Comparative Evaluation of HPLC and Nephelometry for HbA1c Measurement: Diagnostic Agreement and Clinical Utility

Running title: HPLC vs. Nephelometry for HbA1c: Accuracy and Reliability

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Abstract

Background: Glycated haemoglobin (HbA1c) represents the estimated average blood glucose levels over a period of 2–3 months and is essential for both diagnosing and treating diabetes. High-performance liquid chromatography (HPLC) and particularly ion-exchange HPLC is indeed widely regarded as the reference and gold standard method for HbA1c assessment, whereas nephelometry is commonly adopted in clinical laboratories because it provides quicker results. This study compares the accuracy, reliability, and clinical applicability of these two methods.

Methods: A total of 50 patients diagnosed with diabetes mellitus and attending the tertiary care hospital were included in this cross-sectional study. For each participant, HbA1c levels were estimated using both ion-exchange HPLC and nephelometric techniques. Data analysis involved descriptive statistics, Pearson's correlation, the intraclass correlation coefficient (ICC), Bland–Altman plots, and receiver operating characteristic (ROC) curve evaluation. A paired t-test was performed to determine statistical significance, and values with $p < 0.05$ were considered statistically meaningful.

Results: The mean HbA1c values obtained through HPLC (6.2% ± 1.5) and nephelometry (6.3% ± 1.4) were closely aligned. A strong correlation was observed between the two techniques ($r = 0.96$, $p < 0.01$), and the intraclass correlation coefficient also indicated excellent concordance (ICC = 0.96). Bland–Altman plotting revealed only a slight bias, with a mean difference of 0.09%. ROC curve evaluation showed that both methods exhibited good diagnostic capability, although HPLC achieved marginally higher sensitivity (90%) and specificity (92%) than nephelometry (88% and 91%, respectively). While nephelometry offered quicker processing, its diagnostic accuracy was slightly lower.

Conclusion: While HPLC demonstrated superior diagnostic accuracy and method agreement, nephelometry offers operational advantages in high-volume settings. Despite high correlation between the two methods, their interchangeability should be approached with caution, particularly in borderline cases. Further studies with larger, diverse cohorts and evaluation of potential confounding factors such as hemoglobin variants are warranted.

Keywords: HbA1c, Diabetes Mellitus, Ion-Exchange HPLC, Nephelometry, Diagnostic Accuracy, Glycated Haemoglobin

Introduction

With rising rates of prevalence across all age groups and regions, diabetes mellitus remains as a major public health challenge worldwide. Approximately 589 million adults aged 20–79 were living with diabetes globally in 2024 as reported by the International Diabetes Federation (IDF) Diabetes Atlas, and this number is expected to increase to 853 million by 2050 (1). A substantial majority—about 81% reside in developing countries, where diagnostic infrastructure can be variable. India, with over 101 million adults living with diabetes, ranks second only to China in disease burden (1). Early diagnosis and consistent monitoring are critical to preventing long-term complications, especially in high-prevalence settings where many individuals remain undiagnosed.

As a biomarker, glycated haemoglobin (HbA1c) plays a crucial role in identifying diabetes and tracking chronic glycaemic management. In contrast to fasting plasma glucose and oral glucose tolerance tests, HbA1c reflects the mean blood glucose concentration over the preceding 8–12 weeks and can be measured without the need for fasting or strict timing (2–4). Both the International Expert Committee and the American Diabetes Association (ADA) recommend HbA1c for diagnostic purposes, with values of $\geq 6.5\%$ confirming diabetes and levels between 5.7% and 6.4% identifying individuals at risk (5,3). Owing to its stability and convenience, HbA1c testing is particularly useful in outpatient and resource-limited settings.

Despite its clinical value, the reliability of HbA1c depends on the precision of the analytical method employed. The ADA specifies that only assays certified by the National Glycohemoglobin Standardization Program (NGSP) and aligned with the Diabetes Control and Complications Trial (DCCT) reference standards (3) should be used for HbA1c measurement. Several analytical methods are available for this purpose, including enzymatic assays, nephelometry, immunoassays, boronate affinity methods, and ion-exchange high-performance liquid chromatography (HPLC), each differing in operational requirements, cost, and analytical accuracy (7).

Ion-exchange HPLC is widely regarded as the gold standard due to its excellent precision and its capability to identify various hemoglobin variants. It separates hemoglobin fractions based on charge and provides detailed chromatographic profiles (8). Nephelometry, an immunoturbidimetric method, measures light scatter resulting from antigen–antibody complex formation. Although suitable for rapid, high-throughput testing, this technique may exhibit reduced analytical specificity in the presence of hemoglobin variants. According to the NGSP, immunoassay-based HbA1c measurements may be influenced by hemoglobinopathies, with the extent of interference varying between assay types (9,10).

Studies such as those by Weykamp et al. and Little et al. have shown that while both methods generally correlate well, significant method-specific biases and interferences may occur, especially in real-world settings [8,11]. Method comparison studies are critical in evaluating these differences, but most are conducted in controlled environments, often without accounting for diverse population characteristics or lab conditions. Real-world data from high-prevalence regions like India remain limited.

Given the clinical importance of precise HbA1c estimation for therapeutic decisions, even small variations between methods may lead to under- or over-treatment. This is particularly important in regions like India, where variability in access to certified testing platforms may result in inconsistent care.

The present study aimed to assess how well nephelometry and ion-exchange HPLC align diagnostically and to examine their practical usefulness in an Indian clinical setting. By analysing their accuracy, precision, and overall agreement, this work seeks to offer meaningful comparisons between the two techniques and guide informed decisions regarding HbA1c testing methods in healthcare systems with high disease burden and varying resource availability.

Methods

A cross-sectional observational study was conducted from October to December 2024 in the Department of Biochemistry associated with the central lab at a tertiary care teaching hospital in Hyderabad, Telangana, India.

A total of 50 adult patients with biochemically confirmed diabetes mellitus, including 28 males (56%) and 22 females (44%), attending the General Medicine outpatient department for follow-up, were recruited. Written informed consent was taken and participant confidentiality was maintained throughout the study. The mean age of the participants was 53.4 ± 8.2 years with a range of 35 to 70 years. Participants with pregnancy, anaemia, chronic kidney disease, liver disease, cardiovascular disease, or hypertension or who declined to give consent were excluded from the study.

Ethics approval was obtained from the Institutional Ethics Committee. Venous blood samples of approximately 5 mL were drawn from all participants using aseptic technique. HbA1c levels were estimated using two methods. The first was nephelometry, performed on the Mispa i2 analyser (Agappe Diagnostics) according to the manufacturer's instructions. The second, ion-exchange HPLC, was carried out using the Meril GluQuant HPLC system (Meril Diagnostics). Internal quality control (QC) was performed prior to sample analysis. Two levels of control (normal and pathological) were run for each method. Mispa HbA1c control reagents (Agappe Diagnostics) were used for the nephelometry assay, and Meril HbA1c control materials (Meril Diagnostics) were used for the HPLC method. All control results were within manufacturer-specified acceptable ranges. HPLC recognised as the gold standard for HbA1c measurement served as the reference method in this study. For comparison, HPLC was assumed to have 100% sensitivity and specificity, and the diagnostic performance of nephelometry was evaluated against it.

Data was analysed using Microsoft Excel and SPSS version 20. Continuous variables are presented as mean ± standard deviation (SD). Pearson's correlation coefficient (r) and the Intraclass Correlation Coefficient (ICC) was used to assess the relationship and consistency between the two methods Agreement between methods was evaluated using Bland–Altman analysis, reporting the mean difference and 95% limits of agreement. The diagnostic performance of the nephelometry method was determined using ion-exchange HPLC as the reference standard. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated using Receiver Operating Characteristic (ROC) curve analysis. A diagnostic cutoff of 6.5% HbA1c was applied, following American Diabetes Association (ADA) recommendations. Statistical significance was defined as a p-value < 0.05.

Results

A total of 50 venous blood samples were analysed for HbA1c using both nephelometry and ion-exchange HPLC methods.

Table 1. Descriptive statistics of HbA1c values (Mean ± SD, Range)

Method	Mean HbA1c (%)	Standard Deviation (SD)	Range (Min-Max)
Nephelometry	6.3	1.4	4.8- 10.5
HPLC	6.2	1.5	4.5 -10.8

As shown in Table 1, the mean HbA1c values and standard deviations were closely aligned between the two methods. HPLC exhibited a slightly wider range, indicating marginally broader variability in measurement.

Table 2. Precision and reproducibility analysis by ICC

Method	Intraclass Correlation Coefficient (ICC)	p-VALUE
HPLC-Nephelometry	0.96	<0.01

A high intraclass correlation coefficient (ICC) of 0.96 ($p < 0.01$) was observed between the two methods, indicating excellent reproducibility and inter – method agreement in HbA1c measurement (Table 2).

Table 3. Bland-Altman analysis (Agreement between methods)

Statistic	Value
Mean Difference (Nephelometry - Ion Exchange)	0.09
Standard Deviation (SD) of Differences	0.39
95% Confidence Interval (CI)	(-0.05, 0.23)

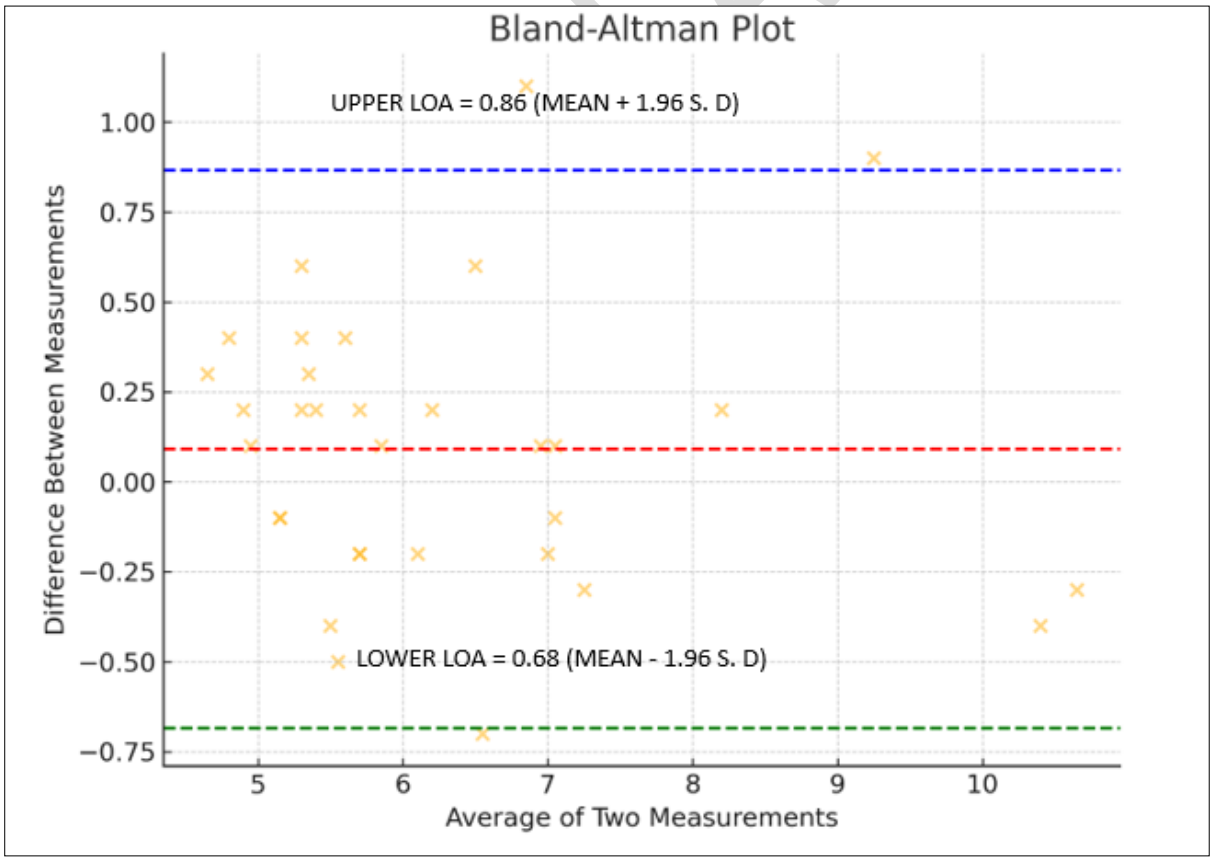

Figure 1. Bland–Altman Plot of Differences Between Nephelometry and HPLC Against Their Mean Values

The Bland–Altman analysis (Table 3 and Figure 1) demonstrated a mean difference of 0.09%, with a standard deviation of 0.39. The 95% confidence interval ranged from –0.05 to 0.23, and

the limits of agreement (LOA) spanned from –0.68% to +0.86%, indicating minimal systematic bias and close agreement between the two methods.

Table 4. Paired t-test and Pearson correlation analysis

Statistic	Value	Interpretation
Paired t-test (p-value)	0.204	No significant difference ($p > 0.05$)
Pearson Correlation (r)	0.96	Strong correlation (close to 1)

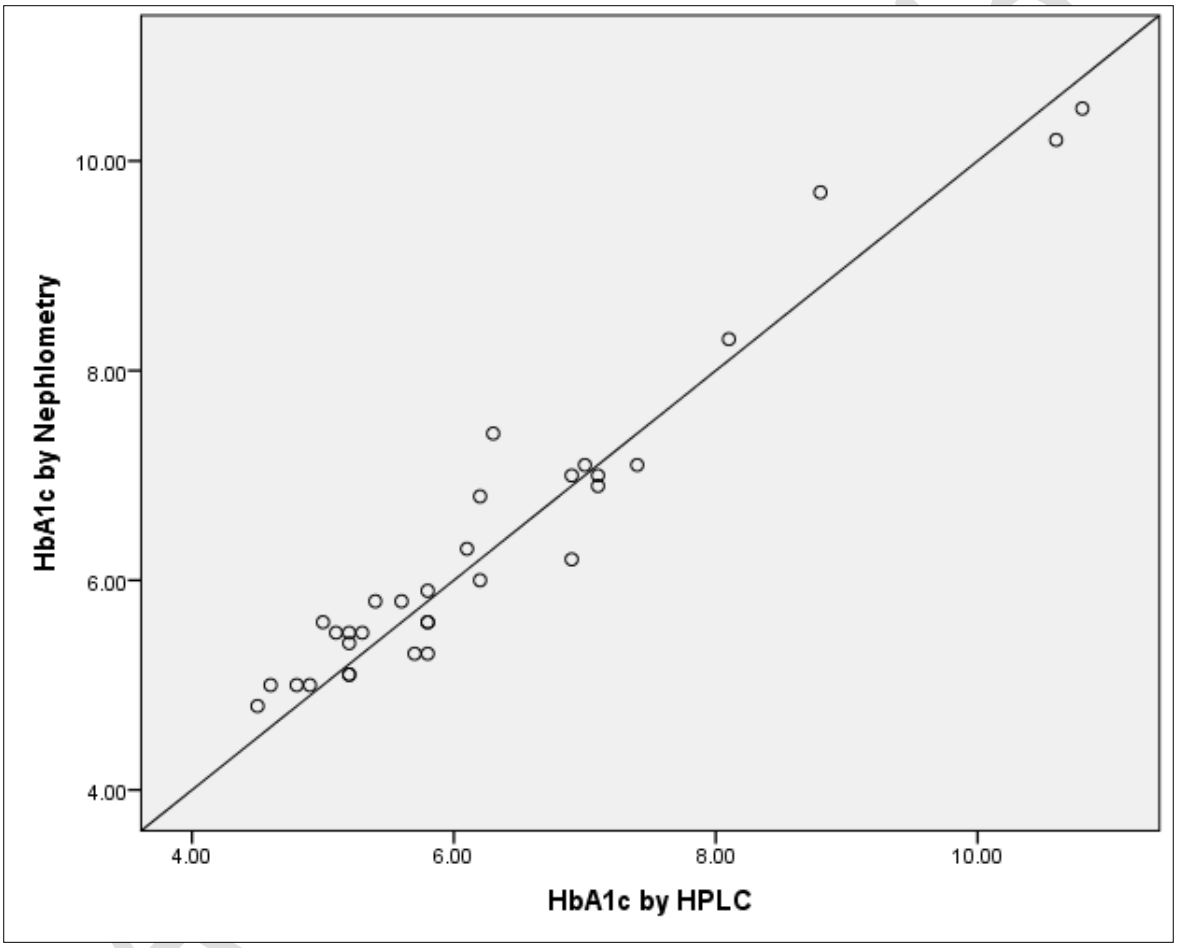

Figure 2. Correlation of HbA1c values between Nephelometry and HPLC methods

The paired t-test yielded a p-value of 0.204, indicating no statistically significant difference between HPLC and nephelometry HbA1c values. The Pearson correlation coefficient was 0.96, signifying a strong positive correlation between the two techniques. These findings are summarized in Table 4 and visualized in the scatter plot (Figure 2).

Table 5. ROC Curve Analysis for Diagnostic Performance

Method	Area Under the Curve (AUC)	Sensitivity (%)	Specificity (%)
Nephelometry	0.92	88%	91%
Ion-Exchange Chromatography	0.94	90%	92%

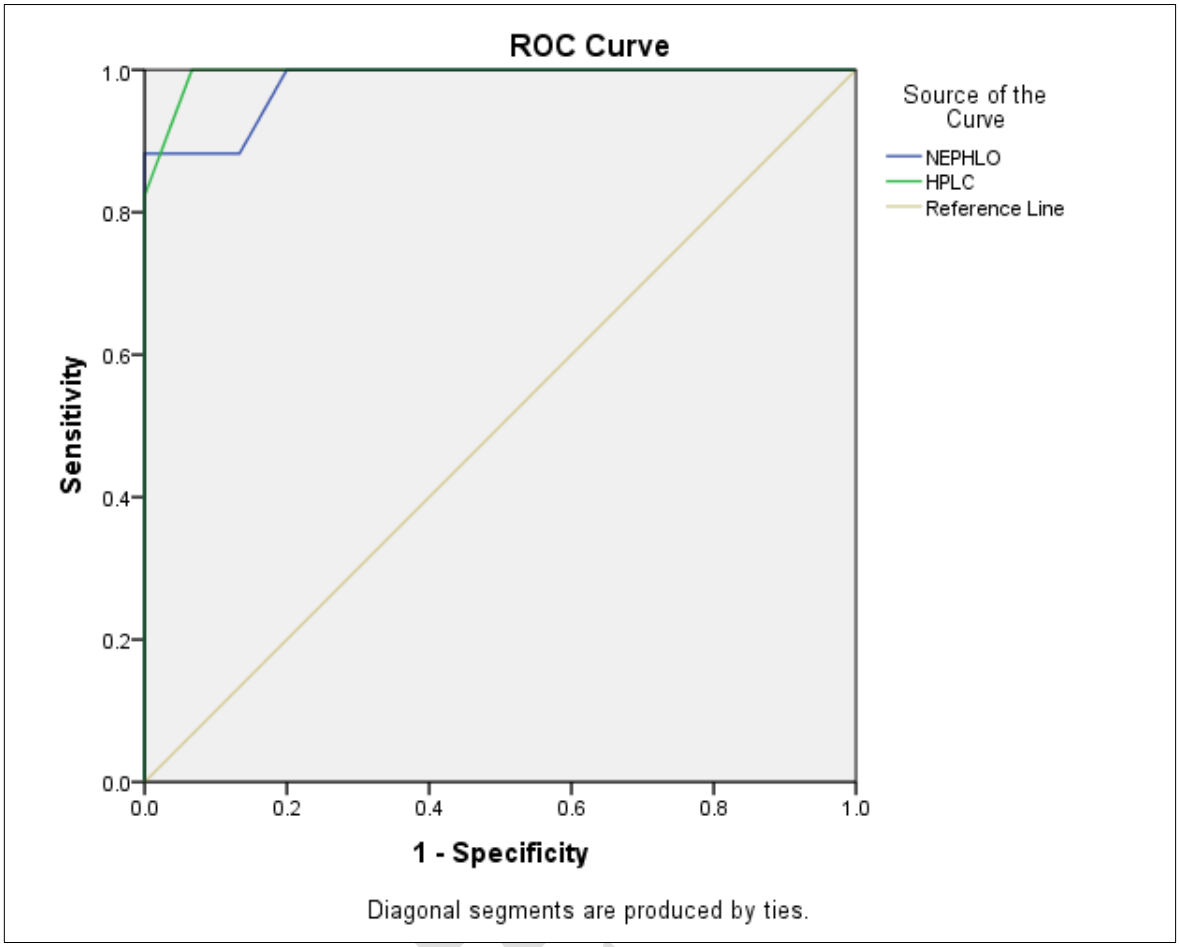

Figure 3. Receiver Operator Curve (ROC) of HbA1c estimation

The ion-exchange HPLC method exhibited an area under the curve (AUC) of 0.94, with a sensitivity of 90% and specificity of 92%, utilising a diagnostic threshold of 6.5% HbA1c (Table 5). The nephelometry-based method yielded a slightly lower AUC of 0.92, with sensitivity of 88% and specificity of 91%. The ROC curves for both methods are illustrated in Figure 3. The HPLC curve (green line) shows a steeper ascent and lies closer to the upper left corner than the nephelometry curve (blue line), indicating marginally superior discriminative ability.

Table 6. Comparison of turnaround time and usability

Parameter	**Nephelometry**	**HPLC Ion-Exchange Chromatography**
Processing Time (minutes)	8 min	12 min
Ease of Use	Moderate	High
Required Sample Volume (mL)	1.0ml	0.8ml

Operational parameters, as presented in Table 6, indicated that nephelometry had a shorter processing time (8 minutes) compared to HPLC (12 minutes). In contrast, HPLC required a smaller sample volume (0.8 mL vs. 1.0 mL) and received higher ratings for ease of use. These features may offer practical advantages in clinical settings where sample conservation and analytical simplicity are important considerations

Discussion

The study aimed to compare the analytical performance and clinical applicability of nephelometry and ion-exchange HPLC for HbA1c measurement in patients with diabetes, focusing on precision, consistency, and diagnostic relevance. HbA1c provides valuable information on long-term glycaemic control and the likelihood of diabetes-related complications, making it a key marker for both diagnosing diabetes and predicting its clinical outcomes (12). Our findings align with previous studies demonstrating that ion-exchange HPLC remains the benchmark method for HbA1c estimation due to its high analytical specificity and accuracy (8,11). The high correlation (r = 0.96) between nephelometry and HPLC suggests that nephelometry may be a viable alternative in settings prioritizing rapid turnaround times, provided haemoglobin variants are not a concern.

The Bland–Altman analysis demonstrated minimal systematic bias, consistent with earlier findings by Rohlfing et al. (13), and underscores the reliability of nephelometry in routine clinical use, especially when hemoglobinopathies are ruled out. Unlike many earlier studies performed in controlled laboratory conditions, the present investigation reflects operational realities in a high-prevalence, resource-constrained setting, enhancing the external validity of our findings.

ROC analysis confirmed the diagnostic advantage of HPLC (AUC = 0.94), particularly for detecting borderline HbA1c values in the prediabetic range (5.7%–6.4%), aligning with previous reports (14,15) that emphasize HPLC's superior discriminative capacity. The slightly reduced specificity observed for nephelometry concurs with point-of-care HbA1c studies (16), where accelerated workflows occasionally compromise diagnostic precision. These discrepancies may arise from nephelometry's indirect measurement principle, which relies on immune complex formation, potentially influenced by sample turbidity, lipid interference, or inflammatory markers. Despite minor diagnostic limitations, nephelometry demonstrated acceptable accuracy and offered operational advantages, including shorter assay time and ease of use—key considerations in high-volume laboratories and primary care settings.

Hence, although HPLC remains preferable in settings requiring rigorous analytical accuracy (e.g., diagnostic confirmation or research laboratories), nephelometry presents a viable option for initial screening and routine monitoring in high-throughput outpatient clinics. This study contributes to the current literature by presenting comparative performance data from a real-world clinical setting in India, a country with one of the highest global burdens of diabetes.". As most prior studies have been conducted in Western populations, our findings help establish the broader applicability of both methods and emphasize the importance of balancing diagnostic precision with operational feasibility in varied healthcare environments.

Limitations

The modest sample size may limit generalizability, and potential confounders such as hemoglobinopathies or recent blood transfusions were not assessed. These factors should be addressed in future studies with larger, more diverse cohorts.

Conclusion

In conclusion, our findings demonstrate excellent agreement between ion-exchange HPLC and nephelometry for HbA1c estimation, with an ICC of 0.96 and a negligible mean difference (0.09%). While HPLC remains the reference standard due to its superior specificity,

nephelometry offers a viable, faster alternative suitable for high-throughput clinical workflows, particularly in resource-constrained environments.

Acknowledgement

The authors extend their heartfelt appreciation to all participants and laboratory staff for their cooperation and assistance with data collection.

Funding source

Self-Funded

Ethical statement

The study received ethical clearance from institutional ethical committee (Ref IEC/MAMS/2024/137)

Conflict of interest

Nil

Author contributions

All authors were instrumental in and made intellectual contributions to the conception, design, and execution of this work.

Data availability statement:

Not applicable.

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