJP who often have coinfections with bacteria, cytomegalovirus, and/or TB [4, 9].

We determined the prevalence of TB on culture, and of respiratory viruses, bacteria, and *P. jirovecii* on sputum multiplex polymerase chain reaction (PCR) in a prospective cohort study of HIV-infected inpatients with WHO danger signs and cough.

METHODS

We conducted a prospective cohort study to improve the evidence base for the WHO algorithm for the diagnosis of TB in seriously ill HIV-infected inpatients. This cohort has been reported in detail elsewhere [10]. In brief, the study was conducted in 2 regional hospitals in Cape Town, South Africa. Inclusion criteria were as follows: adults (≥18 years of age), HIV-infected, screened within 24 hours of admission, current cough (any duration), and 1 or more WHO danger signs (respiratory rate >30 breaths/minute, heart rate >120 beats/minute, temperature >39°C, and inability to walk unaided). Performance of sputum real-time multiplex PCR was an additional inclusion criterion for the current study (this was performed consecutively in the last 284 enrolled participants). Exclusion criteria were anti-TB therapy (either current, completed in the previous month, or interrupted in the past 6 months), exacerbation of congestive cardiac failure or chronic obstructive pulmonary disease, and being unable to produce a spontaneous or induced sputum sample. Cotrimoxazole prophylaxis and TB preventive therapy (limited to isoniazid in our national treatment program) were permitted.

Demographic and clinical data were recorded on standardized case record forms and entered into a database.

In keeping with WHO recommendations, all participants were commenced on empiric broad-spectrum antibiotics (typically ceftriaxone or amoxicillin-clavulanate) either at the referring clinic or on admission to the hospital emergency unit. Chest radiographs were retrospectively reviewed by a specialist radiologist who was blinded to the diagnoses.

Sputum induction, with hypertonic saline in an ultrasonic nebulizer, was done in participants unable to spontaneously produce sputum. One sputum sample was sent for quantitative, multiplex, real-time PCR with FTDResp33 (Fast-Track Diagnostics, Esch-sur-Alzet, Luxembourg) to identify potential respiratory pathogens (for details of sample preparation, see Dube et al [11]); there was no minimum volume required. Standard curves were derived using plasmid standards supplied by the manufacturer, and genome copy number was extrapolated from these curves. We used a cutoff of $>10^5$ copies/mL to distinguish between bacterial colonization and infection [12]. The limit of quantification ranged from 1.12×10^3 to 1.6×10^8 copies per reaction. Two sputum samples were sent for smear microscopy with auramine staining and liquid mycobacterial culture (BACTEC MGIT 960; Becton, Dickinson and Company, Franklin Lakes, New Jersey), and the Xpert MTB/RIF assay (Cepheid, Sunnyvale, California) was done on one sample. Sputum multiplex PCR was done after study completion, but Xpert MTB/RIF and mycobacterial cultures were done in real time and results made available to the treating clinicians. Mycobacterial blood culture (BacT/Alert MP; bioMérieux, Durham, North Carolina) was done in all participants. Where appropriate, other extrapulmonary specimens were sent for liquid mycobacterial culture.

Case definitions were as follows: TB, culture of *Mycobacterium tuberculosis* from any site; PJP, >1000 copies/mL of *P. jirovecii* DNA in sputum on real-time PCR [13, 14]; CAP, cough ≤14 days and pulmonary consolidation on chest radiograph confirmed by a radiologist.

Statistical Analysis

Analyses were performed using Stata version 13 (StataCorp, College Station, Texas) and EpiInfo version 7.1.4.0 software. Distributions of nonparametric data were expressed as median and interquartile range (IQR). Odds ratios (ORs) and 95% confidence intervals (CIs) were determined pairwise to compare proportions of pathogens by case definition, excluding participants who fulfilled both of the relevant case definitions.

Ethics Approval

The study was approved by the Human Research Ethics Committee, University of Cape Town. Eligible participants signed informed consent before enrollment into the study. Confused participants were enrolled and given the option to continue with participation once oriented; their data were removed from the study if consent was declined.

RESULTS

We enrolled 284 participants from January 2012 to October 2014; participant enrollment is shown in Figure 1. Almost all potentially eligible patients were able to produce an induced or spontaneous sputum sample. Participants' baseline characteristics and diagnoses are shown in Table 1. Most participants had >1 WHO danger sign. Criteria for the 3 case definitions were fulfilled in 220 participants. Fifty participants fulfilled >1 case definition: 4 had CAP, PJP, and TB; 39 had CAP and TB; and 7 had CAP and PJP. The 64 participants with "other diagnoses" were medical officer discharge diagnoses of TB (n = 4), CAP (n = 43), and PJP (n = 7) that did not fulfill our case definitions, and 10 with miscellaneous conditions. Chest radiographs were not available for reporting in 32 participants.

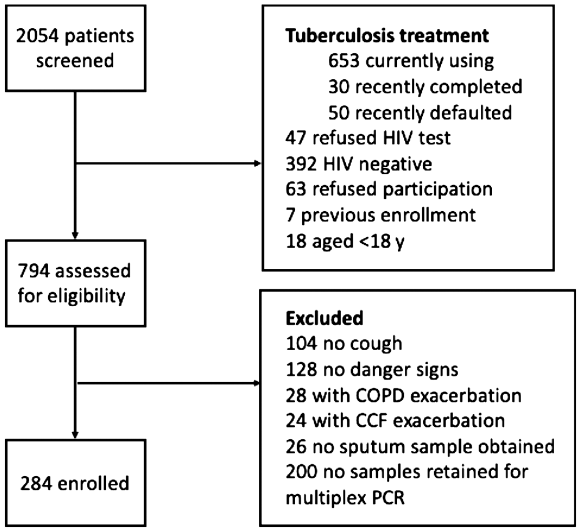

Figure 1. Flow diagram for participant enrollment into the study. Abbreviations: CCF, congestive cardiac failure; COPD, chronic obstructive pulmonary disease; HIV, human immunodeficiency virus; PCR, polymerase chain reaction.

Sputum Xpert MTB/RIF was performed on 281 participants, 147 of whom had culture-positive TB: Xpert was positive in 138 (5 false positives) with a sensitivity of 90.5% (95% CI, 84.5%–94.7%) and a specificity of 96.3% (95% CI, 91.5%–98.8%). The distribution of viruses and bacteria detected by sputum multiplex PCR is shown by diagnosis in Table 2. The prevalence of any bacteria >10^5 copies/mL by case definition was as follows: CAP, 52% (95% CI, 42%–62%); PJP, 34% (95% CI, 19%–54%); and TB, 41% (95% CI, 34%–49%). Participants with CAP were more likely to have bacteria >10^5 copies/mL than participants with PJP (OR, 3.06 [95% CI, 1.09–8.62]) or TB (OR, 1.94 [95% CI, 1.01–3.72]); this association was strengthened when limited to *Streptococcus pneumoniae* infection—CAP vs PJP (OR, 3.80 [95% CI, .47–30.76]) and CAP vs TB (OR, 6.06 [95% CI, 2.45–15.02]). Bacteria >10^5 copies/mL were similar in participants with TB and PJP (OR, 1.49 [95% CI, .57–2.41]). The prevalence of any virus by case definition was as follows: CAP, 67% (95% CI, 57%–75%); PJP, 85% (95% CI, 66%–94%); and TB, 73% (95% CI, 65%–79%). Participants with PJP were more likely to have viruses than participants with CAP (OR, 7.12 [95% CI, .89–56.75]) or TB (OR, 2.35 [95% CI, .66–8.39]).

Table 1. Baseline Characteristics and Diagnoses of 284 Human Immunodeficiency Virus–infected Inpatients With World Health Organization Danger Signs and Cough

Characteristic	No. (%)
Female sex	182 (64)
Age, y, median (IQR)	36 (30–42)
CD4 count, cells/μL, median (IQR)	97 (35–219)[a]
Currently on ART	108 (38)
Duration on ART, y, median (IQR)	2.4 (0.2–4.4)[b]
Sputum induced	201 (71)
WHO danger signs	
Respiratory rate >30 breaths/min	210 (74)
Heart rate >120 beats/min	224 (79)
Temperature >39°C	46 (16)
Unable to walk unaided	146 (51)
Diagnoses	
TB culture positive from any site	148 (52)
TB culture positive from sputum	119 (42)
CAP	100 (35)
PJP	26 (9)
Other	64 (23)

Data are presented as no. (%) unless otherwise indicated.

Abbreviations: ART, antiretroviral therapy; CAP, community-acquired pneumonia; IQR, interquartile range; PJP, *Pneumocystis jirovecii* pneumonia; TB, tuberculosis; WHO, World Health Organization.

[a]1 missing value.

[b]4 missing values.

The distribution of genome copy number by virus is shown in box and whisker plots (Figure 2) for viruses detected in ≥10 participants (different species of coronavirus and parainfluenza virus were combined for this analysis). Detection of >1 pathogen was very common as illustrated by Figure 3A for all 284 participants and by Figure 3B for the 100 participants with CAP.

DISCUSSION

We found a high prevalence (47%) of probable bacterial infection on multiplex real-time PCR of sputum in a prospective cohort study of HIV-infected inpatients with WHO danger signs and cough. The proportion of bacterial infections, especially with *S. pneumoniae*, was higher in participants with CAP than in those with PJP or TB. Sputum multiplex PCR could become a useful diagnostic tool in HIV-infected inpatients who frequently receive antibiotics before or immediately on admission, as recommended by WHO: however, its value is currently limited as quantitative cutoffs have only been established for a few bacterial pathogens and validation has not been done in this patient population. We found an even higher prevalence (71%) of respiratory virus detection, but it is unclear whether these viruses were causing infection as there are no accepted quantitative PCR cutoffs for diagnosing respiratory virus infections. Viruses were more commonly detected in participants with PJP than in those with CAP or TB, but the confidence intervals were wide due to the relatively small number of participants with PJP.

Human metapneumovirus was the commonest viral pathogen detected in our cohort. Human metapneumovirus infection is predominantly seen in early childhood, but a South African epidemiological study showed that it was strongly associated with an increased risk of hospitalization among HIV-infected adults [15]. There are limited and contradictory data on the role of human metapneumovirus infection in HIV-infected adults: it was very uncommon in a Brazilian study of CAP in inpatients [16], but was commonly associated with respiratory infections in a Canadian outpatient study [17]. Cytomegalovirus was also

Table 2. Bacteria (>10^5 Copies/mL) and Viruses (Any Genome Copy Number) Identified on Sputum Multiplex Polymerase Chain Reaction by Diagnoses in 284 Human Immunodeficiency Virus–infected Inpatients With World Health Organization Danger Signs and Cough

Pathogen	TB Any Site (n = 148)	TB Sputum (n = 119)	CAP (n = 100)	PJP (n = 26)	Other Diagnoses (n = 64)
Bacteria (no.)					
Any bacteria (133)	61	44	52	9	35
Bordetella pertussis (0)	0	0	0	0	0
Chlamydophila pneumoniae (1)	0	0	1	0	0
Haemophilus influenzae (64)[a]	30	24	24	4	18
Klebsiella pneumoniae (12)	7	5	3	1	4
Legionella spp (0)	0	0	0	0	0
Moraxella catarrhalis (24)	14	9	11	3	4
Mycoplasma pneumoniae (7)	6	5	3	0	0
Salmonella spp (0)	0	0	0	0	0
Staphylococcus aureus (37)	23	15	9	1	8
Streptococcus pneumoniae (48)	13	10	24	1	15
Viruses (no.)					
Any virus (203)	108	89	67	22	44
Adenovirus (6)	5	5	2	0	1
Bocavirus (0)	0	0	0	0	0
Coronavirus 229 (3)	1	0	3	0	0
Coronavirus 43 (9)	4	4	4	0	2
Coronavirus 63 (3)	2	1	1	0	0
Coronavirus HKU (1)	0	0	1	0	0
Cytomegalovirus (58)	29	29	17	5	15
Enterovirus/parechovirus (3)	0	0	0	1	2
Influenza A (12)	7	7	6	1	2
Influenza B (0)	0	0	0	0	0
Influenza C (0)	0	0	0	0	0
Human metapneumovirus A/B (126)	71	52	39	16	27
Parainfluenza virus 1–4 (10)	6	6	6	1	1
Respiratory syncytial virus A/B (5)	2	2	2	0	3
Rhinovirus (21)	12	12	8	0	2

All data are presented as no.

Abbreviations: CAP, community-acquired pneumonia; PJP, *Pneumocystis jirovecii* pneumonia; TB, tuberculosis.

[a]Includes 3 *Haemophilus influenzae* type B.

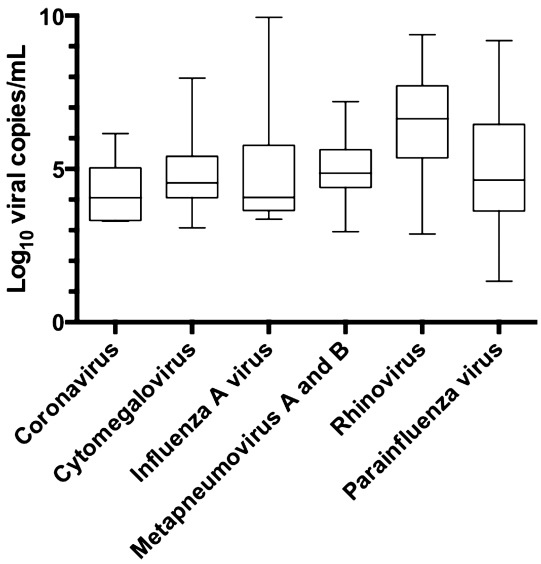

Figure 2. The distribution of genome copy number by virus is shown in box and whisker plots for viruses detected in ≥10 participants (different species of coronavirus and parainfluenza virus, respectively, were combined for this analysis). The horizontal line indicates the median, the box indicates the interquartile range, and the bars indicate the range.

commonly detected in our study and was detected in 17 of 26 participants with PJP. The role of cytomegalovirus as a respiratory pathogen is unclear in HIV-infected patients, especially in patients with PJP, who generally respond to therapy for PJP without an antiviral drug directed against cytomegalovirus [1].

The prevalence of *Haemophilus influenzae* in our participants with CAP was higher than is generally reported [1], but only 1 participant with CAP had *H. influenzae* type B detected.

In the expanded WHO algorithm for seriously ill HIV-infected patients, the use of antibiotics to cover both typical and atypical bacteria "should be considered" [18]. However, the role of atypical bacteria is uncertain in HIV-infected patients with CAP [1], partly due to the limitations of serological diagnosis in immunocompromised patients. A Kenyan study of inpatients with CAP found *Mycoplasma pneumoniae* in 2.7% of HIV-infected patients, and detected no cases of *Legionella* or *Chlamydia* [7]. By contrast, a South African study of inpatients with CAP (81.4% of whom were HIV seropositive) found *M. pneumoniae* in 17%, *Chlamydia pneumoniae* in 4.9%, and *Legionella pneumophila* in 0.9% [8]; the high prevalence of *M. pneumoniae* they reported could be explained if the study was

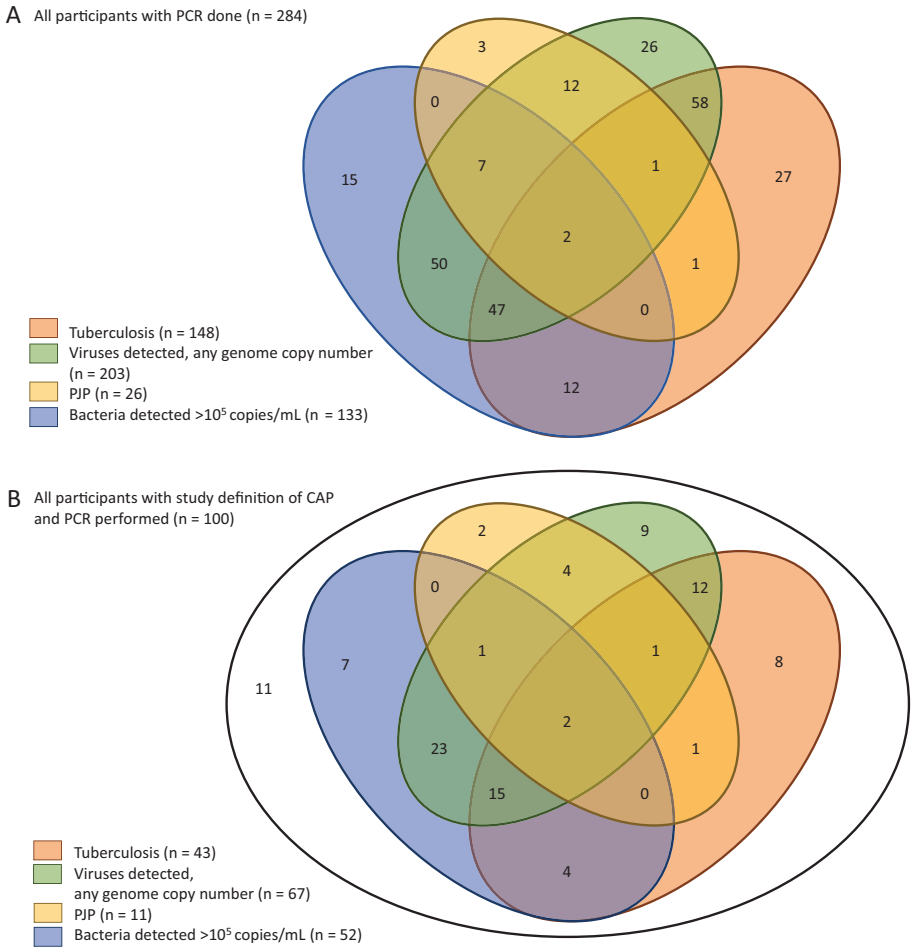

Figure 3. Venn diagrams of pathogen detection in all 284 participants (*A*) and in 100 participants fulfilling the case definition of community-acquired pneumonia (*B*). Abbreviations: CAP, community-acquired pneumonia; PCR, polymerase chain reaction; PJP, *Pneumocystis jirovecii* pneumonia.

done during an epidemic, which is likely as cyclical epidemics are a feature of *M. pneumoniae* epidemiology. We found that atypical bacteria were uncommon in seriously ill HIV-infected inpatients in our study using a molecular approach, which offers rapid diagnosis of atypical bacteria and should not be affected by immunostatus, unlike serology. South African surveys of inpatients (more than half of whom were HIV-infected) with severe respiratory illness found that *M. pneumoniae* was detected in 73 of 4703 (1.9%), which was not related to HIV status, *C. pneumoniae* in 11 of 2793 (0.4%), and *Legionella* species in 21 of 1805 (1.2%) using molecular approaches [19–21], supporting our finding that atypical bacteria are uncommon causes of respiratory infection in HIV-infected inpatients.

Our study has several limitations. First, we are unable to distinguish viral colonization from infection as there are no accepted quantitative PCR cutoffs for diagnosing respiratory virus infections. We are also unable to distinguish between viral upper and lower respiratory tract infections. Second, the cutoff we used for bacterial infection is derived from studies using quantitative culture but has not been well validated for all bacterial pathogens [12], and has not been validated in HIV-infected patients with CAP, PJP, or TB. Third, the quantitative real-time PCR cutoff for *P. jirovecii* that we used as the reference standard for PJP was based on 2 studies that reported cutoffs of 1900 copies/mL [13] and 1300 copies/mL [14] to distinguish between colonization and infection. Because there is currently no accepted cutoff [22], we arbitrarily used 1000 copies/mL, which was within 0.3 $\log_{10}$ copies/mL of the cutoffs of the other 2 studies (the lowest value in participants meeting our case definition was 1858 copies/mL). However, a meta-analysis found that real-time PCR has a sensitivity of 97% and a specificity of 93% for the diagnosis of PJP in HIV-infected patients [22], indicating that this test is a reasonable reference standard. Fourth, our findings were in inpatients with WHO danger signs and cough, in a population with a high prevalence of TB, and with a high proportion requiring sputum induction; therefore, our findings may not be generalizable to other populations of HIV-infected patients. Sputum induction is a low-cost intervention that could be widely implemented in resource-limited settings to facilitate identification of respiratory pathogens, including *M. tuberculosis*.

In conclusion, we found a high prevalence of probable bacterial infection on multiplex PCR of sputum in HIV-infected inpatients with WHO danger signs and cough. More research needs to be done to establish quantitative cutoffs for bacterial and especially viral infection in patients with and without HIV infection, and to establish if multiplex PCR can safely guide therapeutic decisions or improve outcomes.

Notes

Author contributions. G. M., M. M., and M. N. designed the study. R. G. and F. D. performed data acquisition. G. M. and R. G. analyzed the data. G. M. produced the first draft. All authors revised the work, approved the final version, and take accountability for all aspects of the work.

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