Interleukin-6 and vitamin D serum levels in patients with obstructive sleep apnea syndrome before and after long-term continuous positive airway pressure treatment

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Abstract

Background: Hypoxia induces the production of adipocyte-derived mediators such as IL-6 in obstructive sleep apnea syndrome (OSAS). Low serum *25-hydroxyvitamin D* (25(OH)D) levels have been linked to OSAS susceptibility. No data exist to assess whether there has been a correlation between vitamin D and IL-6 serum levels. The effect of CPAP therapy on IL-6 or 25(OH)D levels has yet to be investigated sufficiently in OSAS. We aimed at determining the serum levels of 25(OH)D and IL-6 in OSAS patients compared to non-apneic controls, investigating a possible correlation between 25(OH)D and IL-6 levels and evaluating the changes in IL-6 and 25(OH)D concentrations after twelve months of CPAP therapy in OSAS patients.

Methods: 15 OSAS patients diagnosed by polysomnography and 15 non-apneic controls were included in the study. Serum IL-6 and 25(OH)D levels were measured before and after twelve-month CPAP therapy in the whole population and OSAS group, respectively.

Results: IL-6 levels were significantly elevated in the OSAS group than the controls. IL-6 levels were positively correlated with OSAS severity, nocturnal hypoxemia, and body mass index (BMI). No difference was detected in 25(OH)D serum levels between groups. We found no correlation between IL-6 and 25(OH)D serum levels in two groups. No effect on IL-6 or 25(OH)D levels was detect after one year of effective CPAP therapy in OSAS patients.

Conclusions: IL-6 levels were correlated with OSAS severity, hypoxemia, and BMI. No correlation between 25(OH)D and IL-6 levels and no effect of long-term CPAP on biomarkers were found in OSAS patients.

Background

Interleukin-6 (IL-6) is a pleiotropic cytokine with both pro- and anti-inflammatory capacities, produced by different cells and tissues, such as leukocytes, adipocytes, and endothelium [1, 2]. Accordingly, elevated concentrations of IL-6 may indicate an ongoing inflammatory response due to systemic or localized infection or chronic inflammatory disease [1–4]. IL-6 plasma levels correlate with NF-κB-dependent endothelial dysfunction, arterial stiffness, and the extent of subclinical atherosclerosis [1, 2]. IL-6 has also been implicated in metabolic regulation and especially in lipid metabolic homeostasis, being predictive of incident type 2 diabetes and obesity [3, 4].

Hypoxia and inflammation share an interdependent relationship [5, 6]. The intermittent hypoxia, one of the hallmark characteristics of obstructive sleep apnea syndrome (OSAS), induce polarization of macrophages, adipose tissue inflammation, and production of adipocyte-derived mediators such as IL-6 [7].

Although there are contradictory data [8], most studies support that IL-6 serves as a reliable reporter or an actual effector of either OSAS or OSAS-associated morbidities [9]. Furthermore, it has been reported that continuous positive airway pressure (CPAP) therapy decreases IL-6 levels in OSAS patients [10].

Nevertheless, the effect of CPAP therapy on IL-6 status in OSAS patients has yet to be investigated sufficiently, given the lack of research in this area.

A low 25-hydroxyvitamin D (25(OH)D) serum concentration has been reportedly linked to OSAS susceptibility. The hypoxia in severe OSAS patients is a proposed mechanism for low serum 25(OH)D levels. The association between vitamin D deficiency and hypoxia in OSAS attributed to mechanisms involving hypoxia-inducible factor 1-alpha (HIF-1α) and vascular endothelial growth factor (VEGF)-related pathways that play a crucial role in inflammation [11]. Furthermore, the effect of CPAP therapy on vitamin D status in OSAS patients remains largely unexamined.

Previous studies documented that serum vitamin D levels were negatively correlated with serum IL-6 levels in stroke individuals or patients with different states of acute or chronic inflammation [12, 13]. However, no data exist to assess whether there has been a correlation between vitamin D and IL-6 plasma levels in OSAS patients.

The present study aimed to evaluate the serum levels of 25(OH)D and IL-6 in OSAS patients compared to non-apneic controls, a possible correlation between 25(OH)D and IL-6 serum levels in OSAS patients and the changes in IL-6 and 25(OH)D concentrations after twelve months of CPAP therapy in OSAS patients.

Methods

Patients

This prospective cohort pilot study was performed on 15 adult patients with OSAS diagnosed by standard polysomnography (PSG) according to the American Academy of Sleep Medicine (AASM) criteria [14] and 15 non-apneic adult controls (apnea-hypopnea index, AHI < 5/hour) who were also evaluated by PSG. The whole study population attended the Department of Respiratory Sleep Disorders of the University of Thessaly in Greece in a twelve-month period (August 2018 - August 2019).

Exclusion criteria for OSAS patients and controls were the following: chronic liver disease or chronic renal failure, diabetes mellitus, thyroid dysfunction, osteoporosis, malignancies, autoimmune or neuromuscular disorders, symptoms or signs of acute or chronic inflammation disorders, calcium or vitamin D supplements, and diuretic treatments.

Anthropometric, spirometric, and sleep characteristics of OSAS patients and controls were recorded at first evaluation. Height, weight were measured using standardized techniques. The body-mass index (BMI) was calculated according to the formula: Weight (kg)/height2(m).

Ethics

Informed consent was obtained from all individual participants included in the study. The Bioethics Committee of the Medical University of Thessaly approved the study (No 2566-31/05/2017).

Polysomnography

The patients referred to the sleep laboratory for suspected OSAS underwent full in-laboratory PSG, attended by an experienced sleep technician, from 22:00 to 06:00 hours, and variables were recorded on a computer system (Alice® 4, Philips Respironics, Murrysville, PA, USA). A standard montage of electroencephalogram, electrooculogram, electromyogram, and electrocardiogram signals was used. Arterial oxygen saturation was recorded by a digital pulse oximeter, and airflow was detected using combined oronasal thermistors. The thoracic cage and abdominal motion were also recorded using piezoelectric bands placed around the chest and abdomen. Respiratory events and electroencephalogram recordings were manually scored according to the standard criteria. Apnea was defined as a ≥ 90% reduction in airflow for at least 10 sec. Hypopnea was defined as a ≥ 30% reduction in airflow for at least 10 sec in combination with oxyhemoglobin desaturation of at least 3% or arousal registered by the electroencephalogram. AHI was defined as the sum of all apneas (> 90% reduction in airflow for > 10 seconds) and all hypopneas (> 30% reduction in AHI was defined as the sum of all apneas (> 90% reduction in airflow for > 10 seconds) and all hypopneas (> 30% reduction in airflow > 10 sec) associated with ≥ 3% O_2 desaturation. The AHI was calculated as the average number of apneas and hypopneas/h of PSG-recorded sleep time. OSAS was defined as AHI ≥ 5/hour accompanied by related symptoms. The OSAS severity was defined as mild (AHI 5–15 events/h), moderate (AHI 15–30 events/h), and severe AHI > 30 events/h) [15].

25(OH)D and IL-6 measurements

Blood sampling for 25(OH)D and IL-6 determination was performed the day after PSG after overnight fasting for at least eight hours in all study participants and was repeated after one year of starting on CPAP therapy in OSAS patients. Blood samples were centrifuged at (3500 rpm for 15 min), and the serum obtained was stored at − 80 °C until the time of analysis.

Serum 25(OH)D levels were measured using the commercially available Beckman Coulter Access Total 25(OH) Vitamin D assay on the Unicel DXI 800 analytic system (Beckman Coulter Inc - normal values > 30 ng/mL) The method is a two-step competitive binding, paramagnetic particle, chemiluminescent immunoassay using sheep monoclonal anti-25(OH) vitamin D antibodies. Briefly, 25(OH) Vitamin D present in the sample complexed with vitamin D binding protein is released from it and binds to the immobilized monoclonal anti-25(OH) vitamin D on the solid phase. After incubation with a 25(OH) vitamin D analogue-alkaline phosphatase conjugate and addition of a chemiluminescent substrate, the light generated is inversely proportional to the concentration of the analyte in the sample.

Concentrations of IL-6 were determined by two-site sandwich quantitative enzyme-linked immunosorbent assay using commercially available kits (IMMULITE®1000). The marker concentration was expressed in picograms per milliliter.

Statistical Analysis

All categorical data were reported as percentages. Differences in categorical data between groups were tested by two-tailed Pearson's chi-square or Fisher's Exact Test; correction for continuity was applied. Numerical data were reported as mean and standard deviation. Parametric data comparing two groups

were tested by two-tailed Student's t-test unpaired t-test, while non-parametric data were analyzed with the Mann-Whitney U test. Bivariate analyses of correlations of numerical data between groups were tested by Pearson's Correlation R. P-values smaller than 0.05 were considered significant. All statistical calculations were performed with IBM SPSS Statistics 20 software and graphs were designed using IBM SPSS Statistics 20 software and GraphPad Prism 6 software.

Results

Demographic, spirometric and sleepiness characteristics of OSAS and non-apneic control groups are presented in Table 1. 15 OSAS patients and 15 non-apneic subjects were included in the study. Among OSAS patients, 6.7% (n = 1) had mild OSAS, 26.6% (n = 4) had moderate OSAS, and 66.7% (n = 10) had severe disease. OSAS patients were significantly more obese and had more nocturnal awakenings than age-and gender-matched controls.

Table 1
Demographic, spirometric and sleepiness characteristics of obstructive sleep apnea syndrome patients and controls

Study group characteristics	OSAS patients (n = 15)	Controls (n = 15)	p-value
Gender	11 (73)	11 (73)	NS
Males n (%)	4 (27)	4 (27)	
Females n (%)			
Age, years	57.2 ± 8.2	55.5 ± 13.7	NS
BMI, kg/m^2	43.8 ± 2.7	37.7 ± 4.7	< 0.002
Current smoking n (%)	9 (60)	3 (20)	NS
FVC (% of predicted)	97.3 ± 13.6	99.5 ± 17.9	NS
FEV1 (% of predicted)	98.3 ± 18.7	93.9 ± 18.4	NS
FEV1/FVC	81.0 ± 6.9	78.7 ± 12.4	NS
ESS	10.2 ± 3.6	8.6 ± 4	NS
Sleep Hours	7.4 ± 1.5	7.3 ± 1	NS
Sleep apnea and snoring n (%)	24 (80)	18 (60)	NS
Night awakenings n (%)	13 (86.7)	4 (26.7)	0.003
Note: Data are expressed as mean ± SD or as frequencies (percentages)			
Abbreviations: BMI: body mass index, ESS: Epworth sleepiness scale, NS: non-significant			

The prevalence of comorbidities in OSAS patients and non-apneic controls are presented in Table 2. The prevalence of multiple comorbidity combinations was not significantly differentiated between the two groups.

Table 2
Comorbidities in obstructive sleep apnea syndrome patients and non-apneic controls

Comorbidities	**OSAS patients (n = 15)**	**Controls (n = 15)**	***p*-value**
Diabetes n (%)	5 (33.3)	1 (6.7)	NS
Hypercholesterolemia n (%)	5 (33.3)	3 (20.0)	NS
Thyroid disease n (%)	6 (40.0)	2 (13.3)	NS
Hypertension n (%)	7 (46.7)	5 (33.3)	NS
Coronary Artery Disease n (%)	4 (26.7)	0 (0)	NS
Gastroesophageal Reflux Disease n (%)	6 (40)	7 (46.7)	NS
Nasal septum deviation n (%)	4 (26.7)	0 (0)	NS
Note: Data are expressed as frequencies (percentages)			
Abbreviations: *NS: non-significant*			

Sleep parameters of OSAS patients and controls are presented in Table 3. As expected, there were significant differences in almost all PSG-derived sleep parameters among the groups.

Table 3
Sleep parameters of obstructive sleep apnea syndrome patients and controls at diagnosis.

Sleep parameters	OSAS patients (n = 15)	Controls (n = 15)	*p*-value
S1%	3.6 ± 1.9	4.0 ± 1.3	NS
S2%	64.2 ± 7.4	42.9 ± 5.1	< 0.001
S3-4%	10.2 ± 4.9	19.7 ± 2.9	< 0.001
REM %	9.8 ± 3.2	19.1 ± 2.4	< 0.001
AI (events/h)	14.4 ± 14.9	0.5 ± 0.8	< 0.001
HI (events/h)	21.7 ± 9.1	3.2 ± 1.2	< 0.001
AHI (events/h)	39.3 ± 16.7	3.9 ± 1.0	< 0.001
ODI (events/h)	36.9 ± 20.4	3.2 ± 2.2	< 0.001
MinSpO_2 %	75.2 ± 14.1	88.5 ± 5.9	< 0.001
Nocturnal awakenings (events/h)	37.6 ± 29.6	8.0 ± 5.9	< 0.001
T < 90%	48.0 ± 17.2	20.3 ± 13.3	< 0.001
Note: Data are expressed as mean ± SD			
Abbreviations: AHI: apnea– hypopnea index, AI: apnea index, HI: hypopnea index, MinSpO2: minimum oxyhemoglobin saturation, NS: non-significant, ODI: oxygen desaturation index, REM%: rapid eye movement, S1%: sleep stage 1, S2%: sleep stage 2, S3-4%: sleep stage 3–4, T < 90%: time with hemoglobin saturation < 90%.			

IL-6 and 25(OH)D serum levels in OSAS patients compared to controls at baseline, and IL-6 and 25(OH)D serum levels in OSAS patients after one year of starting on continuous positive airway pressure (CPAP) therapy are presented in Table 4.

Table 4
IL-6 and 25(OH)D serum levels in obstructive sleep apnea syndrome patients AND controls

Serum levels (At baseline)	OSAS patients (n = 15)	Controls (n = 15)	*p*-value
IL-6 (pg/mL)	3.4 ± 1.0	2.3 ± 0.3	< 0,001
25(OH)D (ng/mL)	24.9 ± 10.2	21.9 ± 5.8	NS
After 12 month-CPAP treatment			
IL-6 (pg/mL)	3.3 ± 1.0	-	-
25(OH)D (ng/mL)	20.4 ± 7.0	-	-

IL-6 levels were significantly higher in OSAS patients compared to the non-apneic control group at baseline (Table 4). IL-6 levels were positively correlated with BMI ($r = 0.06$, $p = 0.001$). There was no correlation between the IL-6 levels and other demographic, sleepiness characteristics or comorbidities. Conversely, the IL-6 levels were positively correlated with apnea–hypopnea index ($r = 0.4$, $p = 0.03$) and hypopnea index ($r = 0.5$, $p = 0.07$). Moreover, the IL-6 levels were negatively correlated with minimum oxyhemoglobin saturation ($r=-0.5$, $p = 0.002$). There was no correlation between the IL-6 and 25(OH)D levels neither in the whole study population nor in the subgroups of OSAS patients and controls.

The 25(OH)D levels were not differentiated between OSAS and control groups. There was no correlation between the 25(OH)D levels and demographic or sleepiness characteristics in two groups. Similarly, no correlation was found between 25(OH)D levels and comorbidities in two groups. The 25(OH)D levels were correlated neither with OSAS severity nor other sleep parameters in OSAS and control groups.

In OSAS patients, the IL-6 levels did not change significantly after twelve months of CPAP treatment (3.4 ± 1.0 vs. 3.3 ± 1.0 pg/mL, $p > 0.05$) (Table 4). Similarly, there was no significant difference in 25(OH)D levels at baseline and after one year of C-PAP treatment (24.9 ± 10.2 vs. 20.4 ± 7.0 ng/mL, $p > 0.05$) in the OSAS group (Table 4). It should be noted that all OSAS patients had good CPAP adherence defined as CPAP usage $\geq$ 4 h/night and 70% of nights or > five nights per week.

Discussion

In this study, we compared the IL-6 and 25(OH)D serum levels between OSAS patients and non-apneic controls, investigated the correlation between 25(OH)D and IL-6 serum levels in OSAS patients and evaluated serum level changes of IL-6 and 25(OH)D after long-term CPAP treatment in OSAS group. We found that the IL-6 levels were significantly elevated in the OSAS group than the control group. The most important findings were that IL-6 levels were positively correlated with OSAS severity and nocturnal hypoxemia. Importantly, IL-6 levels were positively correlated with BMI. No difference was detected in 25(OH)D serum levels between OSAS and non-apneic individuals. Furthermore, we found no correlation between IL-6 and 25(OH)D serum levels in two groups. In this pilot study, there was no effect detected on IL-6 or 25(OH)D levels in OSAS patients after one year of effective CPAP therapy.

Our findings support that patients with OSAS have higher pro-inflammatory cytokine basal levels. OSAS in both adults and children has been found to promote a persistently low-intensity inflammatory state, expressed by IL-6 [9]. The concentration of IL-6 has been comprehensively evaluated as a contributor to the regulation of sleep in brain areas such as the hypothalamus and hippocampus [16]. However, contradictory data also exist, found no significant difference in IL-6 serum levels between OSAS patients and controls [17]. Notably, it has been demonstrated that IL-6 levels in human serum exhibited significant circadian variation [18]. Conflicting results have been obtained more recently, found no significant 24-hour variation of serum IL-6 in a small number of severe OSAS males [19].

Our findings accord with earlier observations, showing that serum IL-6 levels were positively correlated with AHI. It has been supported that IL-6 measurements might be used in OSAS treatment follow-ups [20].

Importantly, a strong correlation between AHI changes and serum cytokine levels has been demonstrated independently of BMI [10]. Another study provided evidence connecting a distinct biomarker profile, including high IL-6 levels after sleep, in patients with moderate/severe OSAS than those with mild/no OSAS [21].

Conversely, another study in a population-based cohort of women indicated that intermittent hypoxia, and not the AHI, is related to systemic inflammation seen in OSAS [22]. OSAS is known as a repeated obstruction of the upper airway during sleep, leading to generalized hypoxia episodes. We found a positive correlation between oxygen desaturation and IL-6 inflammation. Hypoxemia manifested as the recurrence of desaturation or as lower levels of average or minimum SpO_2 is the main contributor to inflammation in OSAS [22, 23]. The intermittent hypoxia and chronic sleep fragmentation induce adipose tissue inflammation, the polarization of macrophages, and the production of IL-6 in both laboratory models and humans [7, 24–30]. Moreover, the variation in IL-6 levels is implicated in the quality and depth of sleep [31]. In our cohort, OSAS patients had significantly more nocturnal awakenings than age- and gender-matched controls.

A wide variety of cells can release IL-6. Biopsies of adipose tissue and blood samples in obese patients with and without OSAS revealed substantial increases in tissue expression and circulating levels of a variety of proinflammatory cytokines, including IL-6 [32]. In OSAS, it has been supported that adipose tissue is responsible for a significant proportion of circulating IL-6 [19, 33]. Consistent with the literature, this research found that OSAS patients who characterized by higher IL-6 levels were significantly more obese than age-and gender-matched non-apneic controls, and IL-6 levels were positively correlated with BMI. Obesity is one of the serious consequences leading to an uneven course of sleep. Obesity has been reported to pose a substantial risk for the development of OSAS. Adipose tissues interact with the respiratory system physiologically and pathologically by producing adipocytokines and regulating metabolic and inflammatory processes [34, 35]. IL-6 seems to contribute to weight gain in patients with OSAS and can modify the risk of obesity-related metabolic disorders [36]. Meanwhile, physical exercise has been found to improve inflammatory profiles [37].

Importantly, it has been demonstrated that increases in inflammatory markers in OSAS patients likely reflect the presence of underlying silent or overt end-organ morbidity [9]. IL-6 induces oxidative stress and promotes NF-κB-dependent endothelial dysfunction [24, 38]. Endothelial function as a prognostic parameter for cardiovascular events. Therefore, IL-6 levels appear to be predictive of future cardiovascular disease [39–46]. Nevertheless, in our cohort, the prevalence of comorbidities and cardiovascular disease was higher in OSAS patients but not significantly differentiated between OSAS and non-apneic groups.

The findings of the current study agree with the study by Kong et al. [47] documented that CPAP treatment does not consistently reduce elevated IL-6 levels. However, this issue has been challenged in research, by studies reported that IL-6 level changes were markedly attenuated by long-term CPAP therapy [10, 32] or sleep apnea surgical procedures [48]. The pooling of eight published reports in adults with

OSAS revealed that plasma IL-6 levels ranged from 1.2 to 131.7 pg/mL before CPAP treatment and significantly decreased to between 0.45 to 66.04 pg/mL after CPAP treatment [48]. However, among those studies, there was significant inter-individual heterogeneity [9] which may be related to the variance for IL-6 genes, environmental and/or lifestyle influences [49, 50]. Potential interactions between OSAS, obesity, and both genetic, environmental, and lifestyle factors, ultimately lead to a cascade of pathophysiological pathways, resulting in increased systemic inflammation, as illustrated by increased levels of IL-6 [9, 50].

There is scarce and conflicting evidence on the relationship between vitamin D deficiency and OSAS. We found no difference in 25(OH)D levels between OSAS patients and controls. This result reflects those of Salepci et al. [51] and Mete et al. [52] who also found no significant difference in 25(OH)D levels between OSAS patients and controls. Furthermore, we found no correlation between serum 25(OH)D status and the severity of OSAS. These findings also accord with earlier observations, which showed that vitamin D status does not alter the OSAS severity [53]. Contradictory data comes from a few studies reported that serum vitamin D levels were negatively associated with the increase in AHI [54, 55]. Consequently, there are still many unanswered questions on the cause and effect of vitamin D deficiency and replenishment in OSAS.

So far, there is only a few studies have assessed the effect of a week and one-year CPAP treatment on serum vitamin D levels in middle-aged OSAS men, concluded that short-term or long-term CPAP treatment improved vitamin D deficiency [56, 57]. Conversely, we found no effect on 25(OH)D levels in OSAS patients after one year of effective CPAP therapy. However, all OSAS patients in this cohort had normal 25(OH)D plasma levels.

Vitamin D plays an important role in the regulation of metabolism homeostasis, given its immunoregulatory functions. The adequate levels of serum 25(OH)D have been associated with lower levels of IL-6 [58] .On the contrary, vitamin D deficiency has been associated with chronic inflammatory states and a proinflammatory cytokine profile and has also been linked with augmented collagen synthesis, oxidative stress, and remodeling [59]. A negative correlation between vitamin D and IL-6 concentrations has been found in heart failure and atherosclerosis [59]. No data existed regarding vitamin D and IL-6 concentrations in OSAS patients. In this study, we found no correlation between IL-6 and 25(OH)D plasma levels in two groups.

Some limitations of the present study need to be acknowledged. With the small sample size, caution must be applied, as the findings might not be extrapolated to all OSAS patients with different clinical features. Furthermore, we did not assess sun exposure, dietary habits or metabolic biomarkers, such as parathyroid hormone, lipids, or glycemic indices that affect 25(OH)D levels. Another weakness of this study is that we did not assess polymorphisms in the vitamin D metabolism pathway (vitamin D receptor, 25-hydroxylase, and 1-α-hydroxylase), which are associated with perturbed vitamin D metabolism. Furthermore, other markers, such as high sensitivity C-reactive protein, were not evaluated. In addition, we were unable to demonstrate an effect of CPAP on biomarkers. This does not exclude the possibility that biomarkers are affected by long-term CPAP use.

Conclusions

Even though the evidence provided here is based on small sample size, we suggest that interment hypoxia in sleep apnea triggers inflammatory responses by activating biomarkers of immunity such as IL-6. IL-6 levels were significantly elevated in the OSAS group than the control group and were positively correlated with OSAS severity and nocturnal hypoxemia. Furthermore, the results agree with the findings of other studies in which IL-6 is linked with obesity. For the first time, the relationship between vitamin D levels and IL-6 in OSAS patients was explored, and we found no association between them. Further studies are required to find out which clinical consequences can be derived from the findings of the present study.

Abbreviations

AASM, American Academy of Sleep Medicine; AHI; apnea-hypopnea index; BMI, body mass index; CPAP, continuous positive airway pressure; HIF-1a, hypoxia-inducible factor 1-alpha; IL-6, Interleukin-6; OSAS, obstructive sleep apnea syndrome; 25(OH)D, 25-hydroxyvitamin D; PSG, polysomnography; VEGF, vascular endothelial growth factor

Declarations

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Bioethics Committee of the Medical University of Thessaly (No 2566-31/05/2017). Informed consent was obtained from all individual participants included in the study.

Consent for publication

The participant has consented to the submission of the case report to the journal.

Availability of data and material

The data that support the findings of this study are available on request from the corresponding author, OSK. The data are not publicly available due to restrictions e.g. their containing information that could compromise the privacy of research participants.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

KIG, CH, DIS, OSK, and CP conceived of the presented idea and designed the study. DIS, OSK, MK, VS, EG and CV, contributed to sample collection, sample preparation and data analysis. OSK wrote the manuscript. DIS contributed equally to the writing of the paper. DIS, OSK and MK carried out the experiment. KIG and CH supervised the study. All authors provided critical feedback and approved the final draft.

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