

## RESEARCH ARTICLE

**TNF- $\alpha$  and TGF- $\beta$  Contributes in Recurrent Otorrhea of Active Mucosal Chronic Otitis Media**Dewi Pratiwi<sup>1,\*</sup>, Marisa Rizqiana<sup>1</sup>, Adisetya Wicaksono<sup>1</sup>, Defitaria Permatasari<sup>1</sup>,  
Ratna Dwi Restuti<sup>2</sup>, Tri Nugraha Susilawati<sup>3</sup>, Sutarno<sup>4</sup><sup>1</sup>Department of Otorhinolaryngology Head and Neck Surgery, Faculty of Medicine, Universitas Sebelas Maret – Moewardi Hospital, Jl. Kolonel Sutarto No.132, Surakarta, Indonesia<sup>2</sup>Department of Otorhinolaryngology Head and Neck Surgery, Faculty of Medicine, Universitas Indonesia – Cipto Mangunkusumo Hospital, Jl. Diponegoro No.71, Jakarta, Indonesia<sup>3</sup>Department of Microbiology, Faculty of Medicine, Universitas Sebelas Maret, Jl. Ir. Sutami No.36, Surakarta, Indonesia<sup>4</sup>Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Jl. Ir. Sutami No.36, Surakarta, Indonesia

\*Corresponding author. E-mail: pratiwidewi81@staff.uns.ac.id

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## Abstract

**BACKGROUND:** Active mucosal chronic otitis media (COM) is prevalent in lower-income countries and is associated with recurrent episodes of otorrhea due to chronic inflammation of the middle ear. Cytokines, which are well-known for their effects on the immune system, play an important role in the inflammatory response and tissue remodeling. The specific contributions of proinflammatory and immunoregulatory cytokines in the pathophysiology of active mucosal COM remain unclear. This study aimed to compare the levels of serum tumor necrosis factor (TNF)- $\alpha$  and transforming growth factor (TGF)- $\beta$  in patients with active mucosal COM vs. healthy subjects.

**METHODS:** Total 20 subjects with active mucosal COM and 20 healthy subjects participated in this study. The levels of serum TNF- $\alpha$  and TGF- $\beta$  were measured using enzyme-linked immunosorbent assay (ELISA).

**RESULTS:** The average level of serum TNF- $\alpha$  in subjects with active mucosal COM was significantly higher compare to the healthy subjects (46.373 $\pm$ 41.76 pg/mL vs. 15.021 $\pm$ 7.16 pg/mL;  $p=0.004$ ). In contrast, the average level of serum TGF- $\beta$  in subjects with active mucosal COM was lower compared to the healthy subjects, although the difference is not statistically significant (9.963 $\pm$ 3.2 ng/mL vs. 11.78 $\pm$ 8.48 ng/mL;  $p=0.552$ ). Further analysis showed that in subjects with active mucosal COM, the levels of serum TNF- $\alpha$  had a medium positive correlation with the level of TGF- $\beta$  ( $r=0.525$ ;  $p=0.018$ ).

**CONCLUSION:** TNF- $\alpha$  and TGF- $\beta$ , which are proinflammatory and immunoregulatory cytokines, may contribute to the pathogenesis of recurrent episodes of otorrhea in an active mucosal COM.

**KEYWORDS:** tumor necrosis factor- $\alpha$ , transforming growth factor- $\beta$ , active mucosal chronic otitis media

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## Introduction

Active mucosal chronic otitis media (COM) is characterized by intermittent or constant discharge of pus from the ear through the tympanic membrane perforation (otorrhea). Ear discharge is notoriously difficult to treat, prone to recur, and

even capable of causing death in some cases.(1) The World Health Organization (WHO) considers active mucosal COM to as neglected tropical disease. It affects 65–330 million individuals globally, mostly in lower-income nations. High-income nations have a modest prevalence of active mucosal COM (<1%), but low and middle-income countries have a much higher frequency (>2%).(1-3) The prevalence of

active mucosal COM in Indonesia is 3.9% of the general population. There is evidence that active mucosal COM is more common in children, but other research shows that it is more common in adults.(4,5)

Many disorders, including active mucosal COM, have immunological and inflammatory responses mediated by a group of glycoproteins called cytokines. The inflammatory response and tissue remodeling in the middle ear mucosa are both mediated by cytokines. Cytokines can be released from numerous cell types found within the middle ear cleft, including all types of epithelial, endothelial, and immune cells.(6) It is possible to utilize these cells as biomarkers to detect or track disease progression, and they may also be employed as treatment-specific criteria in the clinic.(7) Middle ear mucosa contains a variety of cytokines, including proinflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , interferon (IFN)- $\gamma$  and IL-6; immunoregulatory cytokines such as IL-2, IL-4, IL-5, IL-10 and transforming growth factor (TGF)- $\beta$  and granulocyte-macrophage colony-stimulating factor (GM-CSF); and other mediators such as receptor activator of nuclear factor kappa-B ligand (RANKL).(8) However, the presence and functions of proinflammatory and immunoregulatory cytokines in the pathogenesis of active mucosal COM remain unclear.

TNF- $\alpha$ , IL-6, IL-1 $\beta$ , IFN- $\gamma$  mRNA and protein levels were higher in the middle ear mucosa of patients with active mucosal COM than in healthy persons.(9) Tissue damage and the shift from acute to chronic otitis media are also possible outcomes of increased levels of these proinflammatory cytokines.(5) TNF- $\alpha$  is one of the most significant inflammatory mediators in otitis media and was detected in human middle ear effusion. Animal models of otitis media induced by *S. pneumoniae* and lipopolysaccharide (LPS) have demonstrated elevated levels of TNF- $\alpha$  in the early stage of otitis media. TNF- $\alpha$  overexpression in otitis media increases the chance of recurrence, longer courses, and chronicity of otitis media. Using TNF- $\alpha$  inhibitors, such as TNF- $\alpha$  -binding protein or inhibitor of TNF- $\alpha$  -soluble receptor, is recommended in clinical practice to prevent chronicity and recurrence of otitis media.(10)

TGF- $\beta$  is an immunoregulatory cytokine with proinflammatory and pro-fibrotic effects. It can control the initiation and regression of inflammation by regulating chemotaxis and activity of inflammatory cells, such as macrophages and granulocytes. In the event of tissue damage, TGF- $\beta$  can help to promote matrix deposition and tissue healing. It can also cause abnormal collagen deposition and fibrosis when present in inflammatory

settings.(11) Consequently, fibroblast proliferation and middle-ear-cleft thickening are two side effects. In the chronic stage of otitis media, fibroblast proliferation continues, and extracellular matrix (ECM) proteins are continually deposited in the middle ear submucosa layer, eventually leading to fibrosis. In the acute stage of otitis media, they contribute to the production of granulation tissue, which continues and intensifies during the chronic stage. TGF- $\beta$  expression is high in secretory otitis media, according to a prior study.(12,13)

The etiology of active mucosal COM included proinflammatory and immunoregulatory cytokines, although their presence and function are as yet unclear. This study aimed to compare the levels of serum TNF- $\alpha$  and TGF- $\beta$  in patients with active mucosal COM vs. healthy subjects and describe the microorganisms in the ear discharge of patients with active mucosal COM.

## Methods

### Study Design and Subjects

A cross-sectional study was conducted from March to May 2021 at the Otorhinolaryngology Clinic, Dr. Moewardi General Hospital, and its affiliated institutions in Surakarta, Indonesia. A total of 40 adult subjects (20 subjects with active mucosal COM and 20 healthy subjects) were recruited into this study.

Subjects were diagnosed with active mucosal COM if they had episodes of recurrent otorrhea within 3 months prior to recruitment. Subjects were excluded from the study if they had medical and/or surgical procedures or illnesses, such as infection and/or inflammation either in an acute or chronic form that may affect the outcome of the study. All subjects signed informed consent and the study protocol was approved by the Research Ethics Committee at Dr. Moewardi General Hospital (No. 372/IV/HREC/2021).

Recurrent otorrhea and hearing loss were the two most prevalent complaints that had been reported by all active mucosal COM subjects. Clinical presentation and otoscopic examination were employed to diagnose active mucosal COM. The decibel level of hearing (dB HL) was assessed using an audiological test. A diagnostic audiometer was used to measure the hearing threshold in each ear at frequencies of 1 kHz, 2 kHz, and 4 kHz.

### Collection and Culture of Bacteria from Exudate

Middle ear exudate was collected under the direction of an otomicroscope using sterile small cotton-tip swabs (Copan

Venturi Transystem 108C use Amies) to avoid contamination by microorganisms from the external auditory canal. Swabs of purulent exudate were cultured overnight at 37°C on blood and MacConkey agar plates. Routine procedures for bacterial organism identification were used to identify the microorganisms.

### Collection of Blood Serum

Blood serum samples were collected from the venous blood of all patients and healthy subjects into SST containers. The fresh blood was allowed to stand at 25°C for 30 min to clot, the blood then centrifuged at 3500 rpm for 15 min. The clarified upper light-yellow liquid layer is the serum. It was preserved on -80°C and moved into -20°C a day before detection.

### Enzyme-linked Immunosorbent Assay (ELISA)

The levels of serum TNF- $\alpha$  and TGF- $\beta$  were measured using ELISA method. The Elabscience Human TNF- $\alpha$  and TGF- $\beta$  ELISA Kit (Elabscience, Hubei, China) utilizes an antibody specific for human TNF- $\alpha$  and TGF- $\beta$  coated on a 96-well plate. Recombinant human TNF- $\alpha$  and TGF- $\beta$  dilutions were used to produce the standard curves. The wavelength at which absorbance may be detected is 450 nm. The overall procedures were conducted in the Biomedical Laboratory, Faculty of Medicine, Universitas Sebelas Maret.

### Statistical Analysis

Data were analyzed using Statistical Package for the Social Sciences (SPSS) Statistic version 25 (IBM Corporation, Armonk, NY, USA). Mean $\pm$ standard deviation of cytokine levels were used in calculations. The significance level for the Kolmogorov–Smirnov and Shapiro–Wilk normality tests was <0.05. The Mann–Whitney test was used to assess if there were significant differences in the blood levels of TNF- $\alpha$  and TGF- $\beta$  between the active-mucosal-COM and healthy subjects. TNF- $\alpha$  and TGF- $\beta$  levels were compared between the active-mucosal-COM and healthy subjects using Pearson rank correlation. Statistical significance was defined as a *p*-value of 0.05.

## Results

### Characteristics of Subjects

Table 1 showed the characteristics of subjects of included in this study. Participants's ages ranged from 19 to 64 years old and the majority of them were in their productive ages; *i.e.*, 36-45 years old (active mucosal COM subjects) and 26-

**Table 1. Characteristics of subjects.**

Variables	n (%)	
	Active Mucosal COM Subjects (n=20)	Healthy Subjects (n=20)
Age (years)		
17–25	5 (25)	0
26–35	2 (10)	17 (85)
36–45	7 (35)	2 (10)
46–55	4 (20)	1 (5)
56–65	2 (10)	0
Gender		
Male	9 (45)	9 (45)
Female	11 (55)	11 (55)
Degree of hearing loss		
Mild	6 (30)	
Moderate	5 (25)	
Moderately severe	5 (25)	
Severe	3 (15)	
Profound	1 (5)	
Otorrhea frequency (per year)		
1–3	0	
4–6	0	
>6	20 (100)	

35 years old (healthy subjects). Most subjects were female (55%) and those with active mucosal COM often had a mild degree of hearing loss (30%). In all cases, the frequency of otorrhea was more than 6 times annually.

### Bacterial Pathogen Detected by Culture

Eighteen samples that were collected from subjects with active mucosal COM yielded positive cultures on blood and MacConkey agar plates, with *Pseudomonas aeruginosa* being the most prevalent organism (90%). The other two samples yielded yeast and these were considered as contaminants originated from skin commensals of the external auditory canal (Table 2).

### Cytokine Levels in Relation to Bacterial Pathogens

The levels of serum TNF- $\alpha$  and TGF- $\beta$  were measured and analysed further in regards to their relationship with bacterial pathogens (Table 3). The average level of serum TNF- $\alpha$  was higher in samples yielding gram-positive bacteria compared to the infected by gram-negative bacteria, although the difference was not statistically significant. However, the average level of serum TGF- $\beta$  was equal among two groups.

**Table 2. Bacterial culture from subjects with active mucosal COM subjects (n=18).**

Microorganisms	n (%)	TNF- $\alpha$ (pg/mL)	TGF- $\beta$ (ng/mL)
Gram-positive			
<i>Corynebacterium striatum</i>	6 (33.3)	58.63 $\pm$ 42.03	9.76 $\pm$ 2.08
<i>Staphylococcus haemolyticus</i>	1 ( 5.5)	33.87	7.79
<i>Staphylococcus xylosus</i>	1 (5.5)	54.88	13.44
Gram-negative			
<i>Pseudomonas aeruginosa</i>	8 (44.4)	40.89 $\pm$ 43.70	10.11 $\pm$ 4.67
<i>Acinetobacter spp.</i>	2 (11.1)	16.75 $\pm$ 7.95	9.94 $\pm$ 1.12

### Serum Cytokines Level

Serum TNF- $\alpha$  and TGF- $\beta$  were detected in all serum samples. The average level of serum TNF- $\alpha$  was significantly higher in subjects with active mucosal COM compare to the healthy subjects ( $p=0.004$ , 95%CI: 19.96-41.42). In contrast, the average level of serum TGF- $\beta$  level in subjects with active mucosal COM subjects was lower than the healthy subjects, although the difference was not statistically significant (Table 4).

### Results of Pearson Correlation Test

We performed Pearson's rank correlation test to assess the correlation between the levels of a proinflammatory cytokines (*i.e.*, TNF- $\alpha$ ) and an immunoregulatory cytokine (*i.e.*, TGF- $\beta$ ) in the serum samples. Figure 1 demonstrated that the level of TNF- $\alpha$  had a medium positive correlation with the level of TGF- $\beta$  in subjects with active mucosal COM subjects ( $r=0.525$ ;  $p=0.018$ ), whilst they had a weak negative correlation in healthy subjects ( $r=-0.341$ ;  $p=0.141$ ).

## Discussion

COM is the most frequent inflammatory diseases of the middle ear among children and adults and is especially problematic in lower-income nations. The condition causes chronic inflammation of the middle ear cleft and the clinical manifestations include otorrhea, permanent perforation, and

hearing loss. COM can be divided into active, inactive or healed COM depending on the clinical disease condition. Based on the histologic features of the middle ear mucosa and the edges of the perforated tympanic membrane, COM is divided into mucosal and squamous COM. Active mucosal COM refers to a permanent defect of the pars tensa with an inflamed middle ear mucosa that produces ear discharge (otorrhea).(14,15)

Our study showed that there is no sex preference for the active mucosal COM with male-to-female ratio was almost equal (45% vs. 55%). This is similar to a previous study in Tanzania that found 54.4% of the study subject with active mucosal COM were male.(16) Another study found that males were more likely to have active mucosal COM (53.5% vs. 46.5%).(17) This is related to male's tendency of working outside the house making them susceptible to infection.(18)

Otitis media is typically thought to be a disease of children and the proportion of active mucosal COM in older subjects tend to decline. Children are more susceptible to otitis media than adults because their eustachian tubes are shorter, more horizontal, and more susceptible to obstruction by enlarge adenoids. Furthermore, in young children, viral infections and allergies are frequent, and both can induce eustachian tube inflammation. It can, however, affect adults and the elderly.(10,19) According to the WHO, the highest prevalence of active mucosal COM occurs in the first year of life (15.40 per thousand), with the lowest incidence

**Table 3. Inflammatory mediators in middle ear culture positive for gram-positive and -negative bacteria.**

Serum Cytokines	Gram-positive (n=8)	Gram-negative (n=10)	p-value
TNF- $\alpha$ (pg/mL)	55.06 $\pm$ 39.87	36.06 $\pm$ 40.00	0.33
TGF- $\beta$ (ng/mL)	9.97 $\pm$ 2.36	10.08 $\pm$ 4.13	0.94

**Table 4. Serum cytokines level in active-mucosal-COM and healthy subjects**

Serum Cytokines	Active Mucosal COM Subjects (n=20)	Healthy Subjects (n=20)	p- value
TNF- $\alpha$ (pg/ml)	46.37 $\pm$ 41.76	15.02 $\pm$ 7.16	0.004
TGF- $\beta$ (ng/ml)	9.963 $\pm$ 3.2	11.78 $\pm$ 8.48	0.552

occurring beyond 65 years of age (2.51 per thousand). This is confirmed by our present study that found the proportion of active mucosal COM in the 56-65 age group was being the lowest incident (10%).

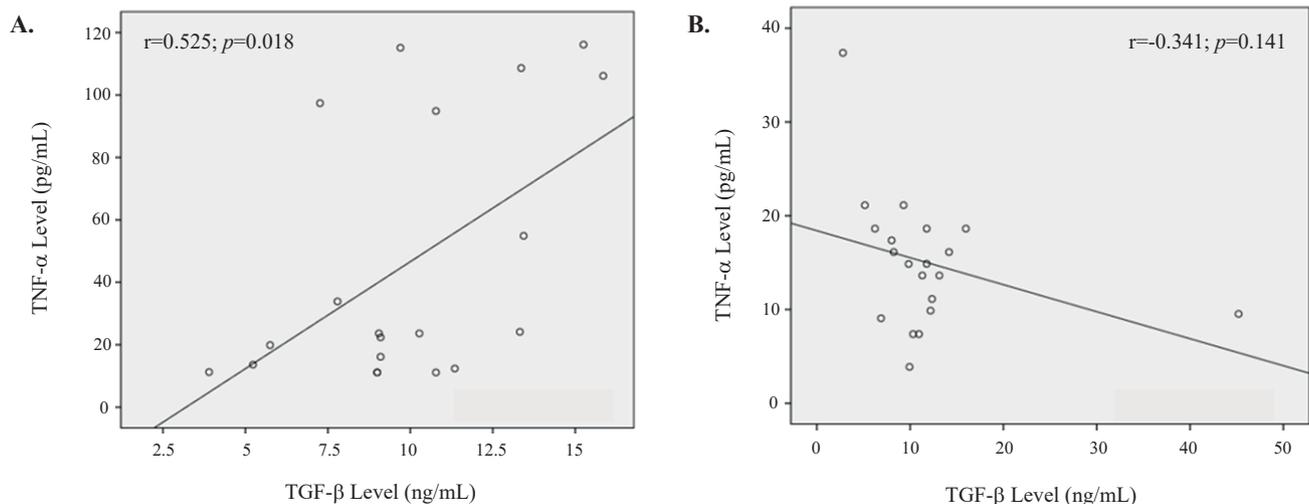
Active mucosal COM is often followed by hearing loss.(20) We found a mild degree of hearing loss (30%) to profound hearing loss (5%) among the cases. Suppurative chronic otitis media can induce both conductive (CHL) and sensorineural hearing loss (SNHL). A pus-filled middle ear is the characteristic of chronic suppurative otitis media and can interfere with sound transmission to the inner ear. The use of topical antibiotics for an extended period or the absorption of chemicals or toxins from active mucosal COM through the round window membrane might also cause sensorineural hearing loss.(21)

Our study found *P. aeruginosa* as the most prevalent pathogen responsible for the development of active mucosa COM which is similar to the results of previous studies.(17,22,23) Other studies from China reported *Staphylococcus aureus* as the most common pathogen in active mucosal COM, followed by *P. aeruginosa*.(24) Different patient populations, regional variations, and antibiotic use before culture might all account for the

discrepancies across studies.(5,24) As fungi live well in humid ears, fungal infections of the middle ear have been found in the cultures of patients with active mucosal COM and who had used topical or systemic antibiotics for an extended period.(25,26)

Gram-negative bacteria accounted for 55.5% of the discharged ear isolates, and gram-positive bacteria accounted for the remaining 40.5%. Another study had similar findings, which found 59.6% gram-negative bacteria in their isolates.(27) The chronic nature of infection, in which gram-negative bacteria from external sources gain access to the auditory canal, suppressing the gram-positive normal flora and eventually becoming dominant, could explain the higher prevalence.(28)

Previous studies showed that leukocytes produce different patterns of proinflammatory cytokines and chemokines when stimulated with gram-positive or gram-negative bacteria.(29) Monocyte/macrophage cytokine release and TLR expression are both triggered by bacteria. TNF- $\alpha$  production is higher in gram-positive bacteria than in gram-negative bacteria. TNF- $\alpha$ , IFN- $\gamma$ , and IL-12 are secreted as a result, whereas only IL-6 and IL-10 are secreted in the presence of gram-negative bacteria. Extravasation



**Figure 1. Pearson correlation between TNF- $\alpha$  and TGF- $\beta$  in the patients with active mucosal COM (A) and healthy subjects (B).**

of polymorphonuclear leukocytes into infected tissue is dependent on TNF- $\alpha$ , which also contributes to prostaglandin and cytokine production, neutrophil activation, and eosinophil and macrophage activation; thus, TNF- $\alpha$  is essential. Patients with active mucosal COM had higher levels of the cytokines TNF- $\alpha$  and TGF- $\beta$ , which were then linked to the kind of bacteria found by culture in the middle ear. We found that the levels of serum TNF- $\alpha$  were greater in cases infected with gram-positive bacteria. This results is similar to a previous study which also demonstrated that gram-positive bacteria is related to the higher levels of TNF- $\alpha$  in the serum.(30)

TNF- $\alpha$  is a cytokine with the ability to promote bone osteolysis. Our study showed that the levels of serum TNF- $\alpha$  levels in patients with active mucosal COM were 3.08-fold greater than in healthy people, while other study found that TNF- $\alpha$  levels in COM with cholesteatome were 3.8-fold greater than in normal skin. This might imply that the increasing level of proinflammatory cytokines such as TNF- $\alpha$  in active mucosal COM causes chronic inflammation to exacerbate, resulting in recurrent otorrhea.(8,31)

TGF- $\beta$  is an immunoregulatory cytokine that plays an important role in the control of inflammatory processes as well as tissue repair. TGF- $\beta$  is thought to be responsible for granulation tissue formation and ossicular fixation in the middle ear.(12) A high expression of TGF- $\beta$  in the serum of patients with active mucosal COM showed that TGF- $\beta$  has an important role in the chronic inflammatory course of active mucosal COM. As TGF- $\beta$  can permeate into middle ear mucosa, it causes higher expression in middle ear discharge in patients with active mucosal COM and is not only produced by the middle ear mucosa. A previous study had suggested that perturbation of the TGF- $\beta$  signaling pathways can lead to chronic otitis media.(32) Prolonged TGF- $\beta$  activation has been linked to the development of chronic inflammation and fibrosis in other parts of the body.(33)

Our study showed no statistically significant difference in the expression of TGF- $\beta$  in between active-mucosal-COM and healthy subjects. However, TGF- $\beta$  might limit the production of mucin elicited by bacteria by downregulating MUC5AC in the middle ear cavity, which may decrease the host's innate immunity to a pathogen, delay the removal of microorganisms, and eventually cause the condition to become chronic.(34)

Nonetheless, our study found a positive correlation between the levels of serum TNF- $\alpha$  and TGF- $\beta$ ; *i.e.*, the higher the level of TNF- $\alpha$ , the higher the level of TGF- $\beta$ . It demonstrates that these two types of cytokines contribute

to the middle ear inflammatory responses, particularly otorrhea in patients with active mucosal COM. Further research into the presence and interplay of proinflammatory and immunoregulatory cytokines in active mucosal COM is needed to offer a more comprehensive picture of the inflammatory process in the middle ear.

Further research is required to determine the association between middle ear secretion TNF- $\alpha$  and TGF- $\beta$  levels in conjunction with active mucosal COM to explain the involvement of cytokines in the pathophysiology of active mucosal COM. Our study is limited to the absence of measurements of cytokine levels in middle ear discharge. Furthermore, due to the lack of pediatric patients, a direct comparison of differences between pediatric and adult patients was impossible.

## Conclusion

The level of TNF- $\alpha$  serum was significantly higher in patients with active mucosal COM than in healthy subjects, but the level of TGF- $\beta$  serum was not. The level of TNF- $\alpha$  had a medium positive correlation with the level of TGF- $\beta$  in subjects with active mucosal COM. These two cytokines may contribute to the pathogenesis of recurrent episodes of otorrhea in an active mucosal otitis media. Further study is needed to explain the roles of other cytokines in the middle ear inflammatory responses.

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## Authors Contribution

DP, RDR, TNS, and S were involved in the planning and supervision of the work, as well as performing the measurements. MR, AW, and DP processed the experimental data, performed the analysis, drafted the manuscript, and designed the figures. DP performed the computations and statistical analysis and also supported MR and AW in interpreting the results and writing the manuscript. Each author discussed the findings and provided feedback on the manuscript.

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