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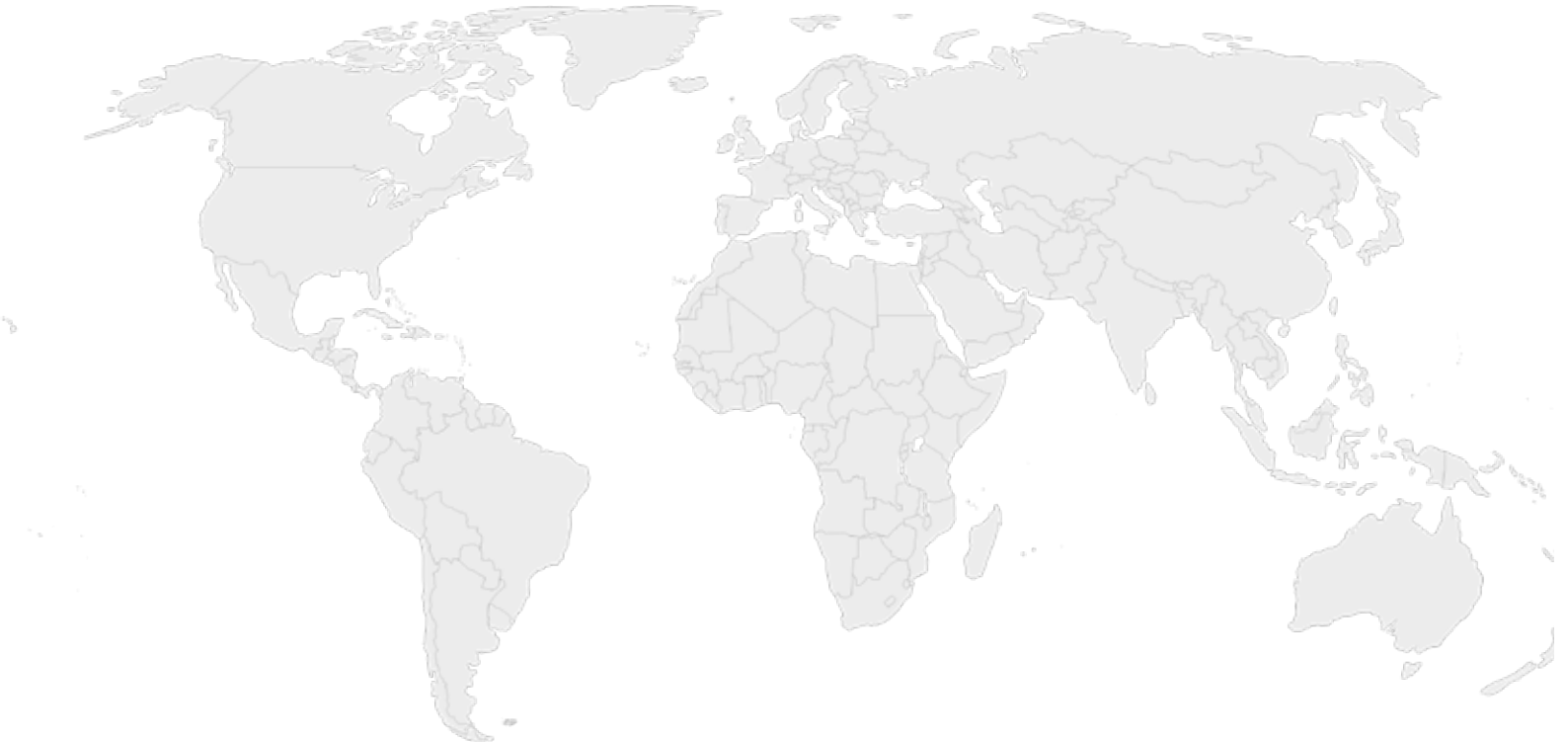

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Research Article

Association of caspase-3 with thyroid hormone levels in patients with hypothyroidism – A hospital-based study

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Keywords

Apoptosis, Caspase-3, Thyroid hormones, Thyroid stimulating hormone

Abstract

Introduction: This hospital-based prospective study examined the relationship between thyroid hormone levels and serum levels of caspase-3, a critical marker of apoptosis, in 183 patients diagnosed with hypothyroidism.

Methods: Serum caspase-3 concentrations were measured using a human caspase-3 ELISA kit. Elevated caspase-3 levels indicate increased apoptotic activity.

Results: In the overall cohort, the serum caspase-3 levels were not significantly correlated with the thyroid hormone level. However, among patients with mildly elevated TSH (Group I), caspase-3 levels were inversely correlated with T_3 ($r = –0.319$, $p < 0.05$) and T_4 ($r = –0.377$, $p < 0.001$). Age-stratified analysis revealed that among younger participants (18–40 years), age independently predicted lower caspase-3 levels ($p = 0.027$), suggesting a decline in apoptotic activity with advancing early adulthood. In older individuals (≥41 years), serum T_4 independently and negatively predicted caspase-3 levels ($p = 0.048$). Partial correlation analysis controlling for age rendered the previous associations non-significant, indicating that age was a confounding factor.

Conclusion: The relationship between thyroid hormones and apoptosis appears to be both age- and severity-dependent. While thyroid hormones are inversely associated with apoptotic activity in mild hypothyroidism, age independently influences caspase-3 in younger individuals and T_4 remains a significant predictor in older adults. These findings underscore the complex interplay between aging, thyroid function, and apoptosis, with implications for thyroid-related pathophysiology across the lifespan.

Introduction

Hypothyroidism is characterized by a deficiency of thyroid hormones and can lead to a wide range of metabolic and systemic disturbances, becoming potentially fatal if left untreated [1]. Thyroid disorders affect an estimated 200 million people globally, with nearly 60% remaining undiagnosed. Thyroid dysfunction is often due to inadequate iodine intake, whereas autoimmune thyroid diseases are more prevalent in regions with sufficient or supplemented iodine intake [2]. Primary hypothyroidism is typically diagnosed by elevated serum levels of thyroid-stimulating hormone (TSH), reflecting the high sensitivity of the hypothalamic–pituitary–thyroid axis to subtle fluctuations in circulating thyroid hormones [3]. The condition may result from autoimmune destruction (e.g., Hashimoto's thyroiditis), iodine deficiency, infiltrative diseases, congenital anomalies, or iatrogenic causes such as neck irradiation, thyroidectomy or radioiodine therapy [4,5]. Transient forms such as subacute, postpartum and silent thyroiditis also contribute to intermittent thyroid dysfunction. [6]. The prevalence of hypothyroidism increases with age, especially in countries undergoing demographic transitions toward aging population, projected to reach 1.4 billion elderly individuals by 2030 [7].

Beyond their classical role in metabolism, thyroid hormones are critical regulators of cellular growth, differentiation and development. These effects are mediated via TSH receptor pathways that regulate proliferative or apoptotic outcomes, depending on the cell type and developmental context [8]. Growing evidence has focused on understanding how thyroid hormone imbalances alters intracellular signalling pathways governing cell cycle regulation and programmed cell death [9]. Apoptosis is a tightly regulated process essential for maintaining tissue homeostasis by eliminating damaged or dysfunctional cells. It can be initiated via intrinsic pathways such as mitochondrial cytochrome c release or extrinsic receptor-mediated mechanisms involving tumour necrosis factor (TNF) signalling. Dysregulated apoptosis contributes significantly to the pathophysiology of various diseases, including cancer, where impaired cell death promotes tumour angiogenesis, survival, and invasion [10].

Caspases, particularly caspase-3, play a central role in the execution phase of apoptosis by cleaving key cellular substrates at specific aspartic acid residues [11,12]. Beyond its apoptotic role, caspase-3 has been implicated in tissue differentiation, regeneration, and neurodevelopment, highlighting its broader physiological relevance [13]. Given the multifaceted roles of caspase-3 and the regulatory influence of thyroid hormones on cellular fate, this study aimed to evaluate serum caspase-3 levels in individuals with hypothyroidism and examine their association with TSH concentrations, while assessing the modulatory effects of age and sex on this relationship.

Materials and Methods

This prospective cross-sectional study aimed to assess serum caspase-3 levels in patients with hypothyroidism. The study protocol was approved by the Institutional Ethical Committee (IEC KMC MLR; Approval number- 07/2024/474). Purposive sampling was used employed to recruit 183 adult patients (aged 18–60 years) whose serum TSH levels exceeding 15 mIU/mL. Exclusion criteria were applied to minimize confounding factors that could independently affect thyroid function or apoptotic activity. Patients with malignancies or those undergoing chemotherapy or steroid therapy were excluded, as these treatments can directly alter caspase-3 expression [14-16]. Individuals with autoimmune thyroid disorders, including Hashimoto's thyroiditis, were excluded to avoid variability from immune-mediated apoptosis [17]. Patients with recent surgery, trauma, or acute infections were excluded as these conditions may activate systemic apoptotic pathways unrelated to hypothyroidism [18,19]. Patients with chronic systemic illnesses were also excluded, as such conditions may affect both thyroid status and apoptosis [20].

Patients included in the study were either hospitalized (inpatients) or managed as outpatients at the time of sample collection, with serum samples obtained during routine clinical investigations. Leftover serum samples collected from the Clinical Biochemistry Laboratory were anonymized, coded, and stored at −80°C until further analysis.

Serum caspase-3 levels were quantified using the KRISHGEN Biosystems Human Caspase-3 ELISA Kit (KBH4804), based on the sandwich ELISA principle for serum or plasma. Patient sera, standards and controls were pipetted into microplate wells pre-coated with capture antibody. After incubation, the wells were washed to remove unbound materials. The detection antibody was then added, followed by a substrate solution. The reaction was stopped with sulfuric acid and absorbance was measured at 450 nm using a microplate reader. Caspase-3 concentrations were calculated from a standard curve generated by plotting known concentrations against optical density values. The KRISHGEN Biosystems kit was selected following a comparison with available commercial assays. The kit was chosen for its high sensitivity and specificity for human serum/plasma samples, with a detection range of 0.16–10 ng/mL, which covers the expected physiological concentration of caspase-3 reported in prior studies. It has been previously used in clinical research, ensuring the comparability of findings, and offering acceptable intra and inter assay variability (<8% and <10%, respectively). Local availability and technical support further supported its selection for the study.

The thyroid hormone parameters (serum total T_3, serum total T_4 and serum TSH) corresponding to the selected samples were retrieved from the Laboratory Information Management System (LIMS). The thyroid hormones were measured through

electrochemiluminescence immunoassay (ECLIA) via the Elecsys Thyroid Assay Kit (Roche Diagnostics). The intra-assay CVs for total T_3 ranged from 1.4% to 2.3% and the interassay CVs ranged from 2.2% to 3.3%. For total T_4, the interassay CVs ranged between 3.5% and 7.2%. The intra-assay CVs of TSH were between 0.7% and 3.4% and the interassay CVs were between 1.8% and 11.2%. Duplicate entries were avoided by cross-checking for age, sex and accession number. Only one record per patient was included.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 29.0 (IBM Corp., Armonk, NY, USA). Categorical variables are presented as frequencies and percentages, while continuous variables are expressed as mean ± standard deviation (SD), as they are normally distributed. The chi-square test was used to assess associations between categorical variables. Comparisons of continuous variables between two groups were performed through independent t tests, whereas comparisons among more than two groups were conducted using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Pearson's correlation analysis was used to examine the bivariate relationships between serum caspase-3 and other variables in the overall cohort.

To account for potential confounding factors and multicollinearity, multiple linear regression models were constructed with serum caspase-3 as the dependent variable. Continuous predictors (age, serum T_3, serum T_4 and serum TSH) were mean-centered prior to analysis. Owing to high collinearity between T_3 and T_4, two separate regression models were fitted-one including T_3 and the other including T_4. Regression assumptions (linearity, homoscedasticity, independence, and normality of residuals) were assessed. Variance inflation factors (VIFs) were examined to assess multicollinearity, with values <5 considered acceptable. A two-tailed p value <0.05 was considered statistically significant.

Results

The study included 183 participants aged 18-60 years (mean ± SD: 42.64 ± 10.67), with 71% females and 29% males. The participants were classified into three TSH groups (15-20, 21-50 and >51 µIU/mL) to represent increasing biochemical severity of hypothyroidism and to assess whether gradations in TSH elevation are associated with differences in apoptotic activity.

Comparison of biochemical parameters

As expected, the serum T_3 and T_4 concentrations decreased progressively, whereas the TSH concentration increased across groups (all $p < 0.001$). Caspase-3 levels did not differ significantly between the TSH groups ($p = 0.632$) (Table 1).

Table 1: Comparison of age, thyroid function parameters (Serum T_3, T_4 and TSH) and serum caspase-3 levels among the three TSH groups.

Parameters	Group I (n=61)	Group II (n=60)	Group III (n=62)	p value#
	Mean ± SD (Min – Max)			
Age	43.74 ± 10.57 (22.00 – 60.00)	42.23 ± 10.01 (19.00 – 58.00)	41.97 ± 11.43 (18.00 – 60.00)	**0.615**
Serum T_3	0.62 ± 0.14 (0.26 – 0.86)	0.40 ± 0.11a** (0.17 – 0.63)	0.06 ± 0.04 b**, c** (0.02 – 0.21)	**<0.001**
Serum T_4	3.94 ± 0.82 (1.83 – 5.29)	2.72 ± 0.73 a** (1.31 – 4.13)	0.49 ± 0.27 b**, c** (0.14 – 1.52)	**<0.001**
Serum TSH	17.65 ± 1.72 (15.00 – 20.80)	31.62 ± 8.04 a* (21.10 – 50.90)	111.99 ± 44.13 b**, c** (51.90 – 254.00)	**<0.001**
Serum Caspase 3	0.43 ± 0.24 (0.09 – 1.28)	0.38 ± 0.26 (0.10 – 1.24)	0.41 ± 0.27 (0.17 – 1.32)	**0.632**

Abbreviations: T_3 – Triiodothyronine; T_4 – Thyroxine; TSH – Thyroid Stimulating Hormone.

#, Comparison of means across multiple groups was performed using ANOVA.

Intergroup comparisons were assessed using Tukey's post-hoc test.

Superscripts a, b and c indicate specific group comparisons, whereas * and ** denote levels of statistical significance.

a = Comparison between Group I and Group II;

b = Comparison between Group II and Group III;

c = Comparison between Group I and Group III; * $p < 0.05$; ** $p < 0.001$

Gender-based

There was no significant difference in the mean serum T_3, T_4, TSH, or caspase-3 levels between males and females ($p<0.05$ for all) (Table 2).

Table 2: Comparison of age, thyroid function parameters (Serum T_3, T_4 and TSH) and serum caspase-3 levels between sexes and age groups.

Comparison of various parameters between genders			
Parameters	**Males (n=53)**	**Females (n=130)**	**p value**
	Mean ± SD (Min – Max)		
Serum T_3	0.35 ± 0.25 (0.02 – 0.86)	0.37 ± 0.26 (0.02 – 0.87)	0.885
Serum T_4	2.37 ± 1.52 (0.20 – 5.23)	2.38 ± 1.61 (0.14 – 5.29)	0.97
Serum TSH	51.49 ± 43.88 (15.50 – 174.00)	55.29 ± 51.43 (15.00 – 254.00)	0.638
Serum Caspase-3	0.40 ± 0.28 (0.16 – 1.32)	0.41 ± 0.25 (0.09 – 1.28)	0.773
Comparison of various parameters between age groups			
Parameters	**18 – 40 years (n=74)**	**41 – 60 years (n=109)**	**p value**
	Mean ± SD (Min – Max)		
Serum T_3	0.32 ± 0.25 (0.02 – 0.87)	0.38 ± 0.25 (0.02 – 0.86)	0.115
Serum T_4	2.16 ± 1.59 (0.16 – 5.29)	2.52 ± 1.55 (0.14 – 5.23)	0.135
Serum TSH	59.80 ± 50.63 (15.00 – 217.00)	50.38 ± 48.19 (15.30 – 254.00)	0.205
Serum Caspase-3	0.41 ± 0.26 (0.19 – 1.28)	0.41 ± 0.25 (0.09 – 1.32)	0.983

Abbreviations: T_3 -Triiodothyronine; T_4 -Thyroxine; TSH - Thyroid Stimulating Hormone.

Comparison of means between two groups were performed using independent t tests.

Age groups

Participants were stratified into 18–40 and 41–60-year groups to reflect early and later adulthood, given known age-related alterations in thyroid physiology and apoptotic activity.

The serum T_3, T_4, TSH and caspase-3 levels did not significantly differ between the two groups ($p > 0.05$). While slight variations existed in the mean values (e.g., the serum T_3 and T_4 levels were slightly greater in the older group and the serum TSH level was slightly greater in the younger group), these differences were not statistically significant [Table 2].

Correlation analysis

Correlation analysis revealed that serum caspase-3 levels were not significantly related to age or serum T_3, serum T_4, or serum TSH levels among the study participants (all p values > 0.05). In the subgroup analysis, significant negative correlations were observed between caspase-3 and serum T_3 ($r = –0.319$, $p < 0.05$) and between caspase-3 and serum T_4 ($r = –0.377$, $p < 0.001$) in TSH Group I only. No significant correlations were detected in Groups II or III or when the data were stratified by sex. In the 18–40-year age group, age was negatively correlated with caspase-3 ($r = –0.262$, $p = 0.024$) (Table 3).

Table 3: Correlations between serum caspase-3 levels and age, and thyroid function parameters (T_3, T_4 and TSH) in the whole cohort and within subgroups.

In all participants		
Parameters	**Serum Caspase -3**	
	r value	**p value**
Age	-0,028	0,702
Serum T 3	-0,044	0,551
Serum T_4	-0,056	0,451
Serum TSH	-0,001	0,984

Based on TSH Groups			
TSH Groups	**Parameters**	**Serum Caspase -3**	
		r value	**p value**
Group I	Age	0,079	0,546
	Serum T_3	-0,319	<0.05
	Serum T_4	-0,377	<0.001
	Serum TSH	0,014	0,917
Group II	Age	-0,217	0,096
	Serum T_3	-0,038	0,772
	Serum T_4	-0,045	0,734
	Serum TSH	-0,068	0,604
Group III	Age	0,027	0,836
	Serum T_3	-0,061	0,635
	Serum T_4	-0,077	0,553
	Serum TSH	0,011	0,932
Based on Age Group			
Age Group	**Parameters**	**Serum Caspase -3**	
		r value	**p value**
18-40 years	Age	-0,262	0,024
	Serum T_3	-0,024	0,841
	Serum T_4	-0,025	0,829
	Serum TSH	-0,014	0,903
41-60 years	Age	0,145	0,133
	Serum T_3	-0,059	0,542
	Serum T_4	-0,078	0,419
	Serum TSH	0,008	0,937
Based on Gender			
Gender	**Parameters**	**Serum Caspase -3**	
		r value	**p value**
Males	Age	0,218	0,117
	Serum T 3	-0,156	0,266
	Serum T_4	-0,162	0,246
	Serum TSH	0,042	0,767
Females	Age	-0,117	0,184
	Serum T_3	0,002	0,981
	Serum T_4	-0,011	0,9
	Serum TSH	-0,019	0,829

Abbreviations: r-Pearson's correlation coefficient; T_3 – Triiodothyronine; T_4 – Thyroxine; TSH – Thyroid Stimulating Hormone.

When controlling for age in the partial correlation analysis, no significant associations were detected between caspase-3 and serum T_3 ($r = –0.055$, $p = 0.462$), serum T_4 ($r = –0.043$, $p = 0.563$), or serum TSH ($r = –0.002$, $p = 0.979$), indicating that age may have confounded the observed relationships.

Multiple Regression Analysis – Whole Cohort

Collinearity diagnostics revealed that serum T_3 and T_4 were highly correlated, with VIF values > 10 and tolerance < 0.2 when they were entered together in the same model. To avoid instability in the parameter estimates, two separate regression models were performed. Model 1 included age (centered), sex, TSH (centered) and T_3 (centered). Model 2 included age (centered), sex, TSH (centered) and T_4 (centered). In both models, none of the predictors demonstrated a statistically significant association with the serum caspase-3 concentration ($p > 0.05$ for all). The VIF values were less than 3, indicating that collinearity did not bias the estimates after centering. These

results suggest that neither demographic variables nor thyroid hormone levels independently predict apoptotic activity, as measured by serum caspase-3, in this cohort (Table 4).

Table 4: Multivariable linear regression models assessing predictors of serum caspase-3 levels in the whole cohort.

Variable	B	SE	β	t	p value	95% CI (Lower, Upper)	VIF
Model 1 (T_3 included)							
Constant	0,389	0,077	-	5,073	<0.001	0.238, 0.540	-
Age_c	-0,001	0,002	-0,021	-0,274	0,784	-0.004, 0.003	1,03
Sex (Female vs Male)	0,013	0,043	0,022	0,294	0,769	-0.073, 0.098	1,04
TSH_c	0	0,001	-0,094	-0,779	0,437	-0.002, 0.001	2,6
T_3_c	-0,118	0,122	-0,117	-0,965	0,336	-0.358, 0.123	2,61
Model 2 (T_4 included)							
Constant	0,388	0,077	-	5,072	<0.001	0.237, 0.539	-
Age_c	0	0,002	-0,02	-0,259	0,796	-0.004, 0.003	1,03
Sex (Female vs. Male)	0,013	0,043	0,023	0,303	0,762	-0.072, 0.099	1,03
TSH_c	-0,001	0,001	-0,127	-1,027	0,306	-0.002, 0.001	2,76
T_4_c	-0,026	0,02	-0,157	-1,26	0,209	-0.066, 0.015	2,77

Abbreviations: B = Unstandardized coefficient; SE = Standard error; β = Standardized coefficient; CI = Confidence interval; VIF = Variance inflation factor; Age_c, TSH_c, T_3_c, T_4_c = centered variables (original value minus sample mean).

Notes: Dependent variable = Serum caspase-3 (ng/mL).

Model 1: Age_c, sex, TSH_c, T_3_c.

Model 2: Age_c, sex, TSH_c, T_4_c.

No predictor was statistically significant ($p > 0.05$) and all the variance inflation factor (VIF) values were < 3, indicating no problematic multicollinearity.

Multiple Regression Analysis – Age-stratified

When stratified by age group, only age (centered) in participants aged 18–40 years was significantly associated with serum caspase-3, with older individuals showing lower levels (Model 1: B = −0.016, 95% CI −0.032 to −0.001, p = 0.038; Model 2: B = −0.016, 95% CI −0.032 to 0.000, p = 0.045). No other predictors, including sex, TSH, or thyroid hormone concentrations (T_3 or T_4), were statistically significant in either age group (all $p > 0.1$). In the 41–60-year age group, no variables were significantly associated with caspase-3. The variance inflation factors (VIF) for T_3/T_4 and their interaction terms exceeded 25 in the younger group and 90 in the older group, indicating severe multicollinearity and limiting the interpretability of these coefficients (Table 5).

Table 5: Multiple Linear Regression for Predictors of Serum Caspase-3 by Age Group.

Age Group	Variable	B	SE	β	p value	95% CI (Lower, Upper)	VIF
Model 1 (T_3 included)							
18–40 years	Constant	-0,008	0,309	-	0,978	-0.626, 0.609	-
	Sex (Female vs. Male)	0,02	0,077	0,031	0,797	-0.134, 0.174	1,042
	Age_c	-0,016	0,008	-0,415	0.038*	-0.032, -0.001	2,866
	TSH_c	0	0,001	-0,095	0,623	-0.003, 0.002	2,797
	T_3_c	-0,692	0,598	-0,677	0,251	-1.884, 0.500	25,54
41–60 years	Constant	0,535	0,293	-	0,071	-0.046, 1.115	-
	Sex (Female vs. Male)	0,003	0,053	0,005	0,962	-0.102, 0.107	1,025

	Age_c	0,013	0,009	0,248	0,162	-0.005, 0.030	3,302
	TSH_c	-0,001	0,001	-0,153	0,334	-0.002, 0.001	2,662
	T_3_c	0,451	0,943	0,448	0,634	-1.420, 2.322	93,55
Model 2 (T_4 included)							
18–40 years	Constant	-0,015	0,325	-	0,964	-0.663, 0.634	-
	Sex (Female vs. Male)	0,02	0,077	0,03	0,798	-0.134, 0.174	1,042
	Age_c	-0,016	0,008	-0,418	0.045*	-0.032, 0.000	3,121
	TSH_c	-0,001	0,001	-0,104	0,608	-0.003, 0.002	3,048
	T_4_c	-0,109	0,096	-0,662	0,26	-0.301, 0.083	25,32
41–60 years	Constant	0,569	0,312	-	0,071	-0.050, 1.189	-
	Sex (Female vs. Male)	0,003	0,052	0,005	0,956	-0.101, 0.107	1,022
	Age_c	0,014	0,009	0,269	0,144	-0.005, 0.032	3,598
	TSH_c	-0,001	0,001	-0,203	0,208	-0.003, 0.001	2,763
	T_4_c	0,075	0,153	0,452	0,626	-0.229, 0.378	92,21

Abbreviations: B = Unstandardized coefficient; SE = Standard error; β = Standardized coefficient; CI = Confidence interval; VIF = Variance inflation factor; Age_c, TSH_c, T_3_c, T_4_c = centered variables (original value minus sample mean).

Sex coded as 0 = female (reference), 1 = male.

*p < 0.05 indicates statistical significance.

Variance inflation factor (VIF) values >10 indicate severe multicollinearity; values between 5–10 suggest moderate multicollinearity.

Notes: Dependent variable = Serum caspase-3 (ng/mL).

Model 1: Age_c, sex, TSH_c, T_3_c.

Model 2: Age_c, sex, TSH_c, T_4_c.

Discussion

In this study, we examined 183 hospitalized patients with hypothyroidism and elevated TSH levels to assess the serum level of caspase-3, as a marker of apoptosis and its relationship with thyroid hormones. Hypothyroidism is increasingly prevalent in India, particularly among older adults and pregnant women, with a reported global prevalence of 2–4% [21]. Despite national salt fortification, factors such as iodine deficiency, maternal hypothyroidism, consanguinity and environmental exposure contribute to India's high burden of thyroid disease [22,23]. Our cohort reflected the expected female predominance (71%), which is consistent with previous studies [24].

The relationship between TSH levels and aging remains controversial, with prior studies showing increased, decreased, or unchanged TSH concentrations in older adults [25]. Such variability may stem from population differences, methodologies, or age-related changes in hypothalamic–pituitary–thyroid responsiveness [26]. In our study, a slight decrease in TSH levels with advancing age was observed, although thyroid hormone concentrations did not differ significantly between age groups. This finding suggests age-related changes in TSH regulation that may occur independently of peripheral thyroid hormone levels.

Thyroid hormones play a critical role in regulating cellular growth, proliferation, and programmed cell death, primarily through TSH receptor–mediated signalling pathways. These regulatory effects are highly dependent on cell type and developmental context [27]. Experimental evidence has shown that TSH can downregulate autophagy and promote apoptosis in chondrocytes, as well as induce apoptosis in thyroid follicular cells to maintain thyroid gland homeostasis [28,29]. Conversely, TSH may also exhibit anti-apoptotic effects by inhibiting Fas-mediated apoptosis and activating pro-survival signalling pathways such as MAPK/ERK and PI3K/Akt [30,31]. These contrasting actions underscore the complexity of TSH-mediated regulation of cellular fate.

The normal serum caspase-3 level has been reported to be approximately 0.2 ng/mL [32]. In our study, all three TSH-based groups showed elevated caspase-3 levels, reflecting increased apoptotic activity in hypothyroid patients. Contrary to our initial hypothesis, caspase-3 did not differ significantly across TSH groups and was not correlated with TSH, even in patients with severe hypothyroidism (TSH > 51 mIU/mL). These findings imply that TSH, despite being a useful marker of disease severity, does not directly reflect apoptotic activity. This may be related to the dual nature of TSH, which has been shown to promote apoptosis in some contexts (e.g., thyroid follicular cells and chondrocytes) [28,29] and inhibits apoptosis in others through Fas antagonism and pro-survival signalling [30,31].

At the bivariate level, thyroid hormones (particularly T_3 and T_4) were strongly associated with caspase-3 levels. In mild hypothyroidism (Group I), both T_3 ($r = –0.319$, $p < 0.05$) and T_4 ($r = –0.377$, $p < 0.001$) were inversely correlated with caspase-3, suggesting that early reductions in thyroid hormones may be linked to increased apoptosis. These results align with experimental data showing anti-apoptotic effects of thyroid hormones across multiple cell types, including cancer cells, cardiomyocytes, and neurons [33-35]. However, these associations were attenuated after adjustment for age, indicating that age acted as a confounding variable.

Age-stratified analyses provided further insight into these relationships. Among younger adults (18–40 years), advanced age was significantly associated with lower caspase-3 levels, suggesting a physiological decline in apoptotic activity with progression through early adulthood. This observation aligns with existing evidence indicating reduced apoptotic turnover with aging, reflecting lower tissue renewal demands [36]. In this age group, thyroid hormones did not remain independent predictors of caspase-3 once age was accounted for. In contrast, among older adults (41–60 years), age itself was not significantly associated with caspase-3 levels, while serum T_4 emerged as an independent predictor. This finding suggests a shift in the relative influence of age and endocrine factors on apoptotic regulation in later stages of adulthood, with thyroid hormone availability playing a more prominent role.

Collectively, these results highlight the complex and age-dependent interplay between thyroid function and apoptosis. Although caspase-3 levels were elevated across all degrees of hypothyroidism, the determinants of apoptotic activity varied by age group. Age predominated as a regulatory factor in younger individuals, whereas thyroid hormone levels - particularly T_4 - were more influential in older adults, underscoring the context-dependent nature of thyroid hormone - apoptosis interactions.

This study has several limitations. The absence of a euthyroid control group limited the ability to establish baseline caspase-3 concentrations for comparison. Additionally, incomplete clinical information regarding disease duration, aetiology, comorbidities and treatment history may have influenced caspase-3 variability. The possibility of euthyroid sick syndrome in hospitalized patients cannot be entirely excluded; however, this was minimized by restricting inclusion to patients with markedly elevated TSH levels (>15 mIU/mL), supporting the diagnosis of true primary hypothyroidism.

Despite these limitations, the study adds valuable clinical evidence to the understanding of thyroid hormone–apoptosis interactions, emphasizing the importance of age stratification when interpreting apoptotic markers in hypothyroidism. These findings provide a rationale for future mechanistic and longitudinal studies to clarify how thyroid dysfunction and aging jointly influence apoptotic pathways across the lifespan.

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Research Ethics

The research related to human use has complied with all the relevant national regulations and institutional policies and in accordance with the tenets of the Helsinki declaration and has been approved by the authors' Institutional Ethics Committee (IEC KMC MLR 07/2024/474).

Informed consent

The Institutional Ethics Committee approved a waiver of informed consent, as anonymized leftover serum samples obtained during routine clinical testing were used.

Author contributions

The authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Use of Large Language Models, AI and Machine Learning Tools

AJE for improving language.

Conflict of interest

The authors state that they have no conflicts of interest.

Research funding

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Data availability

The raw data can be obtained upon request from the corresponding author.

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Research Article

Association of Free Testosterone and DHEA-S with Dyslipidemia in Women with Polycystic Ovarian Syndrome- a case-control study

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Keywords

androgen, DHEA-S, dyslipidemia, free testosterone, infertility, insulin resistance, PCOS

Abstract

Background: Polycystic ovarian syndrome (PCOS) is a multisystem disorder presenting with menstrual irregularities, infertility, and features of hyperandrogenism. Hyperandrogenism predisposes to the critical clinical features of PCOS. This find aimed to study the association of androgenic hormones such as dehydroepiandrosterone sulfate (DHEA-S) and free testosterone with lipid profile in PCOS women.

Methods: This case-control study was conducted in the Department of Biochemistry at a tertiary care hospital in Chennai. Patients were recruited from the Department of Obstetrics and Gynecology. Participants were aged 18-40 years. Blood samples were collected for analysis of lipid profile, DHEA-S, and free testosterone. DHEA-S and free testosterone were analyzed by ELISA. Ethics approval and written informed consent were obtained. Based on the distribution of the data, appropriate statistical tools were used. P-value ≤ 0.05 was considered statistically significant.

Results: Most of the participants were aged between 21 and 30 years. HDL-c was decreased in PCOS patients compared to healthy individuals; however, no statistically significant difference was found. Free testosterone showed an association with triglyceride. The areas under the curves of DHEA-S and free testosterone were 0.638 and 0.765, respectively.

Conclusion: DHEA-S and free testosterone showed good area under the curves. But free testosterone performed better with a higher area under the curve as well as its association with triglyceride. The cut-off values to diagnose PCOS were 3.0 µg/mL and 2.5 pg/mL for DHEA-S and free testosterone, respectively, with adequate sensitivity and specificity. Since free testosterone performed better in ROC curve than DHEA-S, free testosterone is considered to be a potential biomarker of identifying hyperandrogenism in PCOS women.

Introduction

Polycystic ovarian syndrome (PCOS) is the most common endocrine disorder in women of reproductive age group [1]. PCOS presents with a worldwide prevalence rate from 2.2% to 22.5% [2]. Whereas the prevalence in India is 28.9% as reported by the National Institute of Health (NIH) criteria, 35.3% by the Rotterdam criteria [3], and 34.3% by the Androgen Excess–PCOS Society (AE-PCOS) criteria [4]. PCOS is caused by genetic, epigenetic and nongenetic causes. In this metabolic condition, hyperinsulinemia with insulin resistance (IR) is highly prevalent among these women. It predisposes to the development of type 2 diabetes mellitus (T2DM), metabolic syndrome (MetS), and cardiovascular disorders (CVD) [5,6]. Generally, the ovaries and the adrenal glands are involved in the synthesis of steroid hormones. Around 90% of dehydroepiandrosterone (DHEA) and its sulphate ester (DHEA-S) are mainly produced in the adrenal glands. Approximately 40-70% of PCOS women have increased levels of androstenedione and DHEA-S. Studies have shown contradictory evidence between IR and DHEA-S levels. DHEA is found to positively influence the antral follicle count and ovarian volume of women undergoing assisted reproduction for primary ovarian insufficiency [7].

Studies indicate that high androgen levels play crucial roles in the pathogenesis of PCOS. High testosterone levels are linked with abdominal fat distribution, IR and glucose intolerance in obese individuals [8]. PCOS women with elevated free testosterone have an adverse metabolic profile compared to PCOS women with normal androgen levels [6]. IR is associated with dyslipidemia as evidenced by decreased levels of high-density lipoprotein cholesterol (HDL-c), increased levels of low-density lipoprotein cholesterol (LDL-c) and triglyceride (TGL) [9]. Various androgen markers like androstenedione, sex hormone binding globulin (SHBG), DHEA-S, free testosterone, and total testosterone have been analyzed to diagnose hyperandrogenism [10].

The metabolic sequelae in women with PCOS could be due to the activation of the renin–angiotensin–aldosterone system (RAAS), a dysfunctional autonomic nervous system, and increased synthesis and activity of androgen receptors [11]. To screen women with suspected hyperandrogenism, levels of DHEA-S and free testosterone are being analyzed. This study aimed to find the association of free testosterone and DHEA-S with dyslipidemia in women with PCOS.

Methods

The case-control study was conducted in the Department of Biochemistry at a tertiary care hospital in South India.

Study participants

The study included 180 women of reproductive age from 18 to 40 years; healthy women did not have the features of PCOS (n=90). Individuals who presented with PCOS (n=90) with diagnosis based on the Rotterdam criteria were treated as cases. Individuals with diabetes mellitus, endocrine, liver, and renal disorders, and women on oral contraceptive pills, anabolic or androgenic drugs, smokers, and alcoholics were excluded from the study. The study participants were recruited from the Department of Obstetrics and Gynecology.

Ethics statement

Ethics approval was obtained from the Institutional Ethics Committee (Ethics No.: CSP-MED/18/AUG/45/109, dated 24-09-2018). The participants provided written informed consent before participating in the study.

Sample collection

Blood samples from the study participants were collected in red-topped vacutainers by trained phlebotomists. These samples were centrifuged at 3200 rpm for 15 minutes. The separated serum was analysed for free testosterone, DHEA-S, and lipid profile.

Analysis of the parameters

Free testosterone and DHEA-S were assayed by using a competitive ELISA from Diametra, Italy. This assay employs a competitive assay format based on the ELISA principle first described by Engvall and Perlmann (1971) and refined in standard immunoassay protocols for small steroid hormones [12]. Lipid profile was analyzed in the hospital central laboratory by standard methods.

Funding statement

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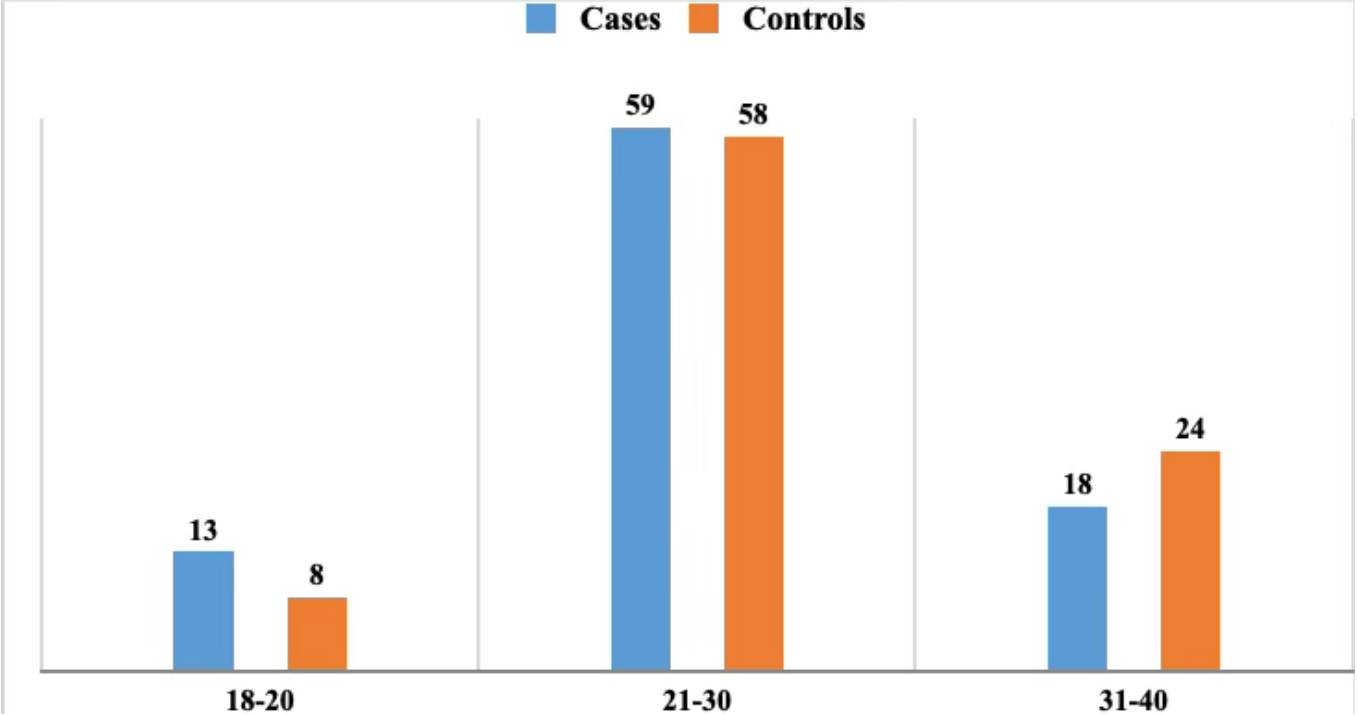
Statistical analysis

The obtained data were subjected to the Kolmogorov-Smirnov test. Based on the type of distribution, mean and standard deviation, or median and interquartile range (IQR) were used. To compare the variables between the groups, Student's t-test or the Mann-Whitney U test was used for continuous variables and the Chi-square test was used for categorical variables. Spearman's Correlation was done to find the association among the variables. A Receiver Operating Characteristics (ROC) curve was analysed and Youden index was used to arrive the cut-off values of DHEA-S and free testosterone. Statistical analyses were performed with SPSS version 16.0. A p-value $\leq$ 0.05 was considered statistically significant.

Results

Figure 1 shows the distribution of the participants according to the age. Among 90 PCOS individuals, 13 belonged to the age 18 - 20 years, 59 belonged to the age 21 - 30 years, and 18 belonged to 31 - 40 years. Among controls, eight belong to 18 - 20 years, 58 belonged to 21 - 30 years, and 24 belonged to 31 - 40 years (Figure 1).

Figure 1: Age-wise distribution of study participants.



Baseline characteristics

The data in Table 1 were compared between PCOS cases and controls given in columns, 3 and 4 respectively. The mean age of the individuals in the case and control groups was 26.77 and 27.56 years, respectively, with no statistically significant difference in age between the groups (p=0.40). There was no statistically significant difference in age distribution among the groups (p=0.35) (Table 1).

Table 1: Comparison of biochemical variables between PCOS women and controls.

Characteristic	Overall (n = 180)	PCOS (n=90)	Control (n = 90)	p-value
Age (year)	27.16 (5.80)	26.77 (5.37)	27.56 (6.21)	0.4
18-20 years n (%)	21 (11.7)	13 (14.4)	8 (8.9)	0.35
21-30 years n (%)	117 (65)	59 (65.6)	58 (64.4)	
31-40 years n (%)	42 (23.3)	18 (20)	24 (26.7)	
DHEA-S, µg/mL	2.3 (1.2-5.0)	2.7 (1.6-5.8)	1.9 (1.0-3.5)	<0.001**
Free testosterone, pg/mL	1.95 (1.0-2.95)	2.3 (1.3-4.2)	1.2 (0.9-2.0)	< 0.001**
Total cholesterol, mg/dL	135.53 (25.30)	134.52 (23.99)	136.53 (26.63)	0.6
TGL, mg/dL	98.84 (33.01)	100.72 (36.79)	96.96 (28.83)	0.4
HDL-c, mg/dL	39.61 (3.75)	39.13 (3.39)	40.09 (4.04)	0.08
LDL-c, mg/dL	76.17 (22.76)	75.21 (21.67)	77.13 (23.88)	0.6
Normal lipids n (%)	65 (36.2)	29 (32.2)	36 (40)	0.27
Dyslipidemia n (%)	115 (63.8)	61 (67.8)	54 (60)	

Total cholesterol, TGL, HDL-c, LDL-c expressed in mean and standard deviation, compared by student's t-test; DHEA-S, Free testosterone: expressed in median and interquartile range, compared using Mann-Whitney U test; Age, normal lipids, dyslipidemia: expressed in frequency and percentage, compared using Chi-Square test; * p-value: statistically significant; **p-value: Highly statistically significant

In the present study, the median values of DHEA-S were 2.7 in PCOS and 1.9 µg/mL in controls, and there was a statistical difference (p<0.001) between the groups. The mean levels of free testosterone were 2.3 in cases and 1.2 pg/mL in controls. There was a high statistical difference between the groups (p<0.001) (Table 1). There was no statistically significant difference between the groups for total cholesterol (p=0.6), TGL (p=0.4), HDL-c (p=0.08), and LDL-c (p=0.6) (Table 1).

Alterations of lipid profile

The lipid profile data were characterized by National Cholesterol Education Program (NCEP)-Adult Treatment Panel (ATP)-III guidelines [13]. Total cholesterol, TGL, and LDL-c were within the acceptable limits in both groups. However, HDL-c was normal in the control group but lower in PCOS. Around 32.2% of individuals in cases and 40% of individuals had normal lipids; whereas, 67.8% of individuals in PCOS and 60% of individuals in controls had dyslipidemia, which was statistically not significant (p=0.27) (Table 1).

Spearman correlation was done for DHEA-S and free testosterone with other parameters, as shown in Tables 2 and 3, respectively.

Table 2: Spearman correlation of DHEA-S with the other parameters.

Parameter	Correlation coefficient ('ρ' value)	p-value
Free Testosterone	0.115	0.12
Total cholesterol	0.007	0.92
TGL	-0.061	0.41
HDL-c	0.06	0.42
LDL-c	0.016	0.82

* p-value: statistically significant; ** p-value: Highly statistically significant

Spearman correlation of DHEA-S with other parameters did not show any statistical difference (Table 2).

Table 3: Spearman correlation of free testosterone with other parameters.

Parameter	Correlation coefficient ('ρ' value)	p-value
Total cholesterol	-0.027	0.71
TGL	0.212	0.004**
HDL-c	-0.123	0.1
LDL-c	-0.067	0.37

* p-value: statistically significant; ** p-value: Highly statistically significant

Table 4: Area under the curve and cut-off values of DHEA-S and free testosterone.

Biomarker	AUC	95% CI	p-value	Sensitivity (%)	Specificity (%)	Optimal Cut-off
DHEA-S	0.765	0.551 – 0.718	<0.001**	59	67	3 µg/mL
Free testosterone	0.765	0.692 – 0.832	<0.001**	68	81	2.5 pg/mL

Free testosterone showed larger AUC compared to DHEA-S. Sensitivity and specificity are larger for free testosterone compared to DHEA-S (Table 4).

Discussion

In the present study, around 65% of the participants in both cases and controls were in 21- 30 years age group. This was further emphasised by the mean age of around 27 years in both groups. DHEA-S and free testosterone were higher in PCOS compared to that of controls. There was no significant difference in lipid profile between cases and controls. All the lipid profile parameters were within the biological reference intervals except HDL-c which was lower in cases. Free testosterone showed correlation with TGL as well as better performance in the ROC curve.

PCOS is a common metabolic disorder prevalent among reproductiv age group women. It is characterized by ovarian dysfunction, hyperandrogenism, and polycystic ovarian morphology. Patients present with menstrual irregularities, infertility, hirsutism, acne, obesity, and features of MetS. Also, hyperandrogenism in PCOS has significant long-term implications, such as increased risks of T2DM, CVD,

endometrial hyperplasia, and psychosocial impacts. Hence early diagnosis and treatment is mandatory.

The study participants were aged between 18 and 40 years, 65% were aged 21 to 30 years in both groups. The mean ages of the individuals in the PCOS and control groups were 26.77 and 27.56 years, respectively (Table 1). The present study findings about age of the participants were in alignment with Vijayan et al 2022. PCOS is prevalent in women of approximately 26 years, and these individuals have higher Anti-Mullerian Hormone (AMH) compared to healthy controls [14]. Gupta et al (2018), found a PCOS prevalence of 8.2% among college girls aged 17 to 24 years [15]. The prevalence (22.5%) is higher in a study conducted in Mumbai, among 600 girls aged 15–24 years [16]. Thus, prevalence varies according to the study population and the criteria used to diagnose PCOS.

PCOS is associated with increased risk of IR, obesity, dyslipidaemia, and MetS. Studies show that among the lipid profile variables serum levels of total cholesterol, LDL-c, and TGL get elevated, while the level of HDL-c gets lowered in PCOS women compared to that of controls [17]. As per Mehreen et al study, symptoms of hyperandrogenism, hyperinsulinemia, and adiposity are commonly seen in PCOS. This is associated with weight gain and male pattern distribution of body fat. Among South Indian adolescents with PCOS, weight gain is common among women with menstrual disorders. The male pattern of body fat distribution in PCOS is due to hyperandrogenism. This facilitates a vicious circle of hyperinsulinemia, hyperandrogenemia, central obesity, and metabolic dysfunction [18]. Hyperandrogenism also influences unfavorable dyslipidemia in these individuals. In addition to hyperandrogenism, other factors such as IR, environmental factors, and genetics play roles in the setting and progression of dyslipidemia. Obesity as measured by anthropometric measurements such as body mass index (BMI) and waist circumference (WC) shows good correlation with dyslipidemia, especially in South Indian PCOS women [19].

According to Parveen et al, BMI, WC, and waist-to-hip ratio (WHR) are elevated in PCOS. Among lipid profile, total cholesterol showed a positive correlation with TGL among PCOS patients [20]. Begum et al, in their study found that 58% of the participants are overweight while 15% are obese, and 25% have normal BMI. In addition, PCOS individuals have higher fasting plasma glucose levels compared to controls [21]. PCOS women with large WC have lower HDL-c levels, apolipoprotein A1 (Apo A1), and albumin levels compared to controls. In PCOS, testosterone levels are associated with higher very low-density lipoprotein (VLDL) and elevated WC compared to controls. The higher testosterone levels, are also associated adversely with insulin levels and homeostasis model assessment for insulin resistance (HOMA-IR) in PCOS cases compared to controls [22].

In this study, there was no statistically significant difference between the groups among lipid profile- total cholesterol ($p=0.6$), TGL ($p=0.4$), HDL-c ($p=0.08$), and LDL-c ($p=0.6$) based on NCEP-ATP III guidelines. Total cholesterol, TGL, and LDL-c were within acceptable limits in both groups. However, HDL-c was below the cut-off level in controls but lower in PCOS. Around 67.8% of PCOS and 60% of controls had dyslipidemia (Table 1). According to Conwell et al, IR is at the crossroads in the pathogenesis of dyslipidemia, glucose intolerance, and hyperandrogenism irrespective of BMI. The surrogate markers of IR include the HOMA-IR, the quantitative insulin sensitivity check index (QUICKI), and the fasting glucose to insulin ratio (FG-IR) [23]. TGL-to-HDL-c ratio of 3.5 predicts the presence of the small dense LDL phenotype, which is considered to be the most atherogenic lipoprotein. This ratio is better than the total cholesterol-to-HDL-c ratio, HDL-c alone, or TGL alone. Thus, it gives an insight into lipoprotein metabolism that increases CVD risk in insulin-resistant patients [24]. According to Macut et al, lipid levels are almost the same in both PCOS women and controls. But PCOS women show characteristic features of IR, such as decreased HDL-c and increased TGL. Impaired clearance of small dense LDL-c and the association of free fatty acids and apolipoprotein E (Apo E) to IR, indicate worsening of metabolic status and premature onset of atherosclerosis [9].

With the onset of IR and hyperinsulinemia, hyperandrogenaemia facilitates the formation of visceral adipose tissue, which exacerbates the secretion of androgen in the ovaries and adrenal glands [25]. The excess androgen hormones produced by the ovaries and adrenal glands are converted to DHEA-S and testosterone. This leads to increased levels of DHEA-S and testosterone in PCOS [26]. Though testosterone circulates in plasma as both free or bound form (bound to SHBG and albumin), free testosterone is found to be a sensitive marker in the diagnosis of PCOS [10]. A study conducted in Belgium showed that 44% of PCOS individuals have high free testosterone levels [10]. In the present study, the median levels of DHEA-S and free testosterone were higher in cases than in controls, and were statistically significant ($p<0.001$) (Table 1). Spearman correlation of DHEA-S with other parameters did not show any statistical difference (Table 2); but free testosterone showed a high statistically significant correlation with TGL ($p=0.004$) (Table 3).

The findings of the study by Christodoulaki et al. revealed that there is a negative correlation between DHEA-S levels and the ovarian volume. The precise pathophysiology behind this association remains unknown. However, this effect might be linked to increased fibrosis of ovarian tissue, which ultimately leads to menstrual disorders and decreased fertility potential [7]. About half of the testosterone is produced by the peripheral conversion of adrenal androgens, and elevated DHEA-S levels also contribute. The DHEA-S/free testosterone ratio might be a more accurate measure of the metabolic effects of androgens. An extra-adrenal mechanism likely to be involved in regulating adrenal androgen release; other than the influence of adrenocorticotropic hormone (ACTH). In PCOS, central adiposity is negatively correlated with serum DHEA-S levels.

PCOS metabolic tests are correlated with higher levels of free testosterone. Thus, DHEA-S/free testosterone ratio is better than the individual hormones to monitor metabolic parameters in PCOS [26].

In the present study, DHEA-S showed an AUC of 0.638 and the cut-off of 3.0 µg/mL with sensitivity and specificity of 59% and 67% respectively. Free testosterone showed a higher AUC of 0.765 with cut-off of 2.5 pg/mL with sensitivity and specificity of 68% and 81% respectively. Since free testosterone showed higher AUC compared to DHEA-S, free testosterone could be a better indicator of hyperandrogenism. (Table 4) Adrenal hyperandrogenism is common in patients with non-classic (B and C) phenotypes of PCOS and is due to higher production of androgens in PCOS patients. However, other factors may increase the adrenal androgen production and influence the clinical expression of the syndrome [27]. In a German PCOS cohort, ROC curve analysis suggests that calculated androgen indices (bioavailable testosterone, free androgen index, free testosterone) are superior to other androgen variables in defining PCOS and represent reliable markers for androgen excess [28].

To investigate the function of free testosterone and androstenedione in the metabolic phenotype of PCOS, Lerchbaum et al. undertook a study in Austria, which showed that PCOS women who had elevated free testosterone with normal androstenedione levels are at a higher risk for metabolic problems [6]. Elevated free testosterone levels are observed in approximately 70% of PCOS patients, especially those diagnosed by the NIH 1990 criteria. The present recommendation is to measure free testosterone concentration and SHBG. Approximately 20% to 30% of patients with PCOS have only high levels of DHEA-S; circulating levels of DHEA-S have limited diagnostic value. Hence, serum androgens, including free testosterone, should be used as an adjuvant tool for the diagnosis of hyperandrogenic disorders [8].

Patients with PCOS should also closely monitor their lipid profiles for the presence of elevated apolipoprotein B100 (Apo B100). A 2.8-fold causative increase in the risk of ischemic heart disease is associated with a non-fasting increase of 1 mmol/L in Apo B lipoprotein remnants, which is correlated with fasting insulin and IR. Dietary control and lifestyle changes are the mainstays for the treatment of dyslipidemia. Additionally, targeted lipid-lowering medications may normalize dyslipidemia; however, this is not yet a recognized treatment for PCOS. The optimal subset of PCOS women who need lipid-lowering therapy and the efficacy of such treatments for dyslipidemic women will need to be determined by future clinical research [29].

Limitations

The PCOS individuals were not classified according to the various phenotypes of PCOS. The lipids and androgens were not compared with insulin resistance. Anthropometric measurements and ovarian morphology were not included in the study.

Conclusion

Most of the study participants were in the third decade. Among the lipid profile markers, HDL-c was lesser than cut-off level among the PCOS individuals. The free testosterone was positively correlated with triglyceride, whereas DHEA-S did not show any association with lipid profile. The ROC curve analysis showed larger area under the curve for free testosterone compared to DHEA-S; thus, indicating that free testosterone could be a sensitive marker for diagnosing PCOS. However, further studies with large sample size is required.

Declarations

Ethics statement

All the 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. Ethics approval was obtained from the Institutional Ethics Committee (Ethics No.: CSP-MED/18/AUG/45/109, dated 24-09-2018). The participants gave written informed consent to participate in the study.

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Conflict of interests

The authors wish to declare that there were no conflict of interests either during the research or while writing this manuscript.

Data availability

Data obtained from the study will be with the corresponding author for a period of five years and the same can be shared for those working in the same field. The data will be destroyed after five years of publication.

Authors' contributions

Conceptualization: Lavanya K, Palaniappan N, Santhi Silambanan, Methodology: Lavanya K, Palaniappan N, Santhi Silambanan, Vinodhini VM, Formal analysis: Lavanya K, Santhi Silambanan, Vinodhini VM, Mahesh Kumar, Investigation: Lavanya K, Palaniappan N, Santhi Silambanan, Resources: Lavanya K, Palaniappan N, Santhi Silambanan, Vinodhini VM, Data curation: Lavanya K, Palaniappan N, Santhi Silambanan, Mahesh Kumar, Writing- original draft: Lavanya K, Palaniappan N, Santhi Silambanan, Writing-Review & editing Supervision: Lavanya K, Palaniappan N, Santhi Silambanan, Vinodhini VM, Mahesh Kumar, Project

administration: Lavanya K, Palaniappan N, Santhi Silambanan, Vinodhini VM, Funding acquisition: Lavanya K, Santhi Silambanan. All the authors have contributed equally in writing the final manuscript.

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Research Article

Nationwide Survey on Knowledge, Attitudes, and Practices regarding Reference Interval Utilization in Clinical Laboratories in Nepal

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Keywords

Reference intervals, KAP study, ISO 15189, Laboratory accreditation, Nepal, Standardization

Abstract

Background: Reference intervals (RIs) are critical for accurate clinical decision-making, yet many laboratories rely on manufacturer-provided RIs without local validation. This study assessed the knowledge, attitudes, and practices (KAP) of clinical laboratories in Nepal regarding RI utilization, highlighting challenges and opportunities for standardization in alignment with ISO 15189:2022 accreditation.

Methods: A nationwide cross-sectional KAP survey was conducted among 56 laboratory professionals. Data were collected via an online questionnaire, covering demographics, RI knowledge, current practices, challenges, and attitudes toward national standardization. Descriptive and inferential statistics (chi-square, Fisher's exact tests) were used for analysis.

Results: While 71.4% of respondents correctly defined RIs as the 2.5th–97.5th percentiles, 28.6% held misconceptions. Most laboratories relied on manufacturer-provided RIs (87.5%) or published literature (67.9%). Key challenges to derive one's own RI included method variability and recruiting reference individuals. Accredited labs (ISO 15189) demonstrated better knowledge of RI (93.3% vs. 63.4%, p=0.032) and higher confidence in using current RI (26.7% vs. 7.3%, p=0.047). Strong interest existed in national RI standardization (92.9%) and training (85.7% preferred hands-on workshops).

Conclusions: This survey of higher tier clinical laboratories in Nepal reveals that while these laboratories generally understand the importance of reference intervals, significant gaps in practice and standardization remain. The findings highlight an urgent need for inclusive strategies that also address the unique constraints of smaller, widespread laboratories, which perform a large proportion of routine testing in the country. The intense interest in a national program presents an opportunity to improve. Multicenter studies and RI validation integration into accreditation are needed to improve diagnostic accuracy.

Introduction

Biological reference intervals (RIs) reported in clinical laboratory test results provide critical information for interpreting a patient's health status and assessing a laboratory's adherence to quality standards. A RI derived from a specific local population using the same analytical platform offers the most accurate interpretation, accounting for biological and analytical variability. Despite this, many laboratories worldwide rely on manufacturer provided RIs for clinical decision making [1].

This reliance is often driven by a lack of knowledge, insufficient resources, or the absence of regulatory requirements mandating the establishment or verification of population specific RIs. In an era of increasing patient engagement and direct-to-consumer testing, discrepancies in RIs may lead to patient confusion and raise concerns among clinicians. Laboratories may respond by quoting published studies, adopting manufacturer RIs, or conducting their studies through direct or indirect methods, to establish or verify RIs [2]. However, each approach carries limitations [3].

Establishing RIs through formal studies is time-consuming, expensive, and methodologically challenging [4]. Factors such as methodological biases, differences in population demographics, and analytical platforms may significantly impact the transferability of RIs across laboratories. Consequently, it becomes essential for laboratories to either establish their RIs or verify adopted ones under local conditions, or for countries to adopt common reference intervals [5].

Improved understanding of RI development and verification processes, better training, and transparent laboratory communication about how RIs are derived are necessary to enhance clinical utility. However, before implementing improvements, it is imperative to assess the current knowledge, attitudes, and practices (KAP) related to RI utilization among clinical laboratories. This study was designed to evaluate KAP regarding reference interval utilization in Nepalese clinical laboratories. Furthermore, as Nepal progresses toward accreditation standards such as ISO 15189:2022, assessing the readiness and attitude of laboratory professionals toward RI utilization can guide stakeholders, including policymakers and professional societies, in developing effective training programs, support mechanisms, and regulatory frameworks to strengthen laboratory quality assurance across the country.

Methods

This study employed a cross-sectional KAP survey to assess the understanding and perceptions of Nepalese laboratory professionals regarding RI utilization. Data was collected for:

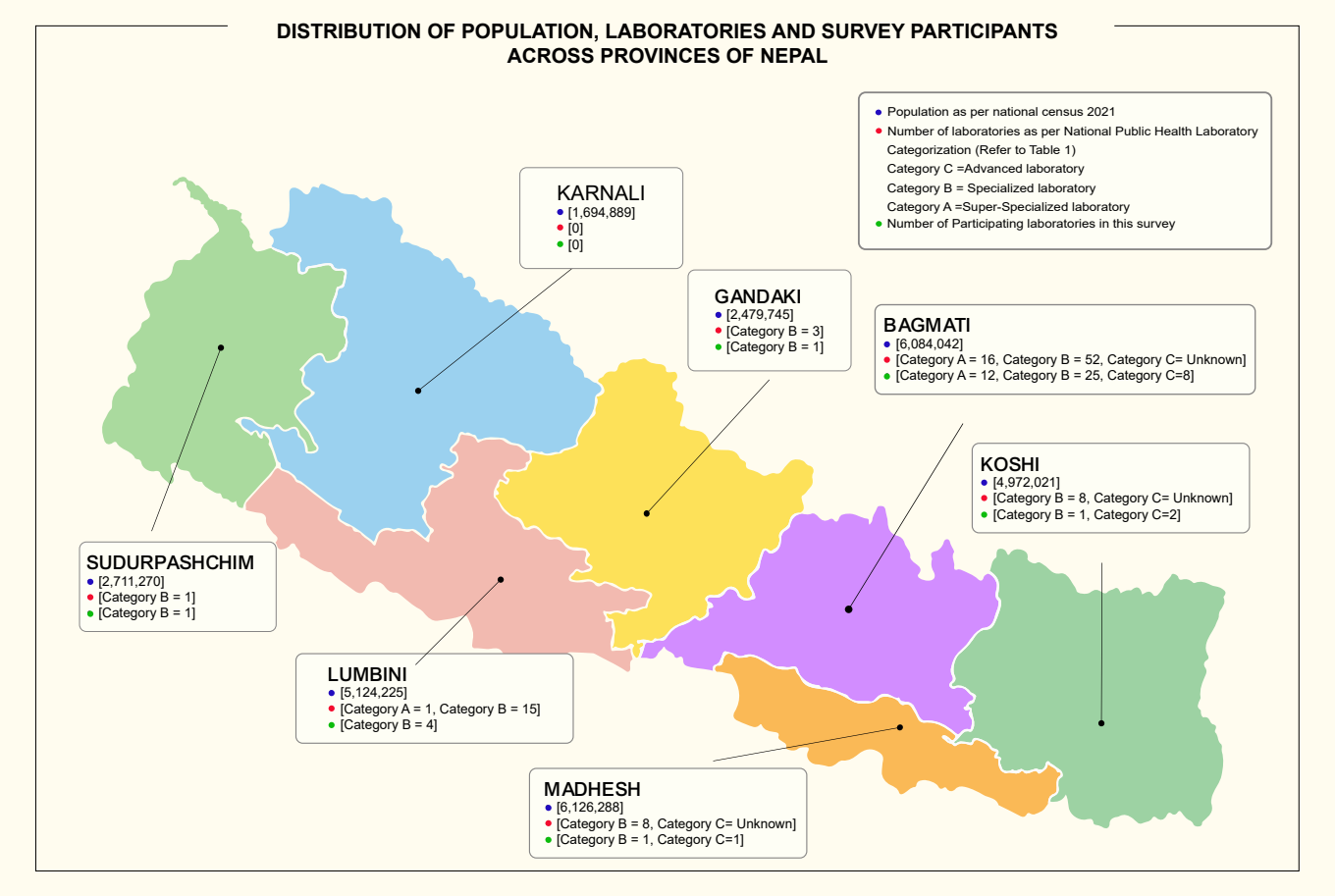
- Laboratory demographics (type, location, years in operation)
- Knowledge about reference intervals
- Current practices in RI utilization
- Challenges faced in establishing RIs
- Attitudes toward national standardization
- Training needs

The study targeted laboratories listed on the National Public Health Laboratory (NPHL)under the Ministry of Health and Population (MoHP) of Nepal, which categorizes clinical laboratories into five levels (A, B, C, D, and E) based on compliance with specific operational standards, including infrastructure, human resources, and test offerings (Table 1). Category A represents the highest standard, while Category E denotes the lowest level of capability and resources.

Study Population and Sampling

The study population consisted of laboratory professionals in laboratories classified under Categories A, B and C. The NPHL, operating under the Department of Health Services within the MoHP, maintains an updated list of laboratories in Categories A and B on its official website [6]. As of the latest update, 17 laboratories were categorized as A, and 91 were classified as Category B. A complete list of Category C laboratories is not available, as they are maintained by respective provincial public health laboratories. To ensure broader participation, the Google survey link containing the questionnaire was emailed to the laboratory in charge of each laboratory. Additionally, the survey link was shared on various social media platforms and the official website of national laboratory societies to encourage participation from professionals working in medical college affiliated laboratories and other relevant institutions. Figure 1 illustrates the number of participants from each province, the respective provincial population, and the number of laboratories registered under the NPHL, highlighting regional disparities and resource distribution.

Figure 1: Number of participants from each province, the respective provincial population, and the number of laboratories registered under the National public health laboratory as of 10 August 2025 [6].



Survey Instrument Development

The questionnaire underwent a validation and pilot testing phase prior to its nationwide deployment. The draft instrument was independently reviewed by external experts in clinical biochemistry and laboratory quality assurance to assess content validity, clarity, relevance, and comprehensiveness. Subsequently, a pilot survey was conducted with 10 laboratory professionals from different laboratory categories (A–C). Their feedback on question wording, flow, technical accuracy, and completion time informed minor revisions to enhance clarity and ensure accurate capture of the intended knowledge, attitude, and practice constructs.

Ethical Considerations

Ethical approval for this study was obtained from the Nepal Health Research Council [Protocol no. 405_ 2024]. Participation in the survey was voluntary, and informed consent was obtained from all respondents before they proceeded with the questionnaire. Data confidentiality and anonymity were maintained throughout the study.

Data Collection and Analysis

The survey was conducted online using a structured questionnaire in Google Survey. The survey questionnaire used in this study can be provided upon request from the corresponding author. Responses were collected over six months, and the data were analyzed using descriptive and inferential statistical methods to identify key trends and associations in the knowledge, attitudes, and practices of laboratory professionals regarding RI utilization in Nepal. Associations between variables (e.g., accreditation status and RI utilization) were assessed using chi-square tests or Fisher's exact test for small samples. A p-value <0.05 was considered significant.

Table 1: Clinical laboratory categories in Nepal, outlining the required test scope, infrastructure, equipment, and staffing for each level of operation.

Category	Test Scope	Space	Equipment & Technology	Staffing
E (Basic)	Basic hematology, biochemistry, AFB stain, routine urine/stool tests, pregnancy test, simple rapid diagnostic test (RDT).	≥ 150 sq. ft.	Basic equipment for listed tests.	≥2 personnel
D (Intermediate)	Expanded hematology, liver enzymes, lipid profile, creatinine, electrolytes, Gram/KOH stain, serology (RPR, Widal, CRP), all RDTs.	≥ 250 sq. ft.	Equipment for designated tests.	≥ 4 personnel (≥ 1 Lab Technologist)
C (Advanced)	Full hematology, cardiac enzymes, thyroid function, bacterial cultures, ELISA, histopathology, cytopathology, CSF/body fluid, semen analysis.	≥ 400 sq. ft.	Equipment for expanded diagnostics.	≥ 6 personnel (≥ 50% with Bachelor's degree, ≥1 with Master's degree)
B (Specialized)	Special coagulation, hormones, tumor markers, anaerobic & fungal cultures, ELISA/CLIA tests.	≥ 1,000 sq. ft.	Specialized test equipment.	≥ 12 personnel (≥ 4 per discipline, ≥ 3 Master's)
A (Super-Specialized)	All B-category tests + molecular diagnostics, flow cytometry, genetic studies.	≥ 2,000 sq. ft.	Advanced diagnostic technology.	≥ 24 personnel (≥ 6 per discipline, ≥ 1 Master's per dept.)

Results

Demographic information

This survey included 56 laboratory professionals, 59% working in Category B laboratories, 21% in Category A, and 19 % in Category C. Each response was collected from a single respondent per laboratory, specifically the quality manager or laboratory in-charge, as they possess the necessary knowledge of RI, their attitude reflects the laboratory's overall stance toward RI, and they are best positioned to describe RI utilization practices within their facility. The majority of participants (82%) were from Bagmati Province, followed by Lumbini Province (7%), Koshi and Madhesh (each 4%), Gandaki and Sudurpaschim, (2%),while no responses were recorded from Karnali Province(Figure 1).Fifty percent of participating laboratories were standalone,12% were from medical college, 18% were private hospital, and the remaining 20% were government laboratories. Most laboratories (69%) had been in operation for more than 10 years, 18% had 1-5 years of experience, and 13% had been operating for 6-10 years. Regarding annual test volume, 69% of laboratories performed more than 50,000 samples, 22% conducted 10,001-50,000 tests, 7% performed 1,001-10,000 tests, and only 2% conducted fewer than 1,000 tests annually. Accreditation under ISO 15189 was reported by 27% of laboratories, while the remaining 73% were not accredited.

Survey Findings

The KAP survey results are presented in Table 2. Although most respondents demonstrated an understanding of the basic RI concept as the 2.5th to 97.5th percentiles, approximately 29% held misconceptions, interpreting RIs as absolute maximum or minimum values, or as simple averages. Standardization remains a challenge, with 87.5% of laboratories relying on manufacturer provided RIs and 67.9% on published literature, while only 16.1% reported developing in-house RIs.

RI updating practices were inconsistent; about half of the laboratories updated RIs only after significant changes, and only 14.3% did so annually. The most frequently reported challenges in deriving population specific RI included variability in test methods (75%) and difficulty recruiting appropriate reference individuals (62.5%). Encouragingly, there was strong interest (92.9%) in a national RI program, suggesting readiness for coordinated efforts toward standardization. Additionally, there was a high demand for training, with 85.7% of respondents seeking hands-on workshops and 64.3% requesting access to international updated guidelines. Confidence in the current RIs was low, with only 12.5% expressing strong confidence and over half (55.4%) indicating uncertainty or lack of confidence in the intervals they use. However, the ISO 15189:2022 accredited labs were more confident in their RI compared to non-accredited labs (Table 3). Also, the accredited labs demonstrated significantly better knowledge of RI (Table 3).

Table 2: Summary of Knowledge, Practices, Challenges, Training Needs, and Attitudes on Reference Intervals.

Title	Category/Aspect	Details/Responses (%)
Knowledge	Understanding of 2.5th and 97.5th Percentiles	Correct: 71.4% Incorrect: 28.6%
	Understanding of Statistical Methods	Correct: 73.2% Incorrect: 26.8%
Practices	Sources of RI	Manufacturer: 87.5%, Literature: 67.9% RI from other labs: 26.8%, In-house: 16.1%
	Frequency of RI Update	When needed: 50%, Instrument change: 23.2% Annual: 14.3%, Every 2–5 years: 19.6%, Never: 7.1%
	Use of Age/Sex-Specific RIs	All analytes: 12.5%, Some analytes: 78.6% None: 8.9%
	Clinician Engagement	Seek laboratory expertise: 53.6%Do not Seek: 32.1%
Challenges in Establishing RI	Method Variability	75.00%
	Patient Recruitment	62.50%
	High Reagent Costs	51.80%
Attitudes	Confidence in Current RIs	Very: 12.5%, Somewhat: 44.6%, Neutral: 25.0% Low: 10.8%
	Interest in National Standardization	Yes: 92.9% No: 3.6%
Training Needs	Hands on Workshops	85.70%
	Access to guidelines	64.30%
	Online Courses	55.40%

Table 3: Comparison of study variables among accredited and non-accredited laboratories.

Variable	Accredited Labs (n=15)	Non-Accredited Labs (n=41)	Statistical Test	p-value
Correct Understanding of RIs (2.5th–97.5th percentiles)	14 (93.3%)	26 (63.4%)	Chi-square Test	0.032*
Use of Percentile Method	12 (80.0%)	29 (70.7%)	Chi-square (χ^2=0.49)	0.484
In-House RI Development	4 (26.7%)	5 (12.2%)	Fisher's Exact Test	0.234
Age/Sex-Specific RIs (All Analytes)	3 (20.0%)	4 (9.8%)	Fisher's Exact Test	0.381
Annual RI Updates	3 (20.0%)	5 (12.2%)	Fisher's Exact Test	0.431
Clinician Engagement	10 (66.7%)	20 (48.8%)	Chi-square (χ^2=1.45)	0.229
Very Confident in RIs	4 (26.7%)	3 (7.3%)	Fisher's Exact Test	0.047*
Top Challenge in RI calculation: Method Variability	9 (60.0%)	33 (80.5%)	Chi-square (χ^2=2.55)	0.11

Discussion

The findings from this study underscore several critical issues in the understanding and application of RIs in clinical laboratories in Nepal. A key concern is the evident knowledge gap among laboratory professionals regarding the fundamental concepts of RIs. Misinterpretations of these intervals can lead to inappropriate clinical decisions, emphasizing the need for educational interventions to reinforce foundational statistical and clinical knowledge [7]. A layperson might understand a RI as a way to distinguish between healthy and unhealthy states. However, it should be noted that RI is a guide to clinicians and they are subject to change and depends on the reference population and the methods used. Some healthy individuals may fall outside the reference interval, and some individuals with health issues may have normal results.

Another key challenge identified in this survey is the widespread reliance on externally sourced RIs, particularly those provided by manufacturers or derived from international literature. Although these sources offer convenience, they often lack relevance for local populations due to variations

in ethnicity, diet, altitude, and other demographic and environmental factors. The limited development of locally tailored, in-house RIs raises serious concerns regarding the accuracy and clinical relevance of laboratory interpretations in the local context. A recent study assessing TSH and fT4 RIs from major manufacturers (Roche, Abbott, Beckman, Siemens) found that these manufacturers provided intervals were often inappropriate, being either too narrow or too wide, thus highlighting the importance of establishing population specific RIs and performing regular verification to reduce the risk of misdiagnosis [8]. There is growing support for the development of indigenous RIs in several countries, including India [9], Pakistan [10], various African nations [11], Australia [12], Scandinavia [13] and the Netherlands [14]. These studies consistently conclude that population specific reference intervals are necessary, as the manufacturer provided reference intervals often differ significantly from the values observed in these populations. The standardization of RI between laboratories also reduces patient risk.

Manufacturer recommended RIs may suffer from several limitations, including reliance on outdated studies, lack of essential demographic or pre-analytical information, use of small sample sizes, exclusion of pediatric data, presence of multiple unexplained intervals, and application of inappropriate statistical methods [1]. While regulatory bodies accept these RI, they may not be clinically suitable. Furthermore, verifying RIs by testing 20 healthy individuals may be insufficient, especially if the intervals are overly broad [1]. Establishing RIs is inherently complex, as the choice of method must account for various biological and technical factors tied to both population characteristics and laboratory instrumentation. Understanding the strengths and limitations of RIs is crucial to enhancing diagnostic accuracy and improving clinical decision making. While this study focused on current practices and awareness of traditional direct methods for establishing RI, the potential of indirect methods warrants consideration, especially within resource-limited settings like Nepal. Indirect techniques such as mining existing laboratory information system data or applying statistical models to routine patient results offer a pragmatic, cost-effective alternative for RI estimation [15]. These approaches can circumvent key barriers identified in our survey, including challenges in recruiting reference individuals and high reagent costs. For many stable analytes, indirect methods present a viable pathway toward population specific RI validation without the operational burdens of prospective studies [15]. Future national standardization efforts and training programs in Nepal should therefore explore and integrate validated indirect methodologies as a scalable strategy to improve the appropriateness and applicability of RI across all tiers of laboratories, particularly in widespread Category C, D and E facilities where resources are most constrained.

The absence of standardized and periodic review processes for RIs in nearly half of the participating laboratories raises concerns about these intervals' continued validity and clinical relevance. Without regular updates in response to changes in instrumentation, methodologies, or evolving population health trends, the accuracy of result interpretation may be compromised. This survey also highlights several practical challenges that hinder RI updates, including limited resources, variability in analytical methods, and difficulty in recruiting appropriate reference individuals. According to ISO 15189:2022, accredited clinical laboratories are required to periodically re-evaluate their RIs [16]. Supporting this, a recent study emphasized the importance of seasonally adjusted RIs and proposed an approach that could help minimize over and under diagnosis [17]. Therefore, RIs should be regularly reviewed, revised, and tailored to meet the specific needs of the population served.

Despite these challenges, there is a strong indication of willingness among laboratories to engage in improvement efforts. The high interest in national standardization programs and hands-on training initiatives reflects a collective readiness for capacity building and practice harmonization. The desire for access to updated international guidelines and structured training also suggests that laboratories recognize the importance of aligning with global best practices.

Laboratory accreditation appears to play a crucial role in fostering better RI practices. Institutions that operate within a quality framework in the current survey are more likely to demonstrate higher confidence in their RIs and better conceptual understanding. This reinforces the value of accreditation which a driver of knowledge and performance improvement. While accredited labs report higher confidence, their practical aspect (e.g., update frequency, customization) does not differ significantly from non-accredited labs. This suggests accreditation improves awareness but not necessarily implementation. Nevertheless, increased awareness can be a positive outcome, but translating that awareness into consistent, practical application across all aspects of laboratory operations requires ongoing effort and commitment.

A coordinated national strategy is essential to advance good laboratory practices in Nepal. This should include regular training for laboratory personnel, stronger collaboration between clinical and laboratory professionals, integration of RI review into routine quality management systems and supporting research initiatives to conduct multicentre studies for the development of population specific RIs. Also, the RI validation should be integrated into accreditation processes, with support provided to laboratories pursuing accreditation. Such efforts will support more accurate diagnostic interpretation, promote evidence based clinical care, and improve patient outcomes.

Our study's modest sample size (n = 56) and the concentration of data from Bagmati Province (82%) limit its generalizability. However, it is essential to note that Bagmati is the capital province of Nepal, home to a significant portion of the country's population and a high density of clinical laboratories. Also our study focuses on higher-tier laboratories (categories A and B), which excludes the majority of lower- tier laboratory

facilities in Nepal that perform a substantial volume of basic routine testing, particularly in rural and remote settings. Therefore, while our findings offer a critical first national assessment of RI utilization among higher-tier laboratories and provide a foundation for policy development, they should be interpreted with recognition of these geographic and tier-based limitations, particularly regarding the widespread lower-tier laboratories that serve much of Nepal's rural population.

Conclusion

This nationwide survey of higher-tier clinical laboratories in Nepal demonstrates that while foundational awareness of reference interval importance is present, substantial implementation gaps persist in practice, standardization, and periodic validation. Despite a strong interest in national RI standardization and targeted training particularly among accredited laboratories, which exhibited more robust knowledge and confidence, widespread reliance on non-validated, manufacturer provided RIs remains a critical concern. These findings emphasize the necessity for structured policy and capacity building initiatives that are inclusive of all laboratory tiers. Moving forward, we recommend integrating RI validation into national accreditation frameworks, supporting multicenter studies to establish population specific reference intervals, and developing scalable training programs to enhance appropriate RI utilization.

Conflict of Interest

None.

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None.

Authors Contribution

VP - Conceptualization, Writing original draft
VP, SP, TB - Preparation of survey questionnaire
SP, DT, AK and NK - Conducted online survey and collected results, Manuscript review and editing
TB - Manuscript review and approval of final version

Ethics approval and consent to participate

This study is in compliance with the ethical principles for medical research involving human subjects, in accordance with the Declaration of Helsinki. Ethical approval for this study was obtained from the Nepal Health Research Council [Protocol no. 405_ 2024].

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Research Article

Assessment of best practices for quality assurance in laboratories in Portuguese-speaking countries

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Keywords

Laboratory best practices, Laboratory survey, Portuguese speaking countries, Quality assurance, Quality management

Abstract

Introduction: Laboratories (labs) play a fundamental role in screening, diagnosis, prognosis, and treatment of diseases. For a laboratory result to be useful, it must have guaranteed quality. In this context, there is no informative data on the best practices adopted by labs in Portuguese-speaking countries (PSC). This information is essential for formulating policies and educational strategies intended for this target audience.

Objective: To identify and assess adherence to laboratory best practices by clinical analysis labs in Portuguese-speaking countries.

Methods: A digital questionnaire consisting of 47 questions on laboratory best practices and quality management was sent to participants in the National Program for External Quality Assessment of Portugal and other labs involved in the Laboratory Quality Improvement Project for PSC-ProMeQuaLab, except from Brazil. Data were collected anonymously between July 7 and September 30, 2024 and analysed with descriptive statistics.

Results: 59 labs (ambulatory and hospital) participated in the study, but 5 institutions did not consent to the disclosure of their data, even if anonymously. Of the 54 included labs, most were from Portugal (39; 72%), followed by Cabo Verde (9; 16%), Guinea-Bissau (4; 7%), São Tomé and Príncipe (1; 2%), and 1 lab did not specify its country of origin. 57% of the labs have an implemented management system, and half of them are certified. Most labs belong to public services (63%), have a professional responsible for the management system (85%), conduct an annual training plan (85%), use quality indicators for the pre-analytical (87%) and post-analytical (83%) phases, and perform internal (70%) and external (89%) quality control. Opportunities for improvement were identified, as only 59% of labs record the causes of rejection of control sample

results, 65% develop a competency matrix, 66% construct control charts, and 72% use quality specifications to assess analytical performance.

Conclusion: Portuguese labs contributed the most to these results. Laboratory best practices are implemented, but there are opportunities for improvement. Conducting training and involving more labs from PSC will contribute to the implementation and harmonization of laboratory best practices, which can contribute to ensuring the quality of results and patient safety.

Introduction

With the aim of disseminating knowledge, building capacity, implementing best laboratory practices, and monitoring quality control measures, the Laboratory Quality Improvement Project – ProMeQuaLab – was established in 2015, focusing on Portuguese-speaking countries. The project emphasizes cooperation among countries so that they can effectively contribute to the improvement of laboratory diagnostics, directly benefiting institutions and, consequently, the general population of the participating countries. The countries involved in the project are Angola, Brazil, Cabo Verde, Guinea-Bissau, Mozambique, Portugal, São Tomé and Príncipe, and Timor [1].

The team responsible for managing a laboratory must establish an effective quality management system that enables the identification and minimization of analytical errors, aiming to ensure reliable and safe results for patients [2]. Quality management through specific indicators allows for mapping laboratory processes, identifying and quantifying errors, and subsequently implementing improvements and corrective actions. The use of indicators in healthcare services enables both internal and external comparisons with other similar services.

Laboratory quality assurance involves a set of management actions that create the necessary conditions for quality control and continuous process improvement. The primary objective of any advancement in the healthcare field is to enhance the safety of the services provided to patients [3,4].

Providing safety to patients means continuously improving all processes that affect them, ensuring the stability and predictability of those processes, and anticipating potential failures whenever possible. This requires a thorough understanding of the complexity of healthcare processes, the associated risk factors, and strict control over all critical stages within a healthcare organization [3].

High-reliability organizations are institutions that operate in high-risk environments, where even minor errors can lead to significant consequences, yet they manage to maintain low failure rates due to the implementation of robust organizational practices. Key characteristics of these organizations include:

1. A continuous concern with failures, aiming to detect and correct even the subtlest errors;
2. Avoidance of oversimplified interpretations, promoting deep and detailed analysis of the underlying causes of problems;
3. Sensitivity to operations, with constant real-time monitoring of activities;
4. A commitment to resilience, ensuring quick and effective responses to unforeseen events;
5. Deference to expertise, prioritizing decision-making based on technical knowledge [3].

At a more advanced stage, laboratory accreditation and certification promote safety, accountability, professional ethics, and efficiency, guiding the delivery of high-quality services. These processes ensure alignment with best practices and provide reliable and safe results for patients.

Clinical laboratories must strictly follow standards, regulations, and best laboratory practices to avoid errors that may affect test results. However, some countries do not have their own regulations, and laboratory accreditation is not mandatory, leaving the adoption of internationally accepted best practices at the discretion of professionals. In this context, there is a lack of informative data on the best practices adopted by laboratories in Portuguese-speaking countries. Such data are essential for the development of targeted policies and educational strategies for this audience.

Therefore, this study aimed to characterize clinical laboratories in Portuguese-speaking countries, focusing on the implementation of the best laboratory practices that are fundamental to ensuring quality in testing procedures.

Methods

A descriptive study was conducted using both qualitative and quantitative approaches to analyze data collected regarding best practices in clinical laboratories of Portuguese-speaking countries (PSC), except Brazil. Data collection took place from July 7 to September 30, 2024, through a digital questionnaire sent to participants of Portugal's National External Quality Assessment Program and other members of the Laboratory Quality Improvement Project for Portuguese-speaking countries [1]. The total number of laboratories reached by the questionnaire is unknown, since each member was asked to further disseminate the survey through the snowball method. To encourage participation, reminder emails were sent to non-respondents. Participation was voluntary and anonymous, except for the identification of the country of origin.

A research questionnaire was developed containing 47 questions, both open-ended and multiple-choice, organized as follows:

12 questions (1 to 7 and 43 to 47): Related to the laboratory's involvement with ProMeQuaLab.

12 questions (8 to 19): Regarding laboratory characteristics.

23 questions (20 to 42): Concerning laboratory best practices.

The study covered all phases of the laboratory process - pre-analytical, analytical, and post-analytical - with the aim of

ensuring greater reliability of results.

Data were collected and electronically tabulated using Microsoft Forms. The responses were counted, and results are presented as absolute numbers, ratios, or percentages.

Results and Discussion

Fifty-nine (59) laboratories participated in the study; however, five (5) did not consent to the disclosure of their data, even in anonymized form. The majority of the laboratories were from Portugal (39 out of 54, 72%), followed by Cabo Verde with 9 participants, Guinea-Bissau with 4, São Tomé and Príncipe with 1, and one laboratory that did not report its country of origin. Four countries (Angola, Brazil, Mozambique, and Timor) were not represented by any institutions in this study.

Of the survey participants, only 10 laboratories (18%) - 3 from Portugal, 6 from other PSC, and 1 that did not disclose its country of origin - have been members of the ProMeQuaLab project for more than two years. On the other hand, 14 laboratories (26%) - 11 from Portugal and 3 from other PSC - reported having visited the ProMeQuaLab website, and three (6%) - 2 from Portugal and 1 from another PSC - had previously participated in one of the project's congress editions. However, 27 laboratories (50%) - 16 from Portugal and 10 from other PSC - reported participating in the training activities promoted by ProMeQuaLab, of which 13 expressed being very satisfied and 14 satisfied with the trainings. Among all participants, seven laboratories (13%) reported having a high level of knowledge about the project's mission, while 19 (35%) stated that they either did not know or were unsure.

Regarding the laboratories' expectations for ProMeQuaLab, 38 out of 54 (70%) expressed a desire for consultancy aimed at continuous improvement; 26 out of 54 (48%) suggested the inclusion of artificial intelligence tools; 36 out of 54 (67%) supported efforts for change and innovation; and 35 out of 54 (65%) and 34 out of 54 (63%) expressed interest in the integration of virtual tools for Internal Quality Control (IQC) and External Quality Assessment (EQA), respectively.

Furthermore, laboratories expressed interest in training in the following areas: bacteriology, parasitology, biochemistry, urinalysis, hematology, microbiology, biorisk management, implementation of external quality control, training for laboratory technicians, inventory management, assay validation, implementation of quality management systems, and training and qualification of managers and human resources.

It was also suggested that ProMeQuaLab should offer more training opportunities and provide support for laboratories in implementing quality control management systems.

Table 1 presents the laboratories' responses, showing that the majority are outpatient-type laboratories (24 out of 54, 44%), with 22 located in Portugal and 2 in other PSC. Additionally, 34 laboratories (62%) offer public services, of which 21 are from Portugal and 12 from other PSC.

Table 1: Responses from all laboratories, only Portuguese laboratories and from other Portuguese-speaking countries laboratories to the questions in this study, grouped into laboratory characteristics, human resources and Quality Management system.

Questions	All Laboratories N (%)	Laboratories from Portugal N (%)	Laboratories from other PSC N (%)
Characterization of the laboratories			
Laboratory Type Public Laboratory Private Laboratory	 34/54 (63.0) 20/54 (37.0)	 21/39 (53.8) 18/39 (46.2)	 12/14 (85.7) 2/14 (14.3)
Service Type Hospital-based Outpatient Outpatient and Hospital-based N/A	 23/54 (42.7) 24/54 (44.4) 4/54 (7.4) 3/54 (5.5)	 13/39 (33.3) 22/39 (56.4) 1/39 (2.6) 3/39 (7.7)	 9/14 (64.3) 2/14 (14.3) 3/14 (21.4) 0/14 (0)
Does it have a laboratory information system? Yes No N/A	 48/54 (88.8) 5/54 (9.3) 1/54 (1.9)	 35/39 (89.7) 3/39 (7.7) 1/39 (2.6)	 12/14 (85.7) 2/14 (14.3) 0/14 (0)
Is a management system implemented? Yes No N/A	 37/54 (68.5) 15/54 (27.7) 2/54 (3.7)	 32/39 (82.0) 5/39 (12.8) 2/39 (5.2)	 5/14 (35.7) 9/14 (64.3) 0/14 (0)

If not, is there a plan to implement a management system? Yes No N/A	10/54 (18.5) 4/54 (7.4) 1/54 (1.9)	2/39 (5.2) 2/39 (5.2) 1/39 (2.6)	7/14 (50.0) 2/14 (14.3) 0/14 (0)
If yes, which management system is implemented? Accredited Certified N/A	8/54 (14.8) 26/54 (48.2) 3/54 (5.5)	9/39 (23.1) 23/39 (58.9) 2/39 (5.2)	0/14 (0) 4/14 (28.6) 1/14 (7.1)
Human Resources			
Number of laboratory assistants? 0 1-5 6-10 >10 S/R	8/54 (14.8) 28/54 (51.9) 8/54 (14.8) 2/54 (3.7) 8/54 (14.8)	6/39 (15.4) 20/39 (51.2) 6/39 (15.4) 2/39 (5.2) 5/39 (12.8)	2/14 (14.3) 8/14 (57.1) 1/14 (7.1) 0/14 (0) 3/14 (21.4)
Number of clinical laboratory technicians? 0 1-5 6-10 >10 S/R	2/54 (3.7) 19/54 (35.2) 11/54 (20.4) 18/54 (33.3) 4/54 (7.4)	1/39 (2.6) 15/39 (38.5) 4/39 (10.2) 16/39 (41.0) 3/39 (7.7)	1/14 (7.1) 4/14 (28.7) 7/14 (50.0) 1/14 (7.1) 1/14 (7.1)
Number of specialists in Clinical Laboratory Science/Clinical Pathology? 0 1-5 6-10 >10 S/R	6/54 (11.1) 19/54 (35.2) 7/54 (13.0) 8/54 (14.8) 14/54 (25.9)	1/39 (2.6) 18/39 (46.1) 7/39 (18.0) 8/39 (20.5) 5/39 (12.8)	4/14 (28.6) 1/14 (7.1) 0/14 (0) 0/14 (0) 9/14 (64.3)
Number of graduates in other fields? 0 1-5 6-10 >10 S/R	5/54(9.3) 6/54 (48.2) 4/54 (7.4) 3/54 (5.5) 16/54 (29.6)	4/39 (10.2) 21/39 (53.9) 3/39 (7.7) 3/39 (7.7) 8/39 (20.5)	1/14 (7.1) 5/14 (35.8) 1/14 (7.1) 0/14 (0) 7/14 (50)
Quality Management System			
Does the laboratory implement an annual training plan? Yes No N/A	46/54 (85.2) 6/54 (11.1) 2/54 (3.7)	34/39 (87.1) 3/39 (7.7) 2/39 (5.2)	11/14 (78.6) 3/14 (21.4) 0/14 (0)
Is a competency matrix implemented? Yes No N/A	35/54 (64.9) 16/54 (29.6) 3/54 (5.5)	30/39 (76.8) 7/39 (18.0) 2/39 (5.2)	5/14 (35.8) 8/14 (57.1) 1/14 (7.1)
Is there a professional responsible for the management system? Yes No N/A	46/54 (85.2) 6/54 (11.1) 2/54 (3.7)	37/39 (94.8) 0/39 (0) 2/39 (5.2)	9/14 (64.3) 5/14 (35.8) 0/14 (0)

Quality Indicators and Specifications			
Does it use quality indicators for the pre-analytical phase? Yes No N/A	47/54 (87.0) 5/54 (9.3) 2/54 (3.7)	33/39 (84.6) 4/39 (10.2) 2/39 (5.2)	13/14 (92.9) 1/14 (7.1) 0/14 (0)
Does it use quality indicators for the post-analytical phase? Yes No N/A	45/54 (83.3) 7/54 (13.0) 2/54 (3.7)	31/39 (79.4) 6/39 (15.4) 2/39 (5.2)	13/14 (92.9) 1/14 (7.1) 0/14 (0)
Are there written procedures for conducting the tests? Yes No N/A	50/54 (92.6) 1/54 (1.9) 3/54 (5.5)	36/39 (92.3) 0/39 (0) 3/39 (7.7)	13/14 (92.9) 1/14 (7.1) 0/14 (0)
Does it use quality specifications to assess analytical performance? Yes No N/A	39/54 (72.2) 11/54 (20.4) 4/54 (7.4)	32/39 (82.1) 4/39 (10.2) 3/39 (7.7)	6/14 (42.9) 7/14 (50.0) 1/14 (7.1)
Which? From the manufacturer Based on EQA (External Quality Assessment) Based on Biological Variation N/A	5/54 (9.3) 19/54 (35.2) 13/54 (24) 17/54 (31.5)	4/39 (10.2) 14/39 (35.9) 12/39 (30.8) 9/39 (23.1)	0/14 (0) 5/14 (35.8) 1/14 (7.1) 8/14 (57.1)

N/A (Not Applicable); PSC (Portuguese-speaking counties)

In our study, as previously mentioned, the results are predominantly from laboratories in Portugal, and the very limited participation from laboratories in other Portuguese-speaking countries significantly restricts any meaningful comparative analysis. Most of the laboratories have an information system and a management system implemented (Table 1). Although the majority of laboratories from other PSC have an information system, most do not have a management system implemented, although they plan to do so. However, due to the very small number of responding laboratories from these countries, these observations must be interpreted cautiously and cannot be generalized. A Quality Management System aims to provide a solid framework for quality in laboratories, contributing to preventive actions [5].

In our research, of the 39 participating Portuguese laboratories, 32 (82%) have some type of management system implemented, with 72% (23/32) certified and 28% (9/32) accredited (Table 1). In comparison, in Brazil, only 922 (3.43%) have at least one accreditation/certification, according to a survey conducted by Pires et al. (2023) [6]. Of these, 183 (19.84%) are accredited by the national standard PALC, 449 (48.70%) by the national standard DICQ, 155 (16.81%) by the national standard ONA, 10 (1.08%) are accredited by the international standard CAP, 121 (13.12%) are certified by the international standard ISO 9001, and only 4 (0.43%) are accredited by the international standard ISO 15189. A study conducted by Kopčinović et al. (2022) [7] in Croatia showed similar results, indicating that only a small portion of laboratories (9%) had accreditation. According to Souza (2016) [8], the pursuit of accreditation is essential for improving laboratory services, as well as the need for international consensus. Standards outline the requirements for a management system to achieve customer satisfaction and continuous improvement in the system's effectiveness. Lescowicz et al. (2018) [9] found no relationship between the size of the laboratory and the organizational quality structure. However, the authors noticed a trend of implementing a quality system in larger laboratories. On the other hand, they inferred that smaller laboratories found it easier to achieve accreditation [9]. However, due to the small number of laboratories that participated in this study, and the predominance of responses from Portugal, comparisons with other countries data are presented only for contextual reference and not for direct comparison.

The research by Lescowicz et al. (2018) [9] found that 40% of laboratories reported a lack of professionals in the market with training in quality assurance. The researchers also reported that one-third of establishments face difficulties in promoting ongoing education for employees due to cost and lack of

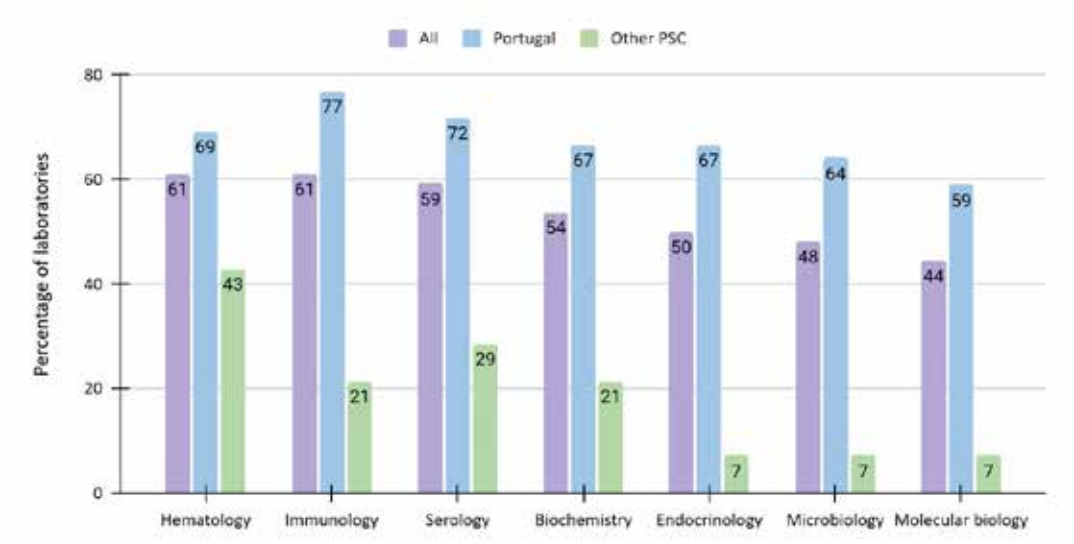
available time. In this context, the mission of ProMeQuaLab seeks to meet the laboratories' demand for training, which is available on the project's website at no cost and can be completed in real-time or at a time convenient for the professionals.

The majority (92%) of laboratories reported having written operational procedures (Table 1), which is a higher proportion than observed among laboratories surveyed in Brazil (70%) [9]. Similar to Brazil (90%) [9] and European countries [10], most of the laboratories in our study use quality indicators for the pre-analytical (87%) and post-analytical (83%) phases.

The proportion of laboratories conducting internal quality control (IQC) in our study (46/54, 85%) was slightly lower than in Brazil, where the rate was 95% [9].

Most laboratories, 38 from Portugal and 8 from other PSC countries, have implemented internal quality control (IQC). Two (4%) laboratories, one from Portugal and one from another PSC country, did not respond, and 6, consisting of 5 from other PSC countries and 1 from an unknown origin, do not perform IQC. As a limitation of our study, it is not possible to state that laboratories that did not respond about performing IQC do not perform the specialties. Among the laboratories conducting IQC, the areas covered are presented in Figure 1.

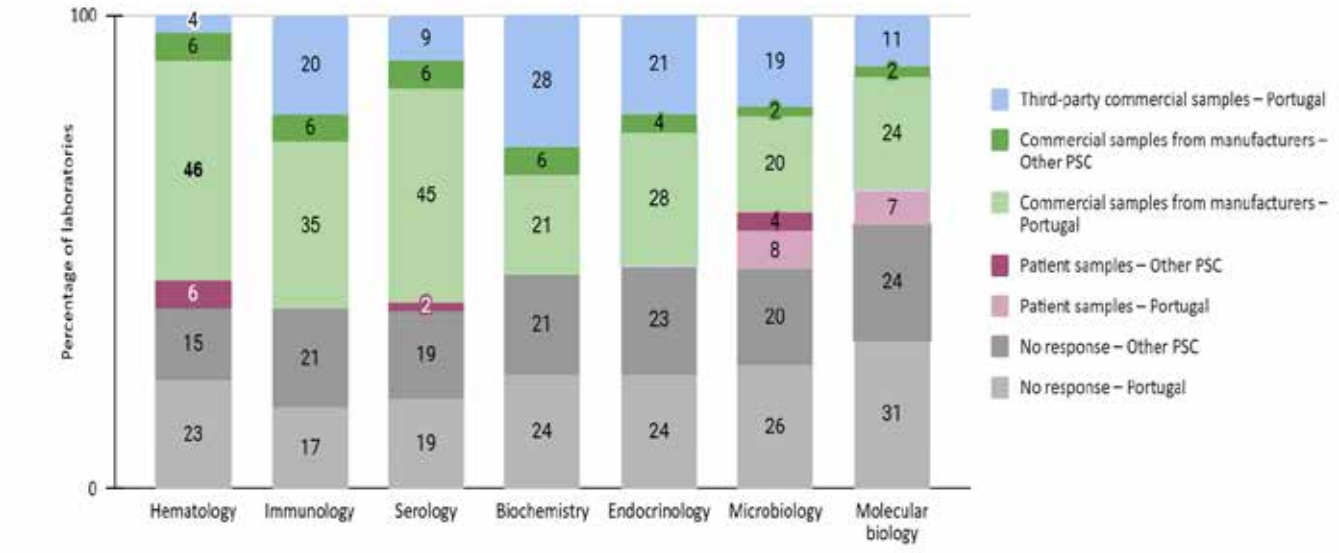
Figure 1: Percentage (%) of laboratories conducting internal quality control in various laboratory areas.



The purple columns represent the percentage among all laboratories in the study, the blue columns represent the percentage among Portuguese laboratories, and the green columns represent the percentage among laboratories from other Portuguese-speaking countries (PSC).

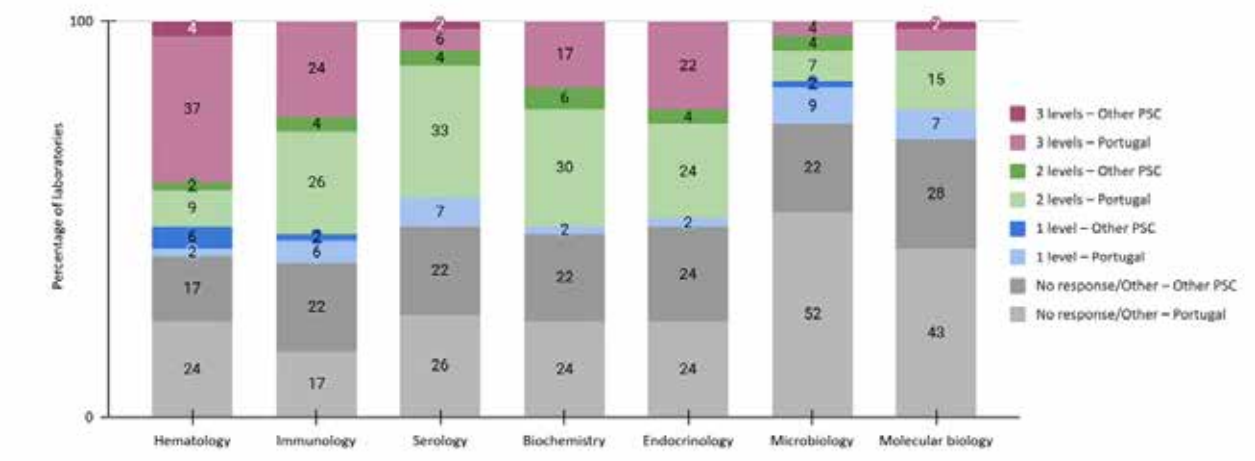
The types of control samples used for IQC by different laboratory areas are shown in Figure 2.

Figure 2: Control materials used in different laboratory areas conducting IQC.



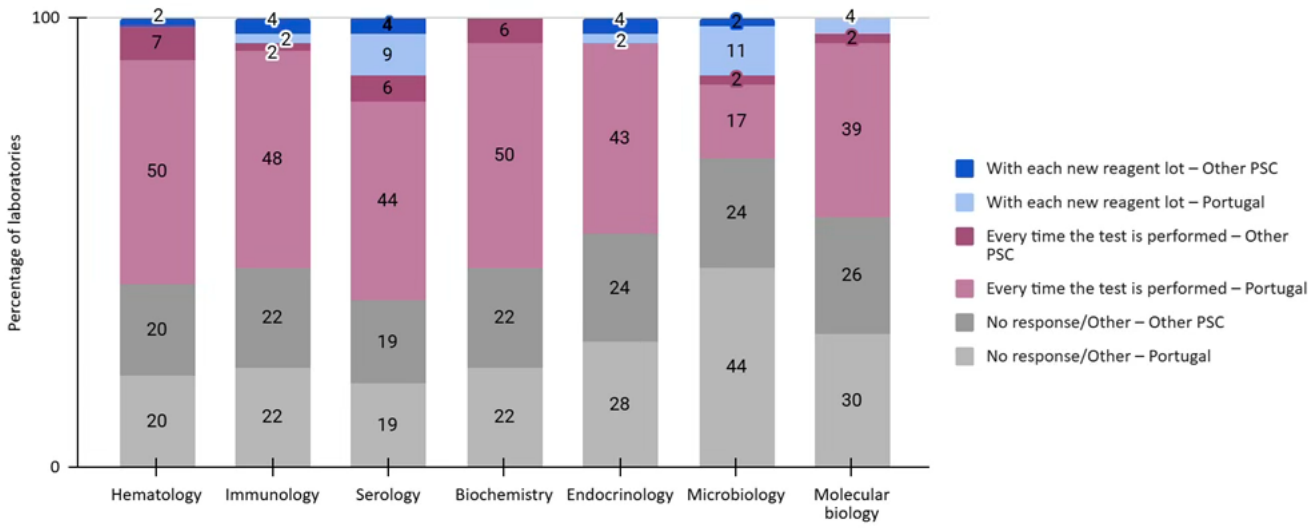
Overall, despite the high number of non-responding laboratories, the areas of hematology, serology, and immunology primarily use control samples from manufacturers, followed by third-party samples in laboratories in Portugal and patient samples in laboratories from other PSC countries.
IQC can be performed with patient samples, commercial samples from the reagent manufacturer, or commercial samples from third parties. It is desirable that IQC be performed whenever the test is executed, with each batch of analyses, and at least once a day using third-party samples. The use of control samples with different concentration levels is also recommended (ISO 15189:2022; 11).
The concentration levels of control samples used in IQC by different laboratory areas are shown in Figure 3.

Figure 3: Concentration levels of control samples used in internal quality control in different laboratory areas.



Despite the high number of non-responding laboratories, in general, the use of 3 concentration levels of control samples is more common in the hematology area, while in other areas, the use of 2 concentration levels is more frequent.
The frequency of IQC implementation by different laboratory areas is shown in Figure 4.

Figure 4: Frequency of internal quality control implementation in different laboratory areas.



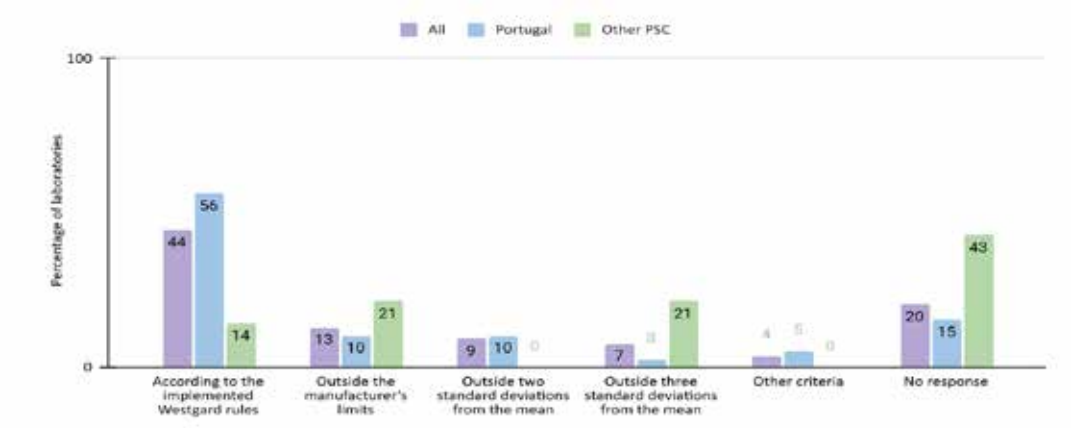
Similarly, to the findings reported by laboratories in a study conducted in Nepal in 2024 by Pant et al. [11], most laboratories in our study perform IQC daily/whenever they conduct the test. In this study, it was observed that most laboratories perform internal quality control on analyses using commercial samples provided by the manufacturer. In contrast, the 2024 study conducted in Nepal showed different results, revealing that the majority of participants (88%) use third-party commercial samples. The use of control samples from the manufacturer is produced under the same conditions as the reagent, which may not allow the detection of changes in analytical performance [11].
More specifically, comparing with the 63% (33/52) of hematology laboratories that reported performing IQC in our study, a survey indicated that 100% of Croatian laboratories perform IQC [12]. Among those who perform IQC in hematology in our study (33), 28 (84%) use commercial samples from manufacturers, compared to 98% of Croatian

laboratories [12].

The majority (36/54, 67%) of laboratories, 32 from Portugal and 4 from other PSC countries, use control charts to monitor control results, 10 laboratories (18%), 6 from Portugal and 4 from other PSC countries, do not use them, and 8 (15%), 1 from Portugal and 6 from other PSC countries, did not respond. The percentage of laboratories that reported monitoring analytical quality through control charts in our study (67%) was similar to the Brazilian rate (75%) [9]. However, although a large part of the laboratories (24/52, 46%) use Westgard rules as criteria for rejecting control samples, 21% (11/52) did not answer the question, suggesting the need to improve knowledge on interpreting control charts.

The majority of laboratories (32/54, 59%), 25 from Portugal and 7 from other PSC countries, also record the causes of control sample rejection results, but 11 (20%), 10 from Portugal and 1 from other PSC countries, do not record them, and 11 (20%), 4 from Portugal and 6 from other PSC countries, did not respond. The criteria used by laboratories to reject control sample results are shown in Figure 5.

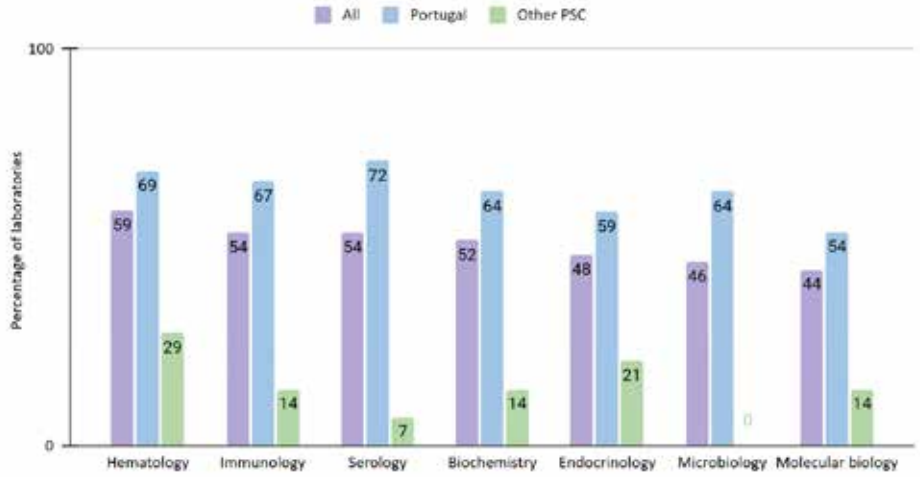
Figure 5: Percentage of laboratories using criteria for rejecting control sample results.



Other criteria: MQC program, Multiple control charts; EWMA, exponentially weighted moving average statistic (EWMA). The purple columns represent the percentage across all laboratories in the study, blue columns represent the percentage of laboratories in Portugal, and green columns represent the percentage of laboratories from other Portuguese-speaking countries (PSC).

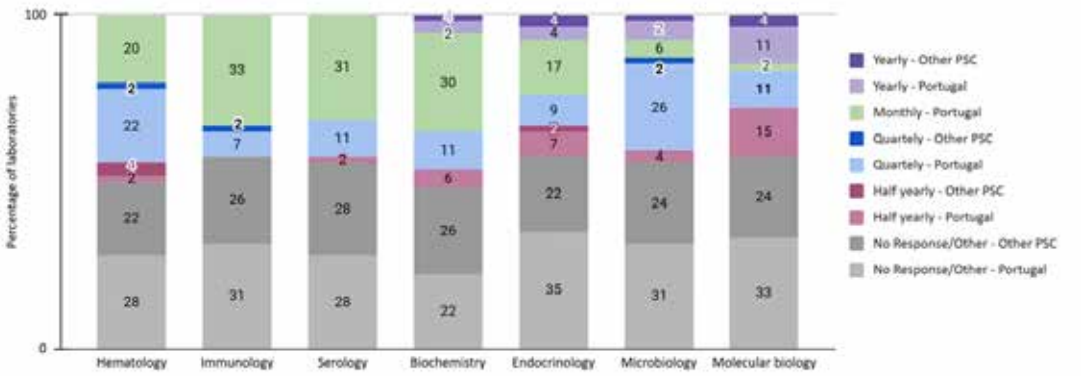
The majority of laboratories (48/54, 89%), 39 from Portugal, 8 from other PSC, and one that did not report its country of origin, participate in EQA programs, while 5 (9%) from other PSC do not participate, and one did not respond. As a limitation of our study, it is not possible to state that laboratories that did not respond about performing EQC do not perform the specialties. The laboratory areas and the frequencies they perform EQA are shown in Figures 6 and 7, respectively.

Figure 6: Percentage (%) of laboratories conducting external quality control in different laboratory areas.



The purple columns represent the percentage across all laboratories in the study, blue columns represent the percentage of laboratories in Portugal, and green columns represent the percentage of laboratories from other Portuguese-speaking countries (PSC).

Figure 7: Frequency of participation in EQA by different laboratory areas.



The purple columns represent the percentage across all laboratories in the study, blue columns represent the percentage of laboratories in Portugal, and green columns represent the percentage of laboratories from other Portuguese-speaking countries (PSC).

Overall, despite the high number of non-responding laboratories, the most common frequency of participation in EQA is monthly, followed by quarterly.

The majority of laboratories indicated that participation in EQA leads to improved analytical performance (39/54, 72%) and detection of systematic errors (34/54, 63%). However, 8 (15%) laboratories, 2 from Portugal and 6 from other PSC countries, did not share this perception and did not answer the question. However, in our study, a higher rate of laboratories (90%) reported conducting EQA compared to Brazilian laboratories (55%). In Brazil, among the challenges to implementing IQC and EQA, cost and the difficulty in finding a single supplier for control samples for all tests were reported [9].

Overall, opportunities for improvement were identified, as only 59% of laboratories register the causes for rejecting control sample results; 65% use a competency matrix, 66% use control charts, and 72% use quality specifications to assess analytical performance.

The main difficulties reported by laboratories for quality control implementation were lack of control materials, human resources, software for analyzing internal quality control results, technicians trained in quality management, team stability, training and capacity building, use of analytical performance specifications, appropriate infrastructure, and the measurement of uncertainty calculations.

Despite efforts to provide free online educational training, such as the webinars of the International Federation of Clinical Chemistry (IFCC), foreign languages can be a barrier to accessing knowledge. A recent survey performed on behalf of the Task Force for Global Education and Learning and Task Force for Laboratory Medicine Practice Guidelines of the IFCC [13] clearly points to topics requested by professionals to complement their training and also guide PROMEQUALAB's actions.

Our study has some limitations, such as the laboratory response rate, the large number of questions to cover all phases of laboratory activities, and since an individualized questionnaire was used, we cannot rule out the possibility that participants provided "desirable" answers instead of reporting their actual practices. The low participation rate significantly affects the generalizability of the results, and this low response rate may be partially explained by the length and complexity of the questionnaire, which contained 47 questions. This extensive format may have discouraged completion, particularly among laboratories with limited human resources or high workload. Shorter or more focused questionnaires may improve response rates in future studies.

Excluding data from Portugal, the results from other PSC laboratories indicate a low level of GLP implementation, which may be associated with several factors, such as financial limitations, access to training and control material, laboratory computerization systems and the guarantee of calibration/ maintenance of laboratory equipment. However, due to the extremely small number of respondents from these countries, these findings cannot be extrapolated or interpreted as representative.

Given the limited number of participating laboratories and the imbalance in representation across countries, the findings and interpretations of this study should be understood as applicable mainly to the Portuguese laboratories included in the dataset.

Conclusions

Despite the existence of international guides for the standardization of procedures, based on our research, it was possible to observe that the degree of implementation of good practices is still partial, and we can infer that there are challenges in the implementation and efficient use of a quality management system in laboratories. These challenges include the need for well-qualified professionals committed to quality management, as well as the increasing requirement for continuous professional development in response to the rapid evolution of laboratory processes.

The small number of laboratories that responded to the survey represents a major limitation and restricts the interpretation of the findings primarily to the Portuguese context. The low response rate may be associated with the length of the

questionnaire - comprising 47 questions - which may have discouraged participation, particularly from laboratories in other Portuguese speaking countries.

Despite the limitation, it was possible to identify opportunities for improvement across all phases of the analytical process in the participating Portuguese laboratories. None of the evaluated requirements - including the implementation and interpretation of internal and external quality control, and the use of quality specifications to assess analytical performance - were met by all laboratories.

These findings highlight the need to enhance dissemination of ProMeQuaLab in PSC, as this project offers free training that may support the harmonization and continuous improvement of laboratory practices. In addition, the results provide valuable insights to guide future training initiatives and encourage develop strategies to increase laboratory participation, potentially contributing to the quality of results and patient safety in PSC countries.

Conflict of Interests

None to declare.

Ethics approval

Not applicable as this study only involved anonymised survey responses.

Research Funding

None received.

Author contributions

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Drawing of Figures and Table: Alice Berlanda Seidler.

Write-up and Review: Alice Berlanda Seidler, Ana Faria, Armandina Miranda, Flávia Martinello, Helena Correia, Maria Elisabeth Menezes, Silvânia Da Veiga Leal.

Consent for Publication

Consent to submit has been received explicitly from all coauthors, as well as from the responsible authorities. Authors whose names appear on the submission have contributed sufficiently to the scientific work and therefore share collective responsibility and accountability for the results.

Data availability

The data included in this study is available upon request to the corresponding author.

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Research Article

Comparison of LDL-C Estimation Using Ridge Regression and Four Established Equations Against Direct Determination of LDL-C in a Northeastern Population in Thailand

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Keywords

Low-density lipoprotein cholesterol (LDL-C), Ridge regression, Machine learning, LDL-C calculation equations, Lipid profile Classification Description: Clinical chemistry, Public health

Abstract

Background: Equations traditionally used for estimating low-density lipoprotein cholesterol (LDL-C) have limitations in accuracy and reliability. This study aimed to compare the performance of established equations with a machine learning approach to determine the most appropriate method for LDL-C estimation.

Methods: A retrospective cross-sectional study was conducted using 14,109 lipid profile records from inpatients and outpatients at Kosumphisai Hospital, Northeastern Thailand (2017–2021). LDL-C was estimated using the Friedewald, Puavilai, National Institutes of Health (NIH), and Martin equations, as well as a Ridge regression model. Direct LDL-C measurement served as the reference standard. Model performance was evaluated using mean absolute error (MAE), the proportion of estimates within ±12% of the direct measurement, and Bland–Altman analysis.

Results: The calculation of LDL-C using Ridge regression provided the highest proportion of estimates within the ±12% error margin (75.37%), the lowest MAE (10.05 mg/dL), and the narrowest 95% limits of agreement (−31.19 to 31.57 mg/dL) in Bland–Altman analysis.

Conclusions: Ridge regression provided greater accuracy and reliability for LDL-C estimation compared with the four established equations. Future research should consider incorporating additional predictors and alternative penalized regression techniques, such as Lasso or Elastic Net, to enhance model robustness.

Introduction

Cardiovascular diseases (CVDs) remain the leading cause of mortality worldwide, accounting for approximately 31% of all global deaths, with ischemic heart disease being a significant contributor [1]. In Thailand, CVDs have been identified as a primary public health concern, contributing to a growing number of hospital admissions and healthcare costs [2]. The accurate assessment of lipid profiles, particularly low-density lipoprotein cholesterol (LDL-C), is essential for the early diagnosis, risk stratification, and management of CVDs. LDL-C is widely recognized as a key biomarker in atherosclerosis progression and is used to guide lipid-lowering therapies, such as statins, to reduce cardiovascular risk [3]. While direct LDL-C measurement using ultracentrifugation or homogeneous assays provides accurate results, these methods are costly, require specialized equipment, and are not routinely available in resource-limited settings, particularly in community hospitals in Thailand.

Due to the limited availability of direct LDL-C measurement, several formulas have been developed to estimate LDL-C using commonly measured lipid parameters. The most widely used equation is the Friedewald equation (LDL-C = TC – HDL-C – TG/5), which assumes a fixed triglyceride-to-VLDL-C (very low-density lipoprotein cholesterol) ratio of 5 [4]. However, this formula is known to be inaccurate in individuals with high triglyceride levels (greater than 400 mg/dL), potentially leading to a misclassification of LDL-C levels.

Alternative equations, such as the Puavilai equation [5], the Martin-Hopkins equation, which utilizes an adaptive triglyceride-to-VLDL-C ratio [6], and the National Institutes of Health (NIH), USA, as the NIH equation [7], have been developed to enhance LDL-C estimation. However, discrepancies between these formulas have been observed, particularly in patients with metabolic syndrome, diabetes, or hypertriglyceridemia, making it challenging to determine the most suitable equation for different populations.

Despite advances in LDL-C estimation methods, no single equation works best for all patient groups, emphasizing the need for more flexible and precise approaches. Machine learning (ML) has emerged as a promising tool in healthcare, capable of detecting complex patterns in medical data [8]. Various ML models, such as random forests, support vector machines (SVMs), and deep learning, have shown better performance in LDL-C estimation compared to traditional equations in certain populations [9-13].

However, most existing studies have been conducted in Western populations, where genetic, dietary, and lifestyle factors differ significantly from those in Southeast Asia, particularly in Thailand. Moreover, ML-based LDL-C estimation models often require large datasets, advanced computational infrastructure, and model interpretability, which can limit their applicability in clinical practice. Additionally, few studies have compared ML models with multiple LDL-C equations within the same setting, creating a gap in understanding their relative performance in real-world scenarios.

An important but often overlooked aspect of LDL-C estimation is its potential effect on health disparities. Traditional LDL-C formulas can introduce bias in certain demographic groups, especially in patients with metabolic disorders, high triglycerides, or unique dietary habits. Likewise, ML models trained on datasets that lack diversity may perform differently when applied to various ethnic or socioeconomic populations. Addressing these disparities is essential to ensure that ML-based LDL-C estimation models are fair and applicable to all groups.

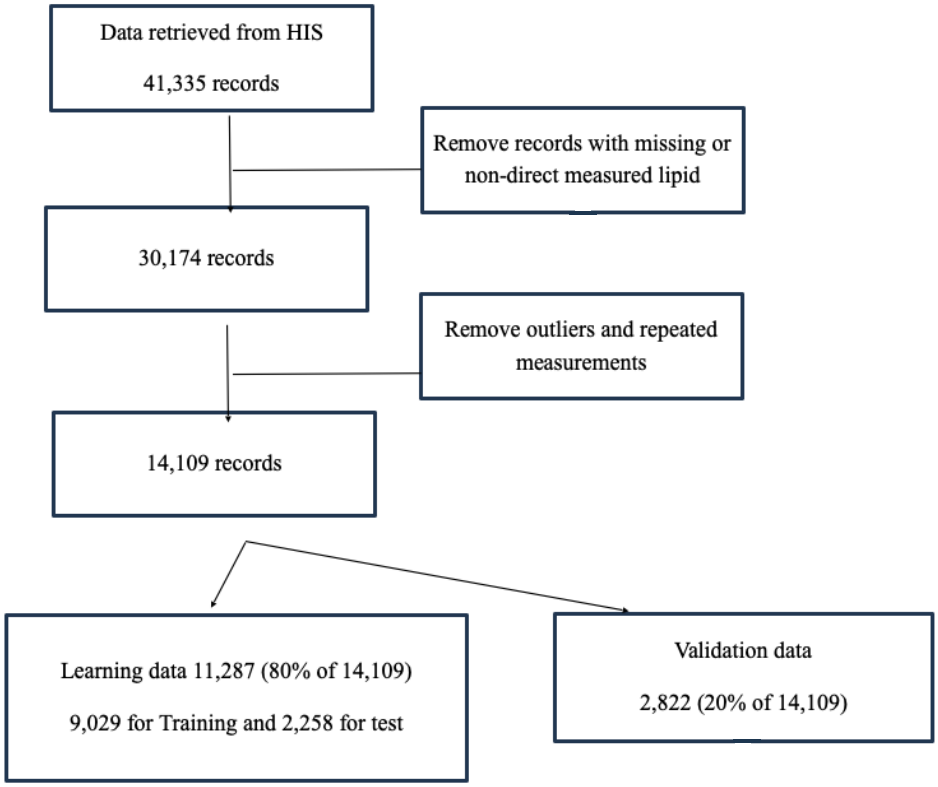
Considering the limitations of traditional LDL-C equations, the potential of ML models, and the lack of research in a Thai population, this study aims to develop and evaluate ML-based LDL-C estimation models using lipid profile data from a community hospital in Northeastern Thailand. Additionally, we compare the performance of the selected ML model with four traditional LDL-C equations (Friedewald, Puavilai, Martin-Hopkins, and NIH) using direct LDL-C measurements to assess their feasibility in clinical settings.

Material and Method

Study Design and Population

This study employed a retrospective cross-sectional design, using lipid profile datasets obtained from both inpatient and outpatient sections of Kosumphisai Hospital, Mahasarakham, a district hospital in the northeastern region of Thailand. Data were extracted from the Hospital Information System (HIS) and included lipid profile measurements from 14,109 individuals gathered between 2017 and 2021. The study population had a mean age of approximately 62 years, with age ranging from about 10–98 years in the training set, 10–93 years in the testing set, and 12–96 years in the validation set. Men accounted for roughly 38% of participants across all three datasets (38.5% in the training set, 37.7% in the testing set, and 38.4% in the validation set). The dataset comprised total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C). Inclusion criteria included individuals with data on TC, TG, HDL-C, and LDL-C. In contrast, exclusion criteria involved individuals with missing lipid profile results and measurements exceeding the linearity limits of the specific assays used. The entire data selection process, including record retrieval, exclusion steps, and dataset partitioning, is summarized in Figure 1.

Figure 1: Flowchart showing the data selection process, including inclusion/exclusion criteria and how the dataset is split for training and validation.



All lipid measurements were obtained from 12-hour fasting plasma samples, which were analyzed using an automated clinical chemistry analyzer (Abbott ARCHITECT, Illinois, USA) according to the manufacturer's instructions. TC and TG levels were measured using enzymatic colorimetric assays, while HDL-C and LDL-C were determined using homogeneous enzymatic assays. All the included patient data was retrieved from the direct LDL-C measurement using, two-step enzymatic colorimetric method. In the first step a polyanion reagent and detergent 1 selectively complex and mask non-LDL lipoproteins (chylomicrons, HDL, VLDL), and cholesterol released from these fractions is consumed by the enzymatic system in a non-chromogenic reaction to eliminate background signal. In the second step detergent 2 liberates cholesterol from the LDL fraction; the liberated LDL-cholesterol is hydrolyzed by cholesterol esterase and oxidized by cholesterol oxidase, and the resulting hydrogen peroxide is detected via peroxidase-catalyzed coupling with 4-aminoantipyrine/TOOS to generate a colored product. The absorbance was read photometrically (primary wavelength ~604 nm) at 37 °C and converted to concentration using HDL/LDL calibrators. The assay is linear from 5 to 600 mg/dL with a limit of detection near 4.5 mg/dL; fresh fasting serum was used, and reagents were handled according to manufacturer recommendations to ensure stability and accuracy [14]. Routine systematic quality control procedures were applied to ensure precision and accuracy.

LDL-C Calculation Equations

LDL-C was estimated using four mathematical equations:

1. Friedewald equation:

LDL-C (mg/dL) = TC – HDL-C – TG/5

2. Puavilai equation:

LDL-C (mg/dL) = TC – HDL-C – TG/6

3. NIH equation:

LDL-C (mg/dL) = (TC/0.948) – (HDL-C/0.971) − (TG/8.56) – [(TG*NonHDL-C)/2140] + (TG2 /16100) − 9.44

where NonHDL-C = TC – HDL-C

4. Martin-Hopkins equation:

LDL-C was determined using the Martin LDL calculator (LDL-Calculator.com).

The accuracy of these formulas was evaluated using direct LDL-C measurement as the reference method.

Principle of Ridge regression

Ridge regression is a regularized linear regression technique that minimizes the sum of squared errors while incorporating a penalty term (L2 regularization) to shrink the regression coefficients [15]. The objective function for ridge regression is defined as:

$$L(\beta) = \sum_{i=1}^{n} (y_i - \hat{y}_i)^2 + \lambda \sum_{j=1}^{p} \beta_j^2$$

where

y_i = observed LDL-C value
y_i = predicted LDL-C value
j = regression coefficients
λ = regularization parameter controlling shrinkage

The penalty term $(\lambda \sum_{j=1}^{p} \beta_j^2)$ prevents overfitting by reducing the magnitude of regression coefficients, making the model more stable when dealing with multicollinearity or small sample sizes [15].

Ridge regression was chosen as the primary machine learning method in this study for several reasons. First, it prevents overfitting through regularization, which reduces model complexity and enhances generalizability to unseen data [16]. This is particularly advantageous when predictor variables such as total cholesterol (TC), triglycerides (TG), and HDL-C tend to be correlated, as ridge regression effectively addresses multicollinearity. Second, the method reduces variance, yielding more stable coefficient estimates and decreasing sensitivity to noise in lipid measurements [17]. Finally, ridge regression is both interpretable and computationally efficient. Unlike more complex non-linear models (e.g., XGBoost or deep learning), it preserves transparency regarding variable contributions and is straightforward to implement in routine clinical practice [18].

Model Training and Evaluation

The remaining data was randomly split into 11,287 (80%) as learning data and 2,822 (20%) as validation data. Afterward, the 80% of 11,287 (n=9,029) was assigned as model training data and 20% of 11,287 (n=2,258) was assigned as the model testing data. Finally, the validation data was used to evaluate all LDL-C methods compared to direct measured LDL-C. The independent variables were TC, TG, and HDL-C, while direct LDL-C measurement was the target variable. A 10-fold cross-validation approach was used to tune the hyperparameter λ by partitioning the dataset into 10 subsets, training the model on 9 subsets while validating on the remaining subset in each iteration. Ridge regression was implemented using Python 3.9 and Scikit-learn 0.23.2.

Statistical Analysis

To assess the accuracy of LDL-C estimation methods, three statistical measures were used:

1. Mean Absolute Error (MAE)

MAE quantifies the absolute difference between estimated and directly measured LDL-C values [19]:

$$MAE = \frac{1}{n} \sum_{i=1}^{n} \left| y_i - \hat{y}_i \right|$$

where:

n = number of data points
y_i = directly measured LDL-C
y_i = estimated LDL-C

A lower MAE indicates a more accurate calculation method.

2. Bland-Altman Analysis

Bland-Altman analysis was used to assess the agreement between estimated and directly measured LDL-C values base on how much the differences between the calculated LDL-C and direct measured LDL-C (20). The mean difference (bias) and limits of agreement (LOA = mean difference ± 1.96 SD) were reported to determine systematic bias and variability between methods.

3. Percentage Within 12% Error of Direct LDL-C

To assess the clinical acceptability of the LDL-C estimation models, the amount of data of estimates within 12% of direct LDL-C measurements was calculated as:

$$\left| \frac{\hat{y}_i - y_i}{y_i} \right| \times 100 \leq 12$$

where:

yi = directly measured LDL-C
yi = estimated LDL-C

A higher number and percentage of calculated LDL-C data within 12% differed from directly measured LDL-C, indicating better clinical agreement. This metric aligns with clinical practice guidelines that suggest LDL-C estimations should be within 12% of direct measurements for reliable cardiovascular risk assessment [21, 22].

Results

The baseline characteristics of the study population are outlined in Table 1. The learning dataset (n = 11,287) was split into a training set (n = 9,029) and a testing set (n = 2,258), while the validation dataset included 2,822 participants. The average age was 61.5 ± 11.7 years in the training set, 61.6 ± 11.6 years in the testing set, and 61.9 ± 11.8 years in the validation set, with similar age ranges across datasets (10–98, 10–93, and 12–96 years, respectively). The percentage of male participants was comparable across groups, representing 38.5% (n = 3,480) in the training set, 37.7% (n = 851) in the testing set, and 38.4% (n = 1,083) in the validation set.

Table 1: Baseline characteristics of the study population.

Variables	Learning data (n = 11,287)				Validation data (n = 2,822)	
	Training (n = 9,029)		Testing (n = 2,258)			
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Age (years)	61.5 ± 11.7	10–98	61.6 ± 11.6	10–93	61.9 ± 11.8	12–96
Male (n, %)	3480 (38.5%)	-	851 (37.7%)	-	1083 (38.4%)	-
TC (mmol/L)	4.87 ± 1.23	1.37–17.48	4.82 ± 1.18	1.37–16.64	4.83 ± 1.18	1.34–20.67
TG (mmol/L)	2.06 ± 1.57	0.27–21.32	2.05 ± 1.50	0.46–15.76	2.08 ± 1.63	0.49–21.11
HDL-C (mmol/L)	1.16 ± 0.31	0.28–3.72	1.15 ± 0.32	0.28–3.10	1.16 ± 0.32	0.28–4.13
Direct LDL-C (mmol/L)	2.96 ± 1.06	0.28–13.33	2.94 ± 1.00	0.49–8.98	2.94 ± 1.00	0.31–9.12

TC: Total Cholesterol; LDL-C: Low-Density Lipoprotein Cholesterol; TG: Triglycerides; HDL-C: High-Density Lipoprotein Cholesterol; Direct LDL-C: Directly measured LDL-C.

Lipid profile distributions were similar across datasets, with minor variations in total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) levels. Table 1 presents the detailed distributions. The mean TC levels were 4.85 ± 1.23 mmol/L in the training set, 4.82 ± 1.18 mmol/L in the testing set, and 4.83 ± 1.18 mmol/L in the validation set, with respective ranges of 1.37–17.48 mmol/L, 1.37–16.64 mmol/L, and 1.34–20.67 mmol/L. The mean TG levels were 2.06 ± 1.57 mmol/L in the training set, 2.05 ± 1.50 mmol/L in the testing set, and 2.08 ± 1.63 mmol/L in the validation set, with respective ranges of 0.27–21.32 mmol/L, 0.46–15.76 mmol/L, and 0.49–21.11 mmol/L. The mean HDL-C levels were 1.16 ± 0.31 mmol/L in the training set, 1.15 ± 0.32 mmol/L in the testing set, and 1.16 ± 0.32 mmol/L in the validation set, with corresponding ranges of 0.28–3.72 mmol/L, 0.28–3.10 mmol/L, and 0.28–4.13 mmol/L. Lastly, direct LDL-C levels remained consistent across datasets, with means of 2.96 ± 1.06 mmol/L in the training set, 2.94 ± 1.00 mmol/L in the testing set, and 2.94 ± 1.00 mmol/L in the validation set, with respective ranges of 0.28–13.33 mmol/L, 0.49–8.98 mmol/L, and 0.31–9.12 mmol/L. The consistency of lipid profile values across datasets supports the reliability of model development, minimizing dataset-related bias.

The performance of the LDL-C estimation models was assessed using Mean ± Standard Deviation (SD) and Mean Absolute Error (MAE), as summarized in Table 2.

Table 2: Comparison of LDL-C estimation methods (Validation data, n = 2,822).

Method	Mean ± SD (mmol/L)	MAE (mmol/L)
Friedewald	2.72 ± 0.96	0,34
Puavilai	2.88 ± 0.95	0,27
NIH	2.83 ± 0.89	0,27
Martin	2.90 ± 0.90	0,28
Ridge regression	2.93 ± 0.92	0,26

MAE: Mean Absolute Error, calculated as the absolute difference between estimated and direct LDL-C values.

Among traditional estimation methods, the Friedewald equation showed the lowest accuracy, with the highest MAE (0.34 mmol/L). In contrast, the Ridge Regression model, developed through machine learning, outperformed all traditional equations, achieving the lowest MAE (0.26 mmol/L), which indicates improved estimation accuracy.

To assess the agreement between estimated LDL-C values and direct measurements, Bland-Altman analysis was performed for each estimation method (Table 3, Figure 2a–e). This analysis evaluated mean bias (the difference between estimated and direct LDL-C values) and 95% limits of agreement (LOA). The Ridge Regression model showed the smallest mean bias (-0.005 mmol/L), indicating its LDL-C estimates closely matched the direct measurements. Conversely, the Friedewald equation exhibited the largest deviation, with a mean bias of -0.213 mmol/L, implying consistent underestimation of LDL-C levels. The 95% LOA indicates the range within which 95% of the differences between estimated and direct LDL-C values fall. Ridge Regression had a fairly narrow LOA (-0.807 to 0.816 mmol/L), suggesting lower variability and better precision, while the Friedewald equation had the widest range (-1.096 to 0.671 mmol/L), reflecting greater inconsistency.

Figure 2 a–e: Bland-Altman plots comparing LDL-C estimation methods with direct LDL-C measurement (Validation data, n=2,822).

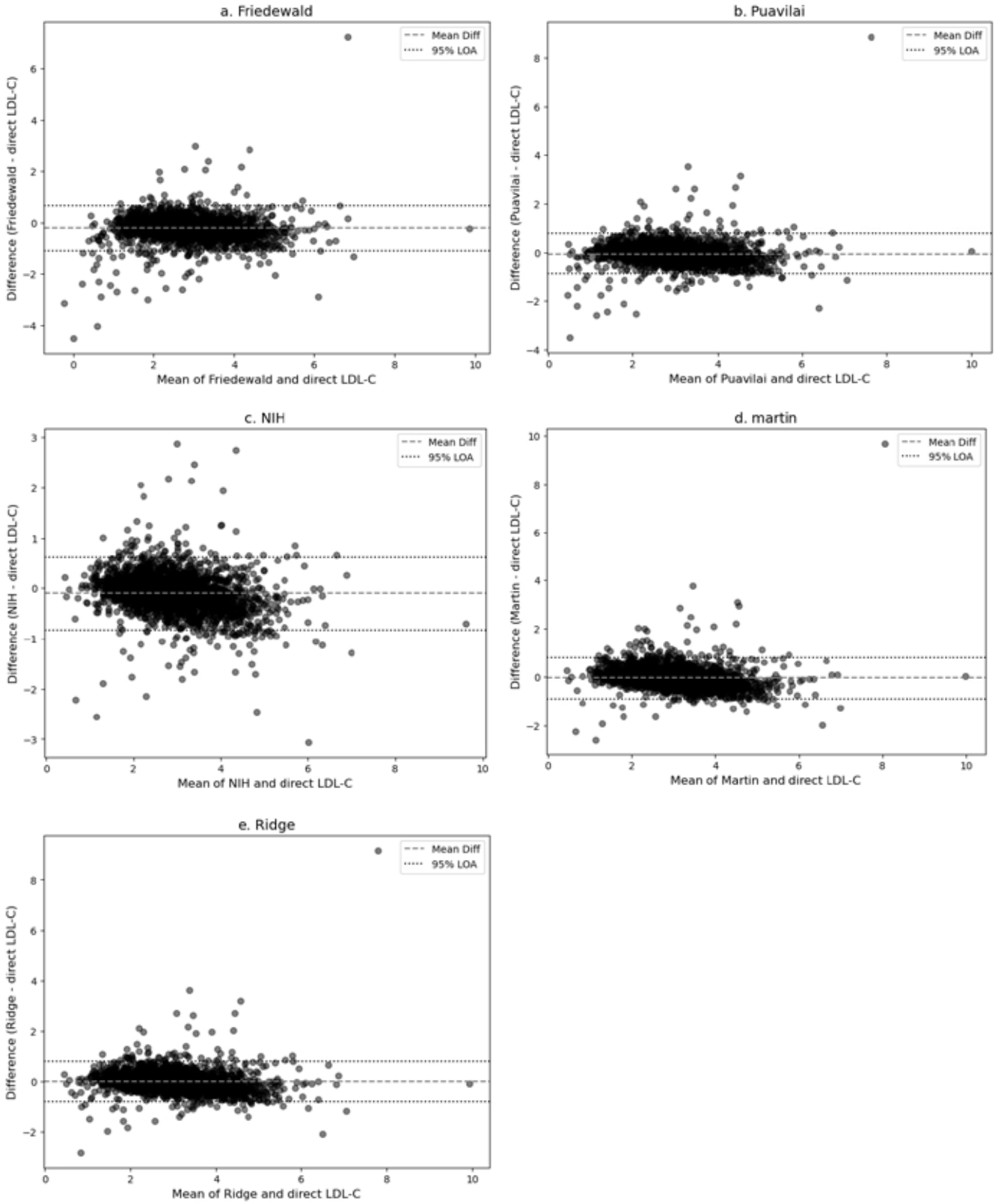


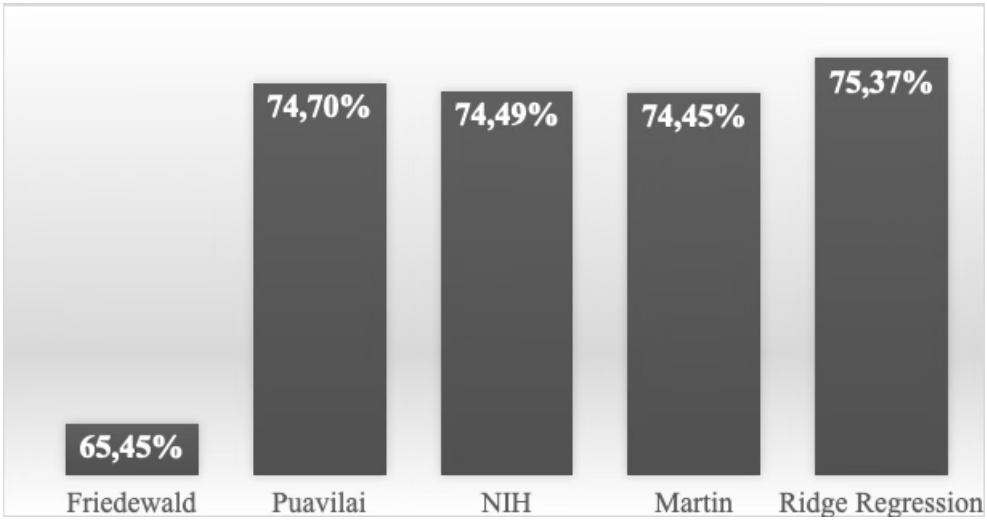
Table 3: Bland–Altman analysis (Validation data, n = 2,822).

Method	Mean Diff (mmol/L)	Lower LOA (mmol/L)	Upper LOA (mmol/L)
Friedewald	-0,213	-1,096	0,671
Puavilai	-0,054	-0,885	0,778
NIH	-0,099	-0,833	0,634
Martin	-0,035	-0,897	0,827
Ridge regression	-0,005	-0,807	0,816

LOA (Limits of Agreement): The range within which 95% of the differences between estimated and directly measured LDL-C values fall. Mean Diff: The average difference between estimated LDL-C values and direct LDL-C measurements. A lower bias indicates closer agreement with the reference measurement.

The percentage of LDL-C estimates within ±12% error was analyzed as a measure of clinically acceptable agreement with direct LDL-C measurement.

Figure 3: Percentage of calculation results within 12% deviated from direct LDL-C.



In Figure 3, the Friedewald equation (65.45%) had the lowest accuracy, showing greater variability. In contrast, the Puavilai (74.70%), NIH (74.49%), and Martin (74.45%) equations demonstrated moderate improvements. Ridge Regression (75.37%) had the highest accuracy; thus, machine learning-based approaches may improve the accuracy of LDL-C estimation. These findings highlight the benefits of alternative LDL-C estimation methods over the Friedewald equation, particularly in improving cardiovascular risk assessment.

Discussion

This study assessed the performance of five low-density lipoprotein cholesterol (LDL-C) estimation methods, including four traditional equations and a machine learning-based Ridge regression model, in a Northeastern Thai population. Our results show that Ridge regression outperforms traditional formulas, offering more accurate and dependable LDL-C estimates.

Consistent with previous studies, our results highlight the limitations of traditional LDL-C estimation methods, particularly the Friedewald equation. Despite its widespread use due to its simplicity, the Friedewald equation is known to underestimate LDL-C in individuals with high triglyceride (TG) levels or low LDL-C concentrations [11, 13, 23-25]. This underestimation is clinically concerning, as it may lead to non-detection of cardiovascular risk and suboptimal treatment decisions. Alternative equations, such as the Martin-Hopkins and Sampson-NIH equations, have attempted to address these limitations by incorporating more sophisticated TG: VLDL-C ratio adjustments or refined algorithms for very-low-density lipoprotein cholesterol (VLDL-C) estimation [6,7]. However, despite these improvements, our study demonstrates that Ridge regression provides superior accuracy and reliability in LDL-C estimation.

The effectiveness of machine learning (ML) models for LDL-C estimation has been corroborated by recent studies. For instance, research in Turkish pediatric populations found that ML models produced more concordant LDL-C estimates than traditional formulas [10]. Similarly, a study in Eastern India found that ML approaches, including XGBoost and Random Forests, outperformed conventional formulas in predicting LDL-C levels [9]. These findings align with our results,

supporting the potential of ML-driven LDL-C estimation across diverse populations. Accurate LDL-C estimation is essential for cardiovascular risk assessment and optimizing lipid-lowering therapies [24]. Errors in LDL-C estimation can lead to misclassification of risk categories, increasing the likelihood of overtreatment or undertreatment. Our findings suggest that integrating Ridge regression into clinical workflows could improve LDL-C assessment precision, enabling more precise treatment strategies.

Machine learning methods often face challenges such as overfitting, multicollinearity, and interpretability. Ridge regression was chosen in this study because it can address these issues while keeping clinical relevance. Unlike deep learning models, Ridge regression provides transparency in how variables contribute, making it easier for clinicians to interpret. It also effectively handles collinear predictors, which is especially important when working with lipid profile data where some variables like total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and LDL-C tend to be correlated. The regularization techniques in Ridge regression help ensure the model remains stable, reducing the risk of excessive prediction variance and improving its ability to generalize [16-18].

While our study offers valuable insights into the clinical use of ML-based LDL-C estimation, several limitations need to be acknowledged. First, our findings are based on a Northeastern Thai population, which may limit their applicability to other ethnicities or regions. Second, seasonal variations in lipid levels, influenced by diet and lifestyle factors, may have affected LDL-C estimates [24]. Third, all LDL-C measurements were obtained from a single laboratory, which could affect the external validity of our results in multi-center settings. Additionally, the timing of lipid testing is often driven by institutional practices, such as annual health checkups or screening programs, which can differ between countries. In Thailand, for example, these services often occur at the start of the fiscal year or during specific seasons, leading to seasonal overrepresentation in the dataset. This clustering may introduce temporal bias into the model's training and evaluation. If other countries follow different testing schedules, the performance of the same LDL-C estimation methods might vary due to differences in seasonal data structures. Therefore, context-specific validation is essential when applying machine learning-based LDL-C models across different healthcare systems.

To further establish the clinical usefulness of regression models, future research should focus on external validation using independent datasets from diverse healthcare settings. This strategy would help confirm the model's ability to work across different patient groups. Improving LDL-C prediction accuracy might be possible by adding more biomarkers, genetic data, or patient-specific clinical parameters. Additionally, examining the robustness of other penalized regression techniques such as Lasso and ElasticNet could enhance model performance across various subgroups. Prospective studies assessing how machine learning–based LDL-C estimation influences clinical decision-making and long-term cardiovascular outcomes would provide valuable insights into its practical application.

Conclusion

Our study shows that a machine learning-based Ridge regression model offers better accuracy in LDL-C estimation compared to traditional equations. This increased precision has important implications for cardiovascular risk assessment and lipid-lowering therapy management. By incorporating machine learning methods into clinical workflows, healthcare professionals can improve LDL-C evaluation, leading to more personalized and effective treatment strategies.

Declaration of Generative AI and AI-assisted technologies in the writing process

We would like to declare that generative AI (ChatGPT-5) was used solely for assistance in checking and refining the English language in this manuscript, including minor translations. The authors entirely generated the content, ideas, and findings presented in the manuscript without AI assistance. After language editing, the authors reviewed and validated the final version to ensure its accuracy and integrity.

Conflict of Interest

The authors declare no conflict of interest.

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Ethical approval

The study was approved by the Khon Kaen University Ethics Committee for Human Research (Approval No: HE651263). Informed consent was not required as no human samples were collected directly for this study.

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Research Article

Association of Hematological Inflammatory Indices with Glycemic Control in Type 2 Diabetes Mellitus- A Cross-sectional study

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Keywords

Diabetes mellitus, Inflammation, Neutrophil Lymphocyte Ratio, Inflammatory indices, Glycemic control

Abstract

Introduction: Type 2 Diabetes Mellitus (T2DM) is a significant global health concern characterized by chronic low-grade inflammation, contributing to various complications. While Glycated Hemoglobin (HbA1c) is a primary tool for assessing glycemic control, hematological inflammatory indices derived from routine Complete Blood Count (CBC) are emerging as promising, low-cost indicators of systemic inflammation. This study aimed to investigate the association between these indices and glycemic control in T2DM patients.

Methodology: This comparative cross-sectional study included 750 individuals, equally categorized into non-diabetic, well-controlled T2DM (HbA1c < 7%), and poorly controlled T2DM (HbA1c ≥ 7%) groups. HbA1c, and eleven hematological inflammatory indices like NLR, MLR, SII, SIRI, etc, were calculated and compared between the groups using JASP software followed by correlation, Receiver Operating Characteristic (ROC) curves, and binary logistic regression.

Results: NLR, SII, SIRI, and AISI consistently showed a rising trend with poorer glycemic control, correlated moderately with HbA1c, and demonstrated moderate predictive performance for uncontrolled diabetes (AUC > 0.6). Subgroup analysis also revealed higher NLR in poorly controlled diabetics and regression analysis proved NLR as an independent predictor of poor glycemic control (adjusted odds ratio = 4.73, $p < 0.001$).

Conclusion: This study demonstrates that hematological inflammatory indices, particularly NLR, SII, SIRI, and AISI, are significantly elevated in patients with poorly controlled T2DM, reflecting a state of chronic systemic inflammation. Among these, NLR shows the strongest and independent association with poor glycemic status, highlighting its potential as a low-cost, accessible adjunct marker for monitoring glycemic control and systemic inflammation in T2DM.

Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by persistent hyperglycemia resulting from insulin deficiency, resistance, or both, and has emerged as a major global health issue [1]. In 2015, the global prevalence of diabetes among adults aged 20–79 years was 8.8%, projected to rise to 10.4% by 2040. According to the International Diabetes Federation, by 2019 approximately 463 million adults, and by 2021 over 536.6 million adults had diabetes, with projections indicating a rise to 783.2 million by 2045, most being Type 2 Diabetes Mellitus (T2DM) [2]. Prolonged hyperglycemia contributes to serious complications such as cardiovascular disease, nephropathy, neuropathy and retinopathy [1].

Chronic low-grade inflammation plays a central role in the pathogenesis and progression of T2DM and its complications [3]. Hyperglycemia activates immune responses, disrupts insulin signaling, and promotes inflammatory cascades, with immune cells like macrophages contributing significantly to these processes [4,5]. Monitoring glycemic control is essential to reduce the risk of complications, typically assessed using HbA1c, fasting plasma glucose, or oral glucose tolerance tests [6]. While HbA1c remains the standard for long-term glycemic control, it has limitations including interference from hemoglobin variants and its inability to reflect acute glucose fluctuations [6–8].

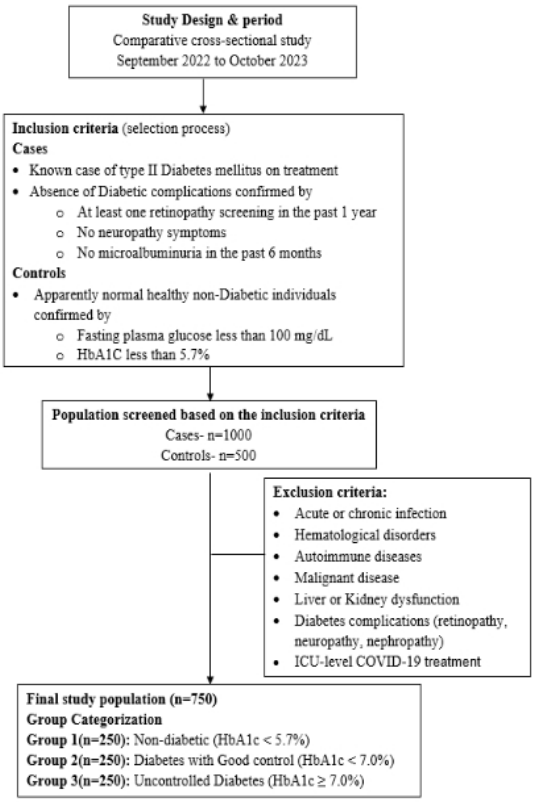
Complete blood count (CBC) derived parameters and indices, calculated from routine hematological tests, particularly white blood cells and platelets are being increasingly investigated as low-cost and easily accessible indicators of systemic inflammation in conditions like malignancy, cardiovascular diseases, autoimmune diseases, metabolic syndrome etc [9]. Some of these indices include the Neutrophil-to-Lymphocyte Ratio (NLR), Platelet-to-Lymphocyte Ratio (PLR), and Monocyte-to-Lymphocyte Ratio (MLR), and composite indices such as the Systemic Immune-Inflammation Index (SII), Systemic Inflammatory Response Index (SIRI). These indices integrate various white blood cell subgroups and platelets, potentially offering a more stable reflection of the body's inflammatory status compared to individual cell counts [9,10]. Several studies have explored the association of these indices with diabetes and its complications. Elevated levels of NLR, PLR, and MLR have been observed in patients with T2DM compared to non-diabetic individuals [11]. NLR has also been associated with microvascular complications like diabetic nephropathy and retinopathy. PLR has been found to be significantly higher in patients with diabetic nephropathy. SII, PLR and SIRI levels were higher in T2DM patients with diabetic retinopathy (DR) compared to those without it, and were identified as independent risk factors for diagnosis and prediction of advanced DR [12]. These findings highlight the potential utility of these hematological inflammatory indices in the assessment of inflammation and its association with glycemic control and complications in T2DM, although their specific relationships and clinical utility warrant further investigation, especially in diverse populations.

This study aims to investigate the association between hematological inflammatory indices and glycemic control in Type 2 Diabetes mellitus patients. Inflammatory indices include Neutrophil-to-Lymphocyte Ratio (NLR), Monocyte-to-Lymphocyte Ratio (MLR), Platelet-to-Monocyte Ratio (PMR), Platelet-to-Lymphocyte Ratio (PLR), Basophil-to-Lymphocyte Ratio (BLR), Eosinophil-to-Lymphocyte Ratio (ELR), Lymphocyte-to-Monocyte Ratio (LMR), and White Cell-to-Platelet Ratio (WPR) and composite indices like Systemic Immune-Inflammation Index (SII), Systemic Inflammatory Response Index (SIRI), and Aggregate Index of Systemic Inflammation (AISI). The objective was to compare these hematological inflammatory indices among groups of individuals with normal glucose tolerance (non-diabetic controls), patients with well-controlled Type 2 Diabetes Mellitus (HbA1c < 7%), and patients with poorly controlled Type 2 Diabetes Mellitus (HbA1c ≥ 7%).

Materials and Methods

This comparative cross-sectional study was retrospectively conducted in the Department of Biochemistry at Sri Ramachandra Medical College and Research Institute, Chennai, Tamil Nadu, India. The study protocol is represented in Figure 1.

Figure 1: Flow chart illustrating the methodology of the study population selection.



The clinical and demographic details were collected from the patient records. The following biochemical and hematological analyses were performed in the central laboratory

- Fasting Blood Sugar (FBS) and Post-Prandial Blood Sugar (PPBS) were measured by Hexokinase method using the Roche Cobas c702 automated chemistry analyzer (Roche Diagnostics GmbH, Mannheim, Germany).
- HbA1c was measured using cation-exchange high-performance liquid chromatography (HPLC) on the Tosoh G8 Analyzer (Tosoh Bioscience, Tokyo, Japan).
- Complete Blood Count (CBC) parameters including total leukocyte count, neutrophils, lymphocytes, monocytes, eosinophils, basophils, and platelets were analyzed using the Sysmex XN-1000 automated hematology analyzer (Sysmex Corporation, Kobe, Japan).

Further the following inflammatory indices were calculated from CBC parameters to evaluate the systemic inflammatory status and its association with glycemic control.

Table 1: Inflammatory indices included in the study along with its calculation formulas.

S.No	Inflammatory indices	Calculation formula
1	Neutrophil Lymphocyte ratio (NLR)	Neutrophil / Lymphocyte
2	Monocyte Lymphocyte ratio (MLR)	Monocyte / Lymphocyte
3	Platelet Lymphocyte ratio (PLR)	Platelet / Lymphocyte
4	Platelet Monocyte ratio (PMR)	Platelet / Monocyte
5	Lymphocyte Monocyte ratio (LMR)	Lymphocyte / Monocyte
6	Eosinophil Lymphocyte ratio (ELR)	Eosinophil / Lymphocyte
7	Basophil Lymphocyte ratio (BLR)	Basophil / Lymphocyte
8	White blood cell Platelet ratio (WPR)	White Blood Cell Count / Platelet Count
9	Systemic Immune-Inflammation Index (SII)	(Platelet × Neutrophil) / Lymphocyte
10	Systemic Inflammatory Response Index (SIRI)	(Neutrophil × Monocyte) / Lymphocyte
11	Aggregate Index of Systemic Inflammation (AISI)	(Neutrophil × Platelet × Monocyte) / Lymphocyte

Statistical Analysis

All statistical analyses were conducted using JASP (Version 0.19, JASP team (2024) JASP (version0.19), University of Amsterdam) and Microsoft Excel. The distribution of continuous variables was assessed using the Shapiro-Wilk test. Data were presented as median with interquartile range [IQR], Kruskal-Wallis test and Dunn's post-hoc test was used to compare hematological and inflammatory parameters across the three groups and Chi-square test was employed for categorical variables. Spearman's correlation was used to assess the strength and direction of association between glycemic parameters and inflammatory indices. For subgroup analysis comparison Mann-Whitney U test or Welch's t-test was used based on the equality of variances. Receiver Operating Characteristic (ROC) curve analysis was performed to evaluate the predictive ability of inflammatory indices for poor glycemic control. In addition, Linear regression was used to assess the direct relationship between NLR and HbA1c. Further, Binary logistic regression was performed to evaluate the predictive value of NLR for poor glycemic control (HbA1c > 7%), both in unadjusted and adjusted models. The adjusted model accounted for age, FBS, PPBS, total WBC count, eosinophils, basophils, and platelets, based on prior correlation analysis. All statistical tests were two-tailed, and significance was defined as a p-value < 0.05.

Results

The final study population of 750 individuals was evenly distributed across three groups based on glycemic control: Group 1 (non-diabetic, HbA1c < 5.7%), Group 2 (well-controlled diabetics, HbA1c < 7%), and Group 3 (poorly controlled diabetics, HbA1c ≥ 7%). The median age significantly increased across the groups ($p < 0.001$), while the proportion of females decreased progressively from 89.6% in Group 1 to 62.4% in Group 3 ($p < 0.001$). Glycemic parameters, including fasting blood sugar (FBS), postprandial blood sugar (PPBS), glycated hemoglobin (HbA1c), and estimated average glucose (EAG), increased significantly from Group 1 through Group 3, indicating worsening glycemic control across the groups as shown in Table 2.

In our study Table 2, the Hematological parameters demonstrated significant difference between the groups. Total WBC count, neutrophils, basophils and platelets increased significantly across the groups while eosinophils, lymphocytes and monocytes showed no statistically significant differences. In post hoc analysis as shown in Table 2, total count and neutrophils were significantly elevated in Group 3 compared to Groups 1 and 2 ($p < 0.001$).

Table 2: Comparison of demographic and hematological parameters between 3 groups in the study conducted from September 2022 to October 2023.

Variables	Group 1 Median [IQR] n=250	Group 2 Median [IQR] n=250	Group 3 Median [IQR] n=250	Overall p-Value
Age (years)	29 [24-37]	40 [30-49.7]	47 [37-56]	<0.0001*#§
Sex (females)	224 (89.6%)	203 (81.2%)	156 (62.4%)	<0.0001
FBS (mg/dL)	92 [86-99]	98 [91-105]	139 [110-194]	<0.0001*#§
PPBS (mg/dL)	107.5 [93-125.7]	121 [101-144.7]	220.5 [152-305]	<0.0001*#§
HbA1c (%)	5.3 [5.1-5.5]	6 [5.8-6.2]	8.6 [7.2-9.9]	<0.0001*#§
eAG (mg/dL)	105 [100-111]	126 [120-131]	175 [146-226]	<0.0001*#§
Total WBC count (cells/µL)	7900 [6770-9395]	8000 [6615-9572]	8920 [7377.5-11097.5]	<0.0001#§
Eosinophils (cells/µL)	197.9 [110.2-311.4]	221.2 [126.4-356.5]	225.5 [137.4-384.2]	0.052
Basophils (cells/µL)	34 [23.1-45.1]	37.4 [21.4-53]	39.5 [26.2-53.6]	0.021#
Neutrophils (cells/µL)	4655.3 [3753.7-5843]	4709.2 [3601.8-5817.2]	5590.4 [4535-7097.7]	<0.0001#§
Lymphocytes (cells/µL)	2317.3 [1964-2806]	2416.9 [1981-2836]	2262.6 [1848-2787.8]	0.131
Monocytes (cells/µL)	447.1 [317.5-565.8]	390 [298-524]	419 [311.6-575.9]	0.161
Platelets (lakhs/µL)	2.84 [2.46-3.35]	3.09 [2.60-3.58]	3.08 [2.51-3.63]	0.002*#

FBS: Fasting blood sugar; PPBS: Postprandial blood sugar: HbA1c: Glycated Hemoglobin; eAG: Estimated average glucose; WBC: White blood cells; ; p-Value <0.01 Highly significant; p-Value <0.05 statistically significant

Post-hoc analysis: *Significant difference between group 1 and group 2; #Significant difference between group 1 and group 3; §Significant difference between group 2 and group 3

Table 3: Comparison of Hematological Inflammatory indices between three groups in the study conducted from September 2022 to October 2023.

Variables	Group 1 Median [IQR] (n=250)	Group 2 Median [IQR] (n=250)	Group 3 Median [IQR] (n=250)	Overall p-Value
NLR	2.05 [1.5-2.5]	1.95 [1.5-2.42]	2.25 [1.8-3.2]	<0.0001#§
MLR	0.19 [0.14-0.25]	0.17 [0.13-0.23]	0.19 [0.13-0.27]	0.006*§
ELR	0.08 [0.05-0.13]	0.09 [0.05-0.15]	0.1 [0.06-0.16]	0.026#
PLR	121.7 [98.4-149.1]	130.5 [99.9-160.8]	131.9 [105.8-165.3]	0.019#
PMR	633.6 [470.3-914.1]	790.9 [570.8-1039.3]	740.8 [493.6-1041.8]	0.001*#
LMR	5.23 [3.93-7.36]	5.96 [4.32-7.90]	5.31 [3.63-7.42]	0.006*§
BLR	0.014 [0.01-0.02]	0.016 [0.009-0.021]	0.017 [0.012-0.024]	0.002#§
SII	564.1 [411.5-765.8]	591.1 [437.9-788.1]	727 [559.7-1040.2]	<0.0001#§
SIRI	885.1 [533.5-1280.6]	776.8 [546.9-1104.6]	1065 [708.8-1608.9]	<0.0001*#§
WPR	0.027 [0.023-0.034]	0.026 [0.021-0.032]	0.029 [0.024-0.036]	<0.0001*§
AISI	2440.9 [1530.3-3762.5]	2312.3 [1498.9-3611.3]	3211 [2049.3-5071.8]	<0.0001#§

NLR: Neutrophil lymphocyte ratio; MLR: Monocyte lymphocyte ratio; ELR: Eosinophil lymphocyte ratio; PLR: Platelet lymphocyte ratio; PMR: Platelet monocyte ratio; LMR:Lymphocyte monocyte ratio; BLR: Basophil lymphocyte ratio: SII: Systemic Immune-Inflammation index; SIRI: Systemic inflammatory response index; WPR: WBC platelet ratio; AISI: Aggregate index of systemic inflammation; p-Value <0.01 Highly significant; p-Value <0.05 statistically significant

Post-hoc analysis: * Significant difference between group 1 and group 2; #Significant difference between group 1 and group 3; §Significant difference between group 2 and group 3

In Table 3, all the calculated hematological inflammatory indices were significantly elevated across the groups (all p<0.05). However, in post hoc analysis, NLR, BLR, SII, SIRI and AISI showed a rising trend with poor glycemic control and significantly elevated levels were observed in Group 3 compared to Groups 1 and 2 (all $p < 0.001$).

Spearman's correlation analysis in Table 4, demonstrated that HbA1c had a moderate positive correlation with NLR ($r = 0.168$, $p < 0.001$), BLR ($r=0.118$, $p <0.001$) SII ($r = 0.233$, $p < 0.001$), SIRI ($r = 0.115$, $p = 0.002$) and AISI ($r = 0.157$, $p < 0.001$). ELR, PLR, and PMR had weaker correlations with glycemic markers, while MLR, LMR and WPR did not correlate with HbA1c. Based on the correlation analysis, NLR was selected for regression analysis to determine its predictive value for poor glycemic control.

Table 4: Spearman correlation analysis between glycemic profile and inflammatory indices included in the study conducted from September 2022 to October 2023.

Variable		Age	FBS	PPBS	HbA1c	eAG
FBS	r-value p-value	0.521 <0.001**	-	-	-	-
PPBS	r-value p-value	0.499 <0.001**	0.717 <0.001**	-	-	-
HbA1c	r-value p-value	0.478 <0.001**	0.578 <0.001**	0.622 <0.001**	-	-
eAG	r-value p-value	0.600 <0.001**	0.742 <0.001**	0.745 <0.001**	0.799 <0.001**	-
NLR	r-value p-value	-0.144 <0.001**	-0.105 <0.001**	-0.003 0.942	0.168 <0.001**	-0.106 0.004*
MLR	r-value p-value	-0.023 0.538	0.036 0.327	-0.018 0.626	-0.009 0.809	-0.164 <0.001**
ELR	r-value p-value	0.209 <0.001**	0.115 0.002*	0.081 0.027*	0.081 0.027*	0.09 0.014*
PLR	r-value p-value	-0.03 0.416	-0.066 0.073	-0.098 0.007**	0.099 0.007**	-0.045 0.218
PMR	r-value p-value	-0.015 0.676	-0.069 0.058	-0.047 0.202	0.099 0.007**	0.118 0.001**
LMR	r-value p-value	0.02 0.579	-0.036 0.33	0.019 0.602	0.01 0.776	0.165 <0.001**
BLR	r-value p-value	0.119 0.001**	0.072 0.048*	0.085 0.020*	0.118 0.001**	0.039 0.286
SII	r-value p-value	-0.153 <0.001**	-0.064 0.081	0.012 0.744	0.233 <0.001**	-0.017 0.645
SIRI	r-value p-value	-0.11 0.002**	0.004 0.910	0.039 0.287	0.115 0.002**	-0.114 0.002**
WPR	r-value p-value	-0.047 0.199	-0.013 0.717	0.097 0.008**	0.05 0.171	-0.041 0.262
AISI	r-value p-value	-0.12 <0.001**	0.020 0.587	0.042 0.246	0.157 <0.001**	-0.051 0.160

FBS: Fasting blood sugar; PPBS: Postprandial blood sugar: HbA1c: Glycated Hemoglobin; eAG: Estimated average glucose; NLR: Neutrophil lymphocyte ratio; MLR: Monocyte lymphocyte ratio; ELR: Eosinophil lymphocyte ratio; PLR: Platelet lymphocyte ratio; PMR: Platelet monocyte ratio; LMR: Lymphocyte monocyte ratio; BLR: Basophil lymphocyte ratio: SII: Systemic Immune-Inflammation index; SIRI: Systemic inflammatory response index; WPR: WBC platelet ratio; AISI: Aggregate index of systemic inflammation;**p-Value <0.01 Highly significant; * p-Value <0.05 statistically significant

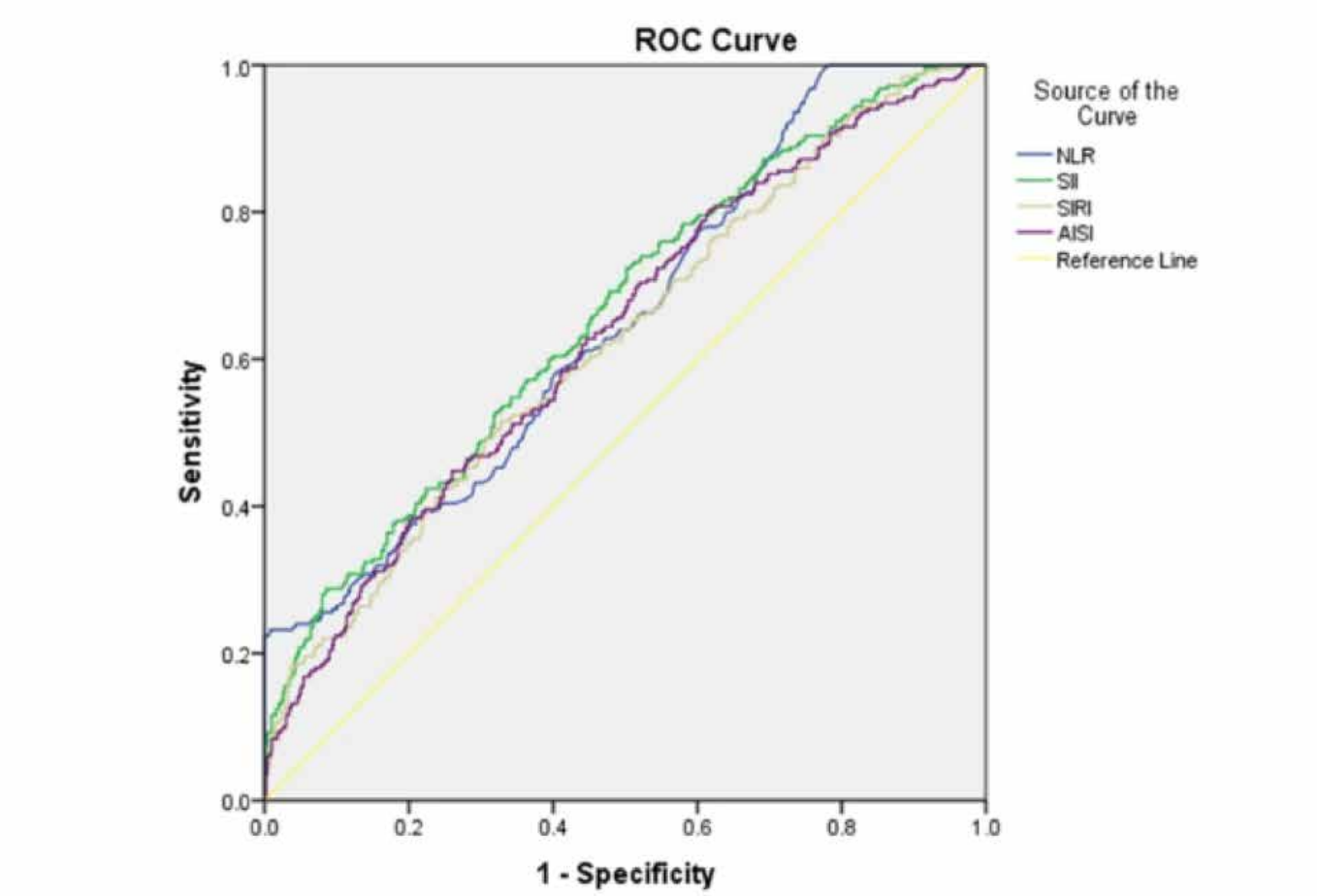
A subgroup analysis (Supplementary Table 1) was conducted within the uncontrolled diabetes group (Group 3) by stratifying individuals based on the severity of the uncontrolled diabetes into sub-group 1 (HbA1c < 8%) and sub-group 2 (HbA1c ≥ 8%). On comparison of the study parameters between the groups, apart from age and glycemic parameters in the study only Neutrophils and NLR showed statistically significant elevation between the groups. Individuals with poor glycemic control had higher NLR values (2.32 [1.89–4.23]) compared to those with moderately controlled diabetes (median 2.19 [1.79–2.98], p = 0.009).

Further Receiver operating characteristic (ROC) curve analysis was performed for all inflammatory indices as shown in Figure 2. The results revealed that only four indices (NLR, SII, SIRI, AISI) had moderate predictive performance. NLR with an optimal cutoff of 1.855 and AUC = 0.651, yielded the highest sensitivity of 76.4% and specificity of 41.0% (p < 0.001) compared to other indices such as SII (AUC = 0.659), SIRI (AUC = 0.625), and AISI (AUC = 0.632).

Out of the four indices that had moderate predictive performance in the ROC analysis, the outcome of logistic regression analysis revealed that only NLR demonstrated statistically significant association with poor glycemic control. In the unadjusted model, elevated NLR was associated with increased odds of uncontrolled diabetes (odds ratio = 2.1, 95% CI: 1.75–2.52, p < 0.001). This association remained strong in

the adjusted model, where NLR was controlled for age, FBS, PPBS, total WBC count, eosinophils, basophils, and platelets, yielding an adjusted odds ratio of 4.73 (95% CI: 3.36–6.65, p < 0.001) (Supplementary Figure 1 and Table 5).

Figure 2: Receiver operating characteristics (ROC) curve analysis of Inflammatory indices for predicting uncontrolled diabetes.



Variable	AUC	Cutoff	Sensitivity	Specificity	p-Value
NLR	0.651	1.8550	76.4%	41.0%	<0.001
SII	0.659	648.06	60.4%	60.0%	<0.001
SIRI	0.625	1030.45	51.6%	67.2%	<0.001
AISI	0.632	2578.06	62.8%	55.4%	<0.001

NLR: Neutrophil lymphocyte ratio; SII: Systemic Immune-Inflammation index; SIRI: Systemic inflammatory response index; AISI: Aggregate index of systemic inflammation; **p-Value <0.01 Highly significant; *p-Value <0.05 statistically significant

Table 5 : Regression analysis for NLR as an independent predictor of uncontrolled diabetes.

Regression model	Variables	Beta coefficient	Odds Ratio	95% CI (odds ratio)	p-value
Linear regression	NLR and HbA1c	0.46	NA	0.35-0.56*	<0.001
Logistic regression (unadjusted)	NLR and HbA1c > 7%	0.74	2.1	1.75-2.52	<0.001
Logistic regression (adjusted)#	NLR and HbA1c > 7%	1.55	4.73	3.36-6.65	<0.001

*The 95% confidence interval in linear regression corresponds to the beta coefficient; #The model was adjusted for Age, Fasting blood sugar, postprandial blood sugar, Total WBC, Eosinophils, Basophils, and Platelets. p-Value <0.01 is considered Highly significant.

Discussion

Diabetes mellitus (DM) is a chronic metabolic disorder marked by sustained hyperglycemia and chronic low-grade inflammation, driving a wide array of vascular complications including cardiovascular disease, diabetic nephropathy, retinopathy, and neuropathy leading to the morbidity and mortality [13]. Among the demographic profiles in Table 2, a significant age gradient was observed, increasing from 29 years [24–37] in Group 1 (non-diabetic) to 40 years [30–49.7] in Group 2 (controlled diabetics), and further to 47 years [37–56] in Group 3 (uncontrolled diabetics) ($p < 0.01$). Further, all glycemic parameters showed statistically significant differences among the groups. HbA1c increased significantly from 5.3% [5.1–5.5] to 8.6% [7.2–9.9] ($9p < 0.01$).

White blood cells (leukocytes) serve as indicators of systemic inflammation and studies have shown elevated total WBC counts in individuals with T2DM compared to non-diabetic controls [9]. In our study table 2, total WBC count increased significantly from 7900 cells/µL [6770–9395] to 8920 cells/µL [7377.5–11097.5] ($p < 0.01$), while neutrophils increased from 4655.3 cells/µL [3753.7–5843] to 5590.4 cells/µL [4535–7097.7] ($p < 0.0001$) and Basophils were significantly higher in Group 3 compared to Group 1 ($p = 0.021$). The lymphocytes, monocytes and eosinophils did not differ significantly across groups (all $p>0.05$).

The mechanistic basis for the leukocyte alterations in T2DM lies in the metabolic disturbances triggered by persistent hyperglycemia [14]. Hyperglycemia induced excessive ROS production and the accumulation of AGEs, stimulates NF-κB and JNK pathways, promoting the transcription of pro-inflammatory genes. Simultaneously, chemokines such as monocyte chemoattractant protein-1 (MCP-1) increase the recruitment of monocytes and other immune cells to sites of tissue injury while IL-6 influences leukocyte production and differentiation contributing to insulin resistance and erythropoietic suppression [15]. Importantly, this chronic inflammatory state linked development of insulin resistance is due to serine phosphorylation of insulin receptor substrate proteins that impairs downstream insulin signaling, exacerbating hyperglycemia and establishing a vicious cycle. WBCs and their inflammatory products are directly implicated in this process. Additionally, in tissues such as the kidneys and retina, leukocyte infiltration contributes to fibrosis, neovascularization, and capillary dropout, all of which are hallmarks of diabetic nephropathy and retinopathy [14-16]. Despite these findings, no single WBC parameter has shown promising outcomes in assessing glycemic control. The Inflammatory indices calculated based on the WBC parameters have shown significant results in conditions like COVID-19, cardiovascular diseases, while the studies of these indices in DM were mostly focused on its vascular complications. In our study, nearly five inflammatory indices from WBC parameters (NLR, MLR, ELR, LMR, BLR), three indices using platelets and WBC (PLR, PMR, WPR) and three composite indices (SII, SIRI, AISI) were compared based on glycemic control in diabetic patients, along with healthy non-diabetic controls. In our study, NLR values were significantly elevated in Group 3 (2.25 [1.8–3.2]) compared to Group 1 (2.05 [1.5–2.5]) and Group 2 (1.95 [1.5–2.42]) with an overall $p < 0.01$. Similar upward trends were observed in SII (564.1 [411.5–765.8] to 727 [559.7–1040.2]; $p < 0.01$), SIRI (885.1 [533.5–1280.6] to 1065 [708.8–1608.9]; $p < 0.01$), and AISI (2440.9 [1530.3–3762.5] to 3211 [2049.3–5071.8]; $p < 0.01$). Statistically significant differences without any particular trend were also found in MLR ($p = 0.006$), ELR ($p = 0.026$), LMR ($p = 0.006$), BLR ($p = 0.002$). These findings were confirmed in the correlation analysis, all the indices correlated with HbA1c all $p<0.05$ except for MLR and LMR. However, in the ROC analysis shown in Figure 2, revealed that only NLR, SII, SIRI, AISI had a moderate predictive performance for uncontrolled diabetes (AUC >0.600). Despite these findings, only NLR and neutrophils exhibited significant difference between the moderate and poorly controlled diabetes patients in the subgroup analysis (all $p<0.05$).

In support of our findings, numerous studies have highlighted the role of the neutrophil-to-lymphocyte ratio (NLR) as a cost-effective reliable indicator of inflammation and its association with diabetic complications [17]. Demirtas et al., Akbas et al., and Li et al., reported significantly elevated NLR levels in diabetic patients with nephropathy or albuminuria [17-19]. While Demirtas et al. found no significant difference in NLR between patients with HbA1c <7% and ≥7%, Prakash et al. observed higher NLR in uncontrolled diabetics (HbA1c >7%) compared to those with better control, despite weak correlation

with HbA1c [17,20]. Mertoglu & Gunay and Yilmaz et al. demonstrated a stepwise rise in NLR from normoglycemic individuals to those with prediabetes and diabetes, with Yilmaz et al. also linking elevated NLR to glucose levels during OGTT in morbidly obese individuals, supporting its role in early disease detection [21,22]. In the context of insulin resistance severity, both Zhang & Liu et al., observed significantly elevated NLR with a positive correlation to HOMA-IR and fasting blood glucose, identifying NLR as an early marker of metabolic dysfunction [23]. Song et al., Verdoia et al. (2015), and Aygun et al.(2015), reported higher NLR levels as independent predictors of diabetic patients with obstructive coronary artery disease (CAD) and peripheral arterial disease (PAD) [24-26]. In diabetic retinopathy (DR), Deng et al. (2025), showed a progressive rise in NLR from 2.19 in No DR to 2.82 in PDR, [10]. Additionally, Lee et al. (2012), demonstrated the long-term predictive utility of NLR in T2DM patients with acute myocardial infarction [27]. Interestingly, a study reported no significant differences in NLR between well and poorly controlled diabetics, likely due to the influence of anti-inflammatory drugs such as aspirin and metformin [11]. In our study, similar to the published literature regression analysis confirmed NLR as an independent predictor of poor glycemic control: linear regression showed a $\beta = 0.46$ (95% CI: 0.35–0.56, $p < 0.001$), and adjusted logistic regression yielded an increased odds ratio of 4.73 (95% CI: 3.36–6.65, $p < 0.001$) from an odds ratio of 2.1 in unadjusted models. Taken together, these findings consolidate NLR's value as a practical, accessible, and low-cost inflammatory marker that reflects systemic inflammation, metabolic stress, and the presence or progression of diabetic complications.

The findings in our study, pertaining to other indices with moderate predictive performance such as SII, SIRI and AISI also align with a growing body of literature. Chen et al. (2024), demonstrated that higher SII quartiles were positively associated with diabetes risk, with BMI and waist circumference acting as partial mediators [28]. Deng et al. (2025), showed that SII was significantly higher across advancing stages of diabetic retinopathy, with a notable linear trend and diagnostic value (AUC > 0.6) [10]. Similarly, Song et al. (2024), reported elevated SII, SIRI, and AISI in T2DM patients with peripheral arterial disease (PAD) with confirmed moderate discriminative ability (all AUC > 0.600) [24]. Wang et al. (2023), and other studies have linked elevated SII and SIRI with increased mortality and risk of diabetic complications including nephropathy, foot infections, hepatic steatosis, and cardiovascular disease [12,29,30]. These findings collectively validate our results and reinforce the role of SII, SIRI, and AISI in detecting worsening glycemic status despite no difference in the subgroup analysis.

In our study, platelet counts significantly increased from 2.84 lakhs/μL [2.46–3.35] in group 1 to 3.09 [2.60–3.58] in group 2, with no significant difference between groups 2 and 3. Among the platelet-related indices, WPR and PLR showed a rising trend, whereas PMR exhibited a biphasic response i.e. elevated in group 2 and subsequently reduced in group 3. Notably, only PLR and PMR correlated positively with HbA1c ($r = 0.099$; $p < 0.007$). Several studies have emphasized the clinical significance of PLR in diabetes. Akdogan et al. (2016), reported significantly higher PLR in diabetic patients and demonstrated strong correlations with HbA1c, disease duration, and atherogenic index [31]. Zhang and Liu. (2024), showed a stepwise increase in insulin resistance across higher PLR tertiles, supporting its role as a marker of chronic inflammation in DM [23]. Deng et al. (2025), observed progressively rising PLR with increasing stages of diabetic retinopathy, although it was not found to be an independent risk factor [10]. Mertoglu and Gunay. (2017), reported a biphasic PLR pattern i.e. lower PLR in prediabetics and newly diagnosed patients, and higher in known diabetics, which mirrors our findings with PMR [21]. PLR was also shown to be associated with diabetic nephropathy and microalbuminuria [17,18]. Additionally, Gao et al. (2024), and Si et al.(2024), linked elevated PLR with DR progression and increased cardiovascular mortality [32,33]. Mechanistically, hyperglycemia-induced platelet activation via oxidative stress and thromboxane A2 promotes inflammation through the release of mediators like Platelet factor 4 (PF4), CD40 ligand (CD40L), and Interleukin-1 beta (IL-1β) contributing to vascular damage [30,34]. Platelet-leukocyte aggregates drive vascular inflammation and endothelial dysfunction, contributing to diabetic macrovascular complications rather than glycemic control.

Other indices like LMR, ELR, and BLR were novel in our study due to the lack of existing literature in diabetes. MLR and LMR showed no correlation with HbA1c, despite differences in comparative analysis, while ELR and BLR exhibited weak to moderate correlation but lacked predictive value in ROC analysis (AUC < 0.5). Lymphopenia is a known marker of chronic inflammation in diabetes, whereas monocyte elevation is gradual and more related to vascular complications than short-term glycemic changes, aligning with the lack of correlation between MLR, LMR and HbA1c in our study. Supporting this, Prakash et al. (2020), found no significant MLR difference between controlled and uncontrolled diabetics (0.2 vs. 0.24; $p > 0.05$), nor any correlation with HbA1c [20]. Zhang & Liu. (2024), reported an MLR–IR association, but no variation across MLR tertiles, limiting stratification [23]. Deng et al. (2025), also observed elevated MLR in PDR, without a linear trend with severity [10]. Ngama et al. (2021), showed no correlation between ELR and HbA1c [35]. BLR findings in our study align with Shen et al. (2024), who found a 2.45-fold increased T2DM risk in the highest BLR quartile, influenced partly by dyslipidemia, potentially explaining the limited discriminative value of BLR in our ROC analysis [36].

The cross-sectional design limits the ability to infer causality between elevated indices and glycemic progression. Lack of data on lipid profiles and co-morbid conditions such as hypertension and Confounding factors such as use of anti-

inflammatory or antiplatelet medications (e.g., aspirin, statins) were not uniformly accounted for, which may influence inflammatory markers. Future longitudinal follow-up studies are required to validate our study findings in development of diabetic complications. Integration of metabolic markers like Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), insulin levels, lipid profile and inflammatory biomarkers can enhance the accuracy and mechanical insight.

Conclusion

This study demonstrates that hematological inflammatory indices derived from routine blood parameters, particularly NLR, SII, SIRI, and AISI are significantly elevated in patients with poor glycemic control, reflecting a state of chronic systemic inflammation in T2DM. Among all indices, NLR showed the strongest and independent association with poor glycemic status, highlighting its potential as a low-cost, accessible adjunct marker for monitoring glycemic control and systemic inflammation in T2DM without complications. While other indices like BLR and PLR also showed significant group-wise differences and correlations with glycemic markers, their predictive performance was less robust, suggesting further evaluation with other confounding factors. Overall, the findings reinforce the utility of inflammatory indices in identifying individuals at risk and tracking metabolic deterioration in diabetes with the novelty of evaluating multiple hematological indices in uncomplicated T2DM. Notably, it is among the first few studies to establish the utility of BLR, ELR, PMR, LMR, and AISI in this context.

Author Contribution

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Writing - review and editing: Kiran S, Karthick E, Sathya Selvarajan, K Sowmya, K.S.Sridharan ; Resources: Kiran S, Karthick E, Sathya Selvarajan, K Sowmya, K.S.Sridharan ; Supervision and final approval: Kiran S, Karthick E, Sathya Selvarajan, K Sowmya, K.S.Sridharan ; Accountability for the research: Kiran S, Karthick E, Sathya Selvarajan, K Sowmya, K.S.Sridharan.

Ethical Approval

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki. Ethical clearance was obtained from the Institutional Ethics Committee (IEC) with reference number CSP/22/SEP/116/486 and waiver of consent was obtained pertaining to the retrospective nature of the study. Confidentiality of the patient data was strictly maintained throughout the study.

Disclosure of Conflict of Interest

The authors declare that there is no conflict of interest concerning this study.

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Supplementary Files

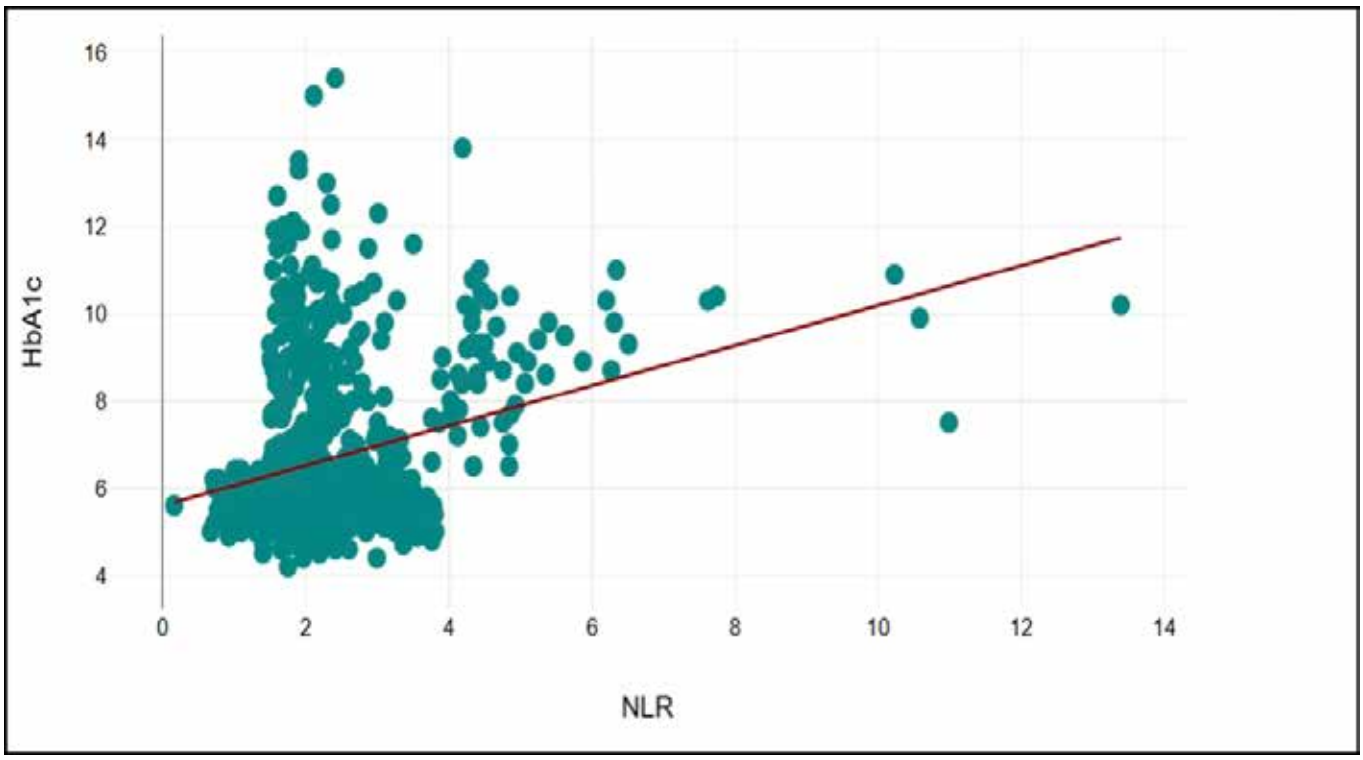
Supplementary Table 1: Subgroup analysis of the Inflammatory indices based on the severity of the uncontrolled diabetes (group 3 HbA1C greater than 7%).

Variable	Group 3 Median [IQR]	Moderate control Median [IQR]	Poor control Median [IQR]	p-value*
Age (years)	47 [37-56]	52 [42-60]	45 [35-52]	<0.001
FBS (mg/dL)	139 [110-194]	124 [108-144]	170 [115-230]	<0.001
PPBS (mg/dL)	220.5 [152-305]	193 [146-231]	280 [174-351]	<0.001
HbA1C (%)	8.6 [7.2-9.9]	7.1 [7.0-7.6]	9.7 [8.9-10.4]	<0.001
EAG (mg/dL)	175 [146-226]	154 [146-169]	220 [186-249]	<0.001
Neutrophils (cells/µL)	5590.4 [4535-7097.7]	5402.7 [4387.5-6724.1]	5717.4 [4758.6-7376.6]	0.016
NLR	2.25 [1.8-3.2]	2.19 [1.79-2.98]	2.32 [1.89-4.23]	0.009

FBS: Fasting blood sugar; PPBS: Postprandial blood sugar: HbA1C: Glycated Hemoglobin; EAG: Estimated average glucose; NLR: Neutrophil lymphocyte ratio; p-Value <0.01 Highly significant; p-Value <0.05 statistically significant

*Comparison was performed using Mann-Whitney U test and Welch test based on the distribution of variance between Moderate control group and Poor control group. Group 3 Data is provided for reference and not used for comparison.

Supplementary Figure 1: Linear regression analysis between NLR and HbA1C in the study.



Review Article

A mini-review of point-of-care C-reactive protein testing in sepsis in the Emergency Department

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Abstract

Timely initiation of appropriate treatment of sepsis in the Emergency Department is crucial in a patient's prognosis. Surviving Sepsis Campaign guidelines suggest improved results in septic shock where antimicrobials are given within 1 hour of presentation. The recognition and treatment of critically ill patients could be expedited using different methods of point-of-care testing as opposed to centralised laboratory testing. C-reactive protein is utilised as a predictor for sepsis prognosis and should be interpreted in conjunction with patient clinical findings due to its low specificity and sensitivity. Some efficient and simple C-reactive protein point-of-care assay kits discussed include immunoturbidimetry, immunonephelometry, lateral flow assays and bioassays, which have advantages over laboratory-based methods, but still require further investigation. A useful tool such as a C-reactive protein point-of-care test could potentially enhance the operational performance of emergency care. Its use in optimizing antibiotic therapy to curb the rate of antimicrobial resistance and improve sepsis outcomes still requires validation. Septic shock management in the Emergency Department continues to be a challenging task and future verification studies and clinical guidelines on biomarkers for point-of-care testing are required to establish its reliability.

Introduction

The Emergency Department (ED) is generally an environment with a high volume of patients and often prolonged waiting times which can impact patient care. There is an increasing need to enhance patient flow through the ED to improve patient outcomes and avoid overcrowding.

Point-of-Care has become increasingly significant in fast-paced settings where timely treatment is essential [1]. Using POC testing, the delay between the onset of symptoms and commencing the relevant therapy could be significantly reduced, although there is not enough evidence available to support this [2]. This could be consequential in critically ill patients, such as in sepsis, where the time of initiation of antibiotics predicts the patient's prognosis and progression to septic shock [1,3,4,5]. Sepsis is an uncontrolled systemic response to an infection resulting in multiple organ dysfunction and sometimes death [6,7]. Aiming to prescribe broad spectrum antibiotics within one hour of presentation reduces mortality according to the Surviving Sepsis Campaign guidelines and the Sepsis 6 pathway, an adaptation of these guidelines [5,8].

To classify acutely ill patients, C-reactive protein (CRP) can be used where the intensity of the pathology is directly proportional to the CRP level in the bloodstream [4,9]. POC CRP testing may expedite triage and streamline ED care [1]. Although this biochemical marker has a low specificity, it has shown to be effective in reducing inappropriate antibiotic prescribing [9]. Despite simple assay availability such as immunoturbidimetry, immunonephelometry and lateral flow tests which may be used under POC conditions, the non-quantitative CRP value and high analytical sensitivity remain an issue [4]. Gaps in verification studies and clinical guidelines currently prevent its clinical use as a POC test [1,4,9].

Diagnosctic value of CRP in Sepsis

C-reactive protein is an immunochemical marker of infection, as well as inflammatory conditions and cardiovascular events, which is predominantly synthesised by the liver [8,10,11,12]. This acute-phase protein rises within 4-6 hours of a stimulus and doubles every 8 hours with a peak at 36-50 hours [9]. This CRP surge during an infection is a response to monocytic mediators known as Interleukin-1 and Interleukin-6 (IL-6). Subsequently, the binding of CRP with phosphocholine activates the complement pathway and has a protective function against bacterial infections [4,12].

Clinical decision making may be guided by CRP levels which can be particularly useful in differentiating between bacterial and viral infections [4]. However, due to the low sensitivity and specificity of CRP testing, interpretation in conjunction with the patient's history and clinical examination is paramount [11,13,14,15]. A prompt and more specific biomarker for sepsis is procalcitonin (PCT) which rises in 3-4 hours after a stimulus [13,15,16]. PCT can predict the patient's prognosis and is valuable in analysing the response to antimicrobials. Although this biomarker can substantially improve the clinical management of sepsis, there are still limitations in reliability. The use of PCT guided antibiotic management for sepsis in adult patients may not be cost-effective in all settings [11,15,16].

Point-of-Care vs Laboratory Based Testing

POC testing is defined as medical testing at or near the site of patient care which allows for rapid result turnaround times (TAT) and therefore, quicker decision making [17,18,19,20]. This is essential in an Emergency care setting where early diagnosis and treatment predicts the outcome of the patient. Typically, the TATs for routine ED blood results processed in a centralised laboratory are estimated to be over 1 hour, whereas POC testing may take between 10-15 minutes [17]. POC methods may therefore minimise diagnostic ambiguity and result in more rational antibiotic prescribing in critically ill patients [17,18]. From patient presentation, sepsis progresses to septic shock by 8% for each hour that antimicrobials are withheld [3]. According to the Surviving Sepsis Campaign Guidelines and the Sepsis 6 pathway, broad-spectrum antibiotics initiated within 1 hour of the recognition of septic shock resulted in improved patient outcomes [8,21]. Process streamlining is vital to be able to take advantage of the expedited results from POC testing. The reduction in TATs can improve patient flow through the ED and could result in a shorter length of stay, contributing to a more efficiently functioning department, however the impact on patient safety still needs to be studied [22].

Point of Care Methods and Operational Considerations

A routine method of measuring serum or plasma CRP, performed under POC conditions and with laboratory analysers, is by immunoturbidimetry. This CRP kit is cost-efficient and simple to use, measuring the interaction between an antibody and an antigen by changing the solution turbidimetry. This assay is cost efficient and although it has a lower sensitivity compared with laboratory-based immunonephelometric assays, it still produces similar results. However, immunoturbidimetric methods require an analytical device which may incur errors on operation. Immunonephelometric assays are complex laboratory tests which are more costly than immunoturbidimetry. They require powerful light sources and therefore are less favoured in comparison [4]. Another simple POC method includes a colourimetric disposable analytical device involving easily accessible resources and minimal reagents, known as the lateral flow test [4,23]. Most lateral flow assays are only qualitative and not economical per test performed as opposed to Enzyme-linked immunosorbent assay (ELISA) and chemiluminescent immunoassay (CLIA) under laboratory conditions. Both immunoturbidimetric and lateral flow assays are typically available within 15 minutes, nevertheless these methods are not yet practical in a POC setting. Using traditional methods, the sensitivity of current ELISA test kits is between 2 and 40ng/L and the limit of detection can reach about 5ng/L. CLIA test

kits have an increased sensitivity of 5ng/L and the test range is estimated to be between 10ng/L and 10µg/L compared to ELISA. Other kits with lower sensitivities and higher detection ranges exist [4].

The development of new user-friendly biosensors and bioassays may compete with laboratory methods, such as ELISA, in terms of accuracy and sensitivity in POC conditions [4,24]. They apply improved analytical specifications for recognizing moderate risk pathologies with a typical threshold of 1mg/L. Assays with light reflectance spectroscopy involving immobilized anti-CRP antibodies had a dynamic range for CRP equivalent to 0.05-200mg/L and limit detection of 1µg/L [4]. A study by Mou et al., 2020 on the detection of infection in the ICU with a newly developed dynamic multiplex POC CLIA was performed. The multiplex included CRP, PCT and Interleukin-6 (IL-6) and showed a sensitivity of 91.7%, 94.4% and 91.6% respectively and a specificity of 63.1%, 81.2% and 66.7% respectively. The cut-off values were 5µg/ML for CRP, 0.2ng/mL for PCT and 50pg/mL for IL-6. This study demonstrates the significance of utilising biomarkers in combination for detecting infection in a clinical setting. [23]

The World Health Organisation states that the development of new POC devices need to meet the ASSURED guideline criteria, which aims for affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable test results to the end-user [19]. POC CRP testing needs further refining due to reduced sensitivity, test TAT and the non-quantitative CRP concentration as a result of its simplicity. Limited published validation studies exist on the performance of these CRP POC assays [4].

Table 1: POC vs Laboratory based simple kit testing methods.

Methods	Estimated TAT (minutes)	Benefits	Limitations
POC			
Immunoturbidimetry [4]	5-15	**AP**: Quantification of values; Similar outcomes to immunonephelometric methods **CP**: Simple to use **Other:** Cost efficient	**AP**: Requires an analytical device **CP**: User-errors
Lateral Flow [4,24,25]	20	**AP**: Easily accessible resources with minimal reagents; No analytical device needed **CP**: Simple to use	**AP**: Reduced analyte concentrations; Inability to conduct a multi-step analysis **CP**: Reduced clinical sensitivity and specificity compared to ELISA due to capillary flow rate; Only qualitative **Other**: Average cost per reagent and chemical is equivalent or more than one ELISA well plate
Biosensors and bioassays [4,25]	2-20	**AP**: Improved accuracy **CP**: Improved sensitivity; Wearable	**AP & CP**: Still in development
Laboratory-based			
ELISA [4]	90-1440	**AP**: Quantification of values **CP**: High sensitivity **Other**: Inexpensive kits	**AP**: Complex analytical process; Professional staff required **AP & CP**: Increased TAT
CLIA [4]	90-1440	**AP**: Quantification of values **CP**: Increased sensitivity compared to ELISA	**AP**: Laboratory based **AP & CP**: Increased TAT

TAT=turn-around time, POC=point-of-care, AP=analytical performance, CP=clinical performance, ELISA=enzyme-linked immunosorbent assay, CLIA=chemiluminescent immunoassay

Limitations and Special Situations

Differentiating between sepsis and non-infectious systemic inflammatory response syndromes is challenging [26]. CRP can be significantly influenced by age, body mass index, cholesterol levels, blood pressure, vigorous exercise, smoking status and heat stroke [4,9,12]. Other conditions such as surgery, burns, trauma, tissue necrosis, advanced cancer, crystal-induced arthritis and auto-immune disorders contribute to the high false-positive rate of CRP [4,9]. In immunosuppressed patients, serum CRP levels have a low specificity for diagnosing bacterial infections which may lead to delayed interventions [27,28].

Although POC testing is convenient and minimises the TAT from testing to treatment, there are shortfalls in analytical accuracy and reliability. Establishing a Quality Management System is essential as actions usually occur immediately after POC testing without verification.

Quality assurance measures and regular calibration need to be implemented to avoid inaccurate values, ensuring no harm is done to the patient. There are no evidence-based guidelines for quality control in POC testing which further limits its use [29,30,31].

A Health Technology Assessment done by the Health Information and Quality Authority (HIQA), 2019, assessed POC CRP testing in acute respiratory tract infections to guide antibiotic prescribing in primary care. The diagnostic test accuracy had mixed evidence for pneumonia and lower respiratory tract infections with a cut-off value of 100mg/L to rule in infection. This was not accurate in ruling out pneumonia using a cut-off value of 20mg/L. HIQA suggested the use of CRP POC testing in combination with clinical history and examination to potentially improve clinical judgement specificity. The analytical performance of two Conformité Européenne (CE) marked semi-quantitative devices were reviewed. This revealed that the accuracy of the tests was moderate to good but decreased after 5 minutes of the reading. In laboratory conditions, most of the CRP POC CE marked devices were accurate. Variations in accuracy and precision were found when the same tests were used in primary care suggesting the need for relevant training and strict standardised operating procedures [32]. Healthcare workers and other device operators should receive professional training in quality assurance processes to ensure result consistency [29,32,33]. Implementing regular internal quality control and utilising devices with less pre-analytical handling are associated with increased accuracy. POC CRP testing was more cost-effective than clinical judgement alone. There is limited evidence on its use in the ED [32].

POC CRP tests have an EU Regulatory requirement on In Vitro Diagnostic Devices (IVDR). Products with CE markings ensure conformity with the regulations. This provides more precise product and clinical data requirements, improved device surveillance, traceability and increased monitoring by authorities [32].

The CRP biomarker is more frequently used due to its wider availability as opposed to PCT, however this should not be used as a standalone biomarker for sepsis triage or therapeutic decision making. Biomarkers have some value in a patient's prognosis, although their independent use is still limited [26]. Utilising CRP as a tool for antibiotic therapy optimisation can be associated with a reduction in unnecessary antibiotic exposure in critically ill patients [32,34]. For more precise results, a combination of biomarkers may potentially be required for sepsis prognosis, and this would necessitate further investigation and guidelines [13,26].

Other Biomarkers in Sepsis

Biomarkers in the ED are generally favoured to develop a prompt diagnosis, formulate differential diagnoses and enhance patient care. POC tests are preferred due to time-sensitive decisions; however, there are still concerns over false-positive results, limited availability and the costs per test [35].

CRP and PCT are familiar biomarkers with diagnostic value in infection. PCT is predominantly assessed using an immunofluorecent assay [36]. It rises 4 hours after a bacterial infection which precedes that of CRP and may be more effective in the detection of early sepsis. Despite its increased specificity compared to CRP, optimal cut off levels still need to be established and its higher costs should be considered [36,37,38,39]. PCT has been more distinctive in determining an infective process over an inflammatory one as opposed to CRP and White Blood Cell counts (WBC). This can guide the correct administration of empirical antibiotics and reduce the total duration of antibiotic therapy although its specific use in the ED was unclear [36,40,42].

Other biomarkers such as IL-6, Blood Urea Nitrogen (BUN) and Presepsin have also been studied. Within only 2 hours of the onset of infection, IL-6 levels increase rapidly. It has been shown to be a more superior diagnostic and prognostic tool in early sepsis than PCT [41,42]. CRP is mostly produced in response to IL-6 and is therefore dependent on the latter. Like CRP, this biomarker is affected by inflammatory conditions such as rheumatoid arthritis and active cancer. As a sole biomarker, it may not accurately diagnose bacterial infections [43].

BUN is a liver by-product of protein metabolism which is filtered by the renal system. Elevated BUN levels are seen with renal organ dysfunction and can be an indicator of early sepsis [41].

Presepsin is a soluble form of lipopolysaccharide CD-14 which is expressed on monocytes, macrophages and dendritic cells. It rises within 2 hours of infection and is valuable in excluding sepsis. It is more sensitive than PCT and CRP in predicting disease prognosis, however there is insufficient data to support its use in the ED [37].

Over-reliance on CRP alone can lead to unnecessary antibiotic administration, especially in the first 12 hours of infection [44]. A definitive diagnosis requires multiple tests including a combination of biomarkers with blood cultures, Polymerase Chain Reaction tests and antibody detection assays where applicable [36,40,44].

Table 2: Biomarker diagnostic performance in bacterial infections.

Study	Year	Biomarker	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	AUC (95% CI)
Ramakrishnan V et al. [40]	2025	CRP PCT WBC	92.86 69.23 92	7.5 57.14 62.79	80.06 88.04 93.34	20.792 28.96 58.07	42.65 61.76 73.5	0.517 0.5 0.631
Nargis W et al. [36]	2014	CRP PCT	85.45 76.36	33.3 72.2	79.66 89.36	42.86 50	72.6 75.34	- -
Harsoor V et al. [39]	2024	CRP PCT	78.9 85.6	71.2 81.3	83.4 90.2	63.5 75.5	- -	0.80 0.89
Yu B et al. [41]	2022	IL-6 BUN	68.3 48.9	82.8 86.4	- -	- -	- -	0.764 0.696
Bruhn R et al. [43]	2025	CRP PCT IL-6	68.1 72 75.3	78.5 67.5 67.5	93.4 91.1 92.4	35.5 34.2 38.3	- - -	0.79 0.76 0.77
Orfanos I et al. [45]	2024	CRP ≥20 mg/L IL-6 ≥50 ng/L	76 93	89 66	55 33	95 98	- -	0.87 0.82

PPV=positive predictive value, NPV=negative predictive value, AUC=area under the curve, CI=confidence interval, CRP=c-reactive protein, PCT=procalcitonin, WBC=white blood cells, IL-6=interleukin 6, BUN=blood urea nitrogen

Clinical Impact and Outcomes

Delays in identifying severe sepsis and septic shock is related to an increased rate of morbidity and mortality. During triage in the ED, a patient with sepsis may initially present as normotensive and with non-specific symptoms and so sepsis could be missed in the first instance [34]. Multiplex biomarker POC testing may more rapidly identify or confirm sepsis prior to receiving laboratory-based results [1,23,34]. This could improve patient outcomes although there are limited POC guidelines to support the administration of antibiotic therapy in the ED [1,34]. Following appropriate POC training and process optimization, streamlined patient care can be implemented in the ED, and therefore a potentially reduced length of stay although limited data is available on this [22, 46].

Laboratory-based testing has an increased TAT, require more staff and costly equipment, yet remains the gold standard for accurate results. Even though POC biomarkers have a high initial cost, they offer bedside testing with reduced TAT and allow for rapid clinical decision making. This may outweigh traditional methods by improving patient waiting times, minimising the duration of hospital stay and overall, more cost effective [47].

According to a study group from the European Society of Clinical Microbiology and Infectious Diseases, antimicrobial stewardship (AMS) guidance tailored for the ED is lacking [48]. The aim of AMS is to responsibly prescribe antimicrobials to enhance patient outcomes, therefore minimising antimicrobial resistance due to its improper use. A considerable number of patients initiated on antibiotics in a hospital setting are seen through the ED which are often resumed as an inpatient [34,48,49]. Biomarker-guided antibiotic therapy has the potential to reduce the overprescription of antimicrobials to patients [34,48,49,50]. Therefore, POC CRP-guided treatment may form part of AMS in the ED to help minimize the threat of antimicrobial resistance particularly in lower respiratory tract infections, although there is minimal evidence available to support this [48,49,51].

Conclusion

CRP is a non-specific biomarker which has its own pitfalls and should be used in conjunction with the clinical history and examination of the ED patient instead of an investigation in isolation. CRP has a low specificity for sepsis compared to non-infectious inflammatory conditions and identification of early sepsis is restricted. Other biomarkers such as PCT or a multiplex of biomarkers may be more favourable in this regard. While limited developments, guidelines and mixed evidence are available for POC CRP testing in sepsis management, this method offers the potential to enhance the operational performance of the ED rather than improve diagnostic precision.

Declaration of Conflict of Interests

I, Natasha Gomes Berlouis, declare that I have no conflicts of interest related to this mini-review. I have not received financial support or other benefits from organizations that may influence the interpretation or conclusions of this mini-review.

Ethical Approvals

No human subjects were involved in this mini-review. All data discussed is obtained from and available in the published literature and studies cited in the references.

CRediT Author Statements

Natasha Gomes Berlouis: Conceptualization, Methodology, Investigation, Resources, Writing-Original Draft, Writing-Review & Editing, Visualization, Project Administration.

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This article does not report new data. All data, summary tables and figures discussed are available in the published literature and studies cited in the references.

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Research Article

Clinicians' Perspectives on the Impact of a Ransomware Attack on a Chemical Pathology Laboratory at a Tertiary Hospital in South Africa

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Keywords

Ransomware, cyberattack, patient safety

Abstract

Objectives: This study aimed to explore clinicians' experiences during a ransomware attack at a public academic hospital in South Africa and assess the perceived impact of chemical pathology laboratory service disruptions on patient care.

Methods: A cross-sectional survey was conducted between September and December 2024. An electronic questionnaire was distributed to clinicians to gather data on their experiences during the ransomware attack, including impacts on patient care and workload. To assess changes in test requesting practices during this period, volume data for both critical (creatinine) and non-critical (vitamin B12) tests from routine annual laboratory reports were analysed.

Results: Among the 58 respondents, 84% reported increased stress levels, while 78% indicated delayed diagnoses during this period. Laboratory test volumes decreased during the attack period compared to previous years, with reductions of 26.8% for creatinine and 34.1% for vitamin B12 tests. Clinicians primarily struggled with result retrieval and reported substantial disruptions to patient care.

Conclusions: This study provides valuable insights into clinicians' perspectives on the impact of a laboratory ransomware attack. The findings highlight the critical need for investment in both cybersecurity infrastructure and comprehensive contingency planning to safeguard patient safety and minimise disruptions during future cyber incidents.

Key Points: This study addresses how ransomware attacks disrupt and impact clinician workflow in resource-limited hospital settings. Medical professionals should develop practical contingency plans for accessing and managing essential laboratory data during cybersecurity incidents to minimise care disruptions. The most significant finding was the dual impact of technical service disruption alongside pronounced clinician psychological stress, creating a compounded effect on healthcare delivery.

Introduction

Health Information Systems (HIS) are both crucial to modern healthcare delivery and increasingly vulnerable to cyber threats. These systems facilitate the easy storage, retrieval, and generation of high-quality information. Health information technology employs computer hardware and software to create these systems, which are essential for sharing healthcare data and supporting informed decision-making [1]. Given the sensitive nature of the data collected by HIS, cybersecurity is a critical consideration. Ensuring robust cybersecurity measures is essential for safe and effective health technology use and maintaining patient safety in the current digital era. Failing to implement adequate cybersecurity measures can leave an institution vulnerable to ransomware attacks. These attacks involve malicious software that renders electronic systems unusable until a ransom is paid, effectively holding the institution hostage [2]. The consequences of such attacks extend far beyond financial concerns.

Cyberattacks on healthcare are not only costly but negatively affect patient care. Additionally, laboratories and healthcare facilities are often unprepared and lack robust contingency protocols for prolonged downtime periods. Multiple authors have attempted to describe the effects of prolonged downtime on the laboratory and stakeholders, including patients [3, 4]. Patient care relies on laboratory data, with up to 70% of clinical decisions regarding diagnosis, treatment, or prevention being made based on this information [5]. Therefore, disruptions in laboratory services, such as those caused by a ransomware attack, can severely impact patient care and compromise patient safety. The National Health Laboratory Service (NHLS) in South Africa, the backbone of the nation's diagnostic infrastructure, delivers more than 80% of the country's pathology services [6]. On June 22, 2024, the NHLS suffered a ransomware attack, which resulted in the inability to utilise information technology (IT)-related systems, including the laboratory information system (LIS) (InterSystems TrakCare® Lab Enterprise, Cambridge, Massachusetts, United States). Consequently, many automated processes reverted to manual operations, while new and historic patient laboratory results were inaccessible. This change led to delays in the turnaround time for patient results and made it challenging for clinicians to access historic results and view new results timeously [7]. There is currently a critical paucity of evidence examining clinicians' perceptions on the impact of laboratory downtime due to ransomware attacks on patient care, particularly in resource-limited settings.

This study aimed to investigate clinicians' perspectives at Tygerberg Hospital (TBH), a public tertiary academic hospital in Cape Town, South Africa, regarding the ransomware attack and to evaluate the perceived consequences of chemical pathology laboratory service disruptions on clinical decision-making and patient management. The findings provide critical insights for developing effective contingency protocols in resource-constrained healthcare environments.

Methods

Ethical Considerations

The study was approved by the Human Research Ethics Committee of Stellenbosch University (N24/08/095) in September 2024. Respondents were provided with detailed information regarding the study purpose, procedures, and voluntary nature of participation before being included in the study, and their completion and submission of the survey was taken as implied consent. All data were collected anonymously to maintain confidentiality.

Study Design

This study used a cross-sectional survey design to evaluate the impact of the June 2024 laboratory ransomware attack on clinicians' workflows, stress levels, and perceptions of laboratory performance at TBH. The survey was distributed electronically across all clinical departments, targeting clinicians of all ranks and specialities who were actively engaged in patient care during the downtime period. Data were collected and managed using Research Electronic Data Capture (REDCap) electronic data capture tools hosted at Stellenbosch University. Access to the database required two-factor authentication and was restricted to study authors.

Study Setting and Population

This study was conducted at Tygerberg Hospital (TBH), a 1384-bed state-owned teaching hospital affiliated with Stellenbosch University. TBH provides healthcare services to approximately 3.6 million people, with its onsite NHLS facility processing an average of 125,000 chemical pathology tests monthly. As a public healthcare institution, TBH predominantly serves communities with limited resources in the Cape Town Metro East sub-districts while also receiving referrals from rural catchment areas throughout the Western Cape province.

The survey targeted clinicians across all levels of training (from medical interns to specialist consultants) employed at TBH who were directly responsible for patient management decisions during the June – July 2024 ransomware attack. This period saw significant disruption to the hospital's normally robust laboratory information systems and services.

Laboratory Response to Downtime

The ransomware attack on the laboratory IT systems necessitated rapid operational adjustments to maintain essential service delivery. A key measure involved prioritising critical tests while temporarily suspending specialised testing. The laboratory published a prioritised test list to guide clinicians and ensure efficient resource allocation during this period.

With electronic systems compromised, alternative result retrieval methods were implemented. Clinicians either contacted the laboratory directly or physically collected paper printouts from analysers. To improve accessibility, these physical result copies were systematically organised by patient ward location.

Three weeks into the incident, limited digital access was restored through Single Patient Viewer (SPV), an alternative electronic platform maintained by the Western Cape Department of Health (DoH). This web-based portal, which functions as an integrated electronic health record system and retains historical data, became a crucial interim solution. Through collaboration between the NHLS and the DoH, select laboratory results were systematically uploaded to SPV, partially restoring clinicians' ability to access patient results electronically.

However, significant limitations persisted. Manual and semi-automated chemistry test result were not integrated into the SPV platform, and technical issues occasionally prevented successful result uploads, creating gaps in the available electronic data that continued to challenge clinical decision-making.

Data Collection Methods

The survey (Supplementary Table 1) was collaboratively developed to evaluate key aspects of clinicians' downtime experience following a ransomware attack affecting the NHLS. The survey collected demographic information, including years of clinical experience and primary clinical setting of the respondents. Participants provided feedback on the impact of the downtime on patient care, including delays in diagnosis, treatment decisions, investigations, patient referrals, surgical scheduling, and hospital discharge. The survey also assessed effects on clinicians themselves, such as increased workload and stress levels. Additionally, respondents evaluated the laboratory's preparedness, communication effectiveness, and result accessibility during the downtime. A five-point Likert scale was used for closed-ended questions, while open-ended questions allowed for additional feedback and recommendations.

Survey development followed a rigorous process. Initial drafts were refined through team discussions. To ensure clarity, the survey underwent pre-testing among laboratory staff to assess question coherence, platform usability, and branching logic. Feedback was used to refine wording, structure, and resolve technical issues. Pre-testing responses were excluded from the final dataset.

Distribution employed a multi-faceted approach to maximise clinician engagement. Following ethics approval, the electronic survey was distributed until 31 December 2024, with fortnightly reminders to enhance response rates. Quick response (QR) codes linking directly to the survey were included on posters placed in hospital wards and outpatient departments. Additional reminders included direct messages to team representatives and authors physically visiting clinical areas. Despite these comprehensive efforts, it remained challenging to determine precisely how many clinicians encountered or interacted with the survey.

To complement survey findings, routine laboratory statistics were analysed to provide insights into test volume reductions during the downtime period. Creatinine and vitamin B12 were purposively selected as sentinel analytes to represent contrasting clinical priorities: creatinine as a high-volume, clinically critical test included on the laboratory's prioritised test list, and vitamin B12 as a non-urgent, non-priority test. Numbers for both tests were compared for the month of July across years 2021 to 2023 to account for seasonal variation and establish a pre-attack baseline against which the ransomware period (July 2024) could be interpreted.

Statistical Analysis

Data collected from the survey were analysed using both descriptive and inferential statistical methods to explore the impact of laboratory downtime on clinicians. Categorical variables, including demographics and responses to Likert-scale questions, were summarised as frequencies and percentages. Likert-scale data were further analysed to calculate agreement levels for key metrics, such as stress levels, diagnostic delays, and perceptions of laboratory performance. Survey data were accessed from REDCap, then underwent systematic extraction, cleaning, and analysis. Routine testing volume data for July across four consecutive years (2021, 2022, 2023, and 2024) were compared using analysis of variance (ANOVA) to assess significant differences between years. All statistical analyses were conducted using R version 4.3.1, with specific analytical and visualisation functions from the dplyr, ggpubr, and ggplot2 packages.

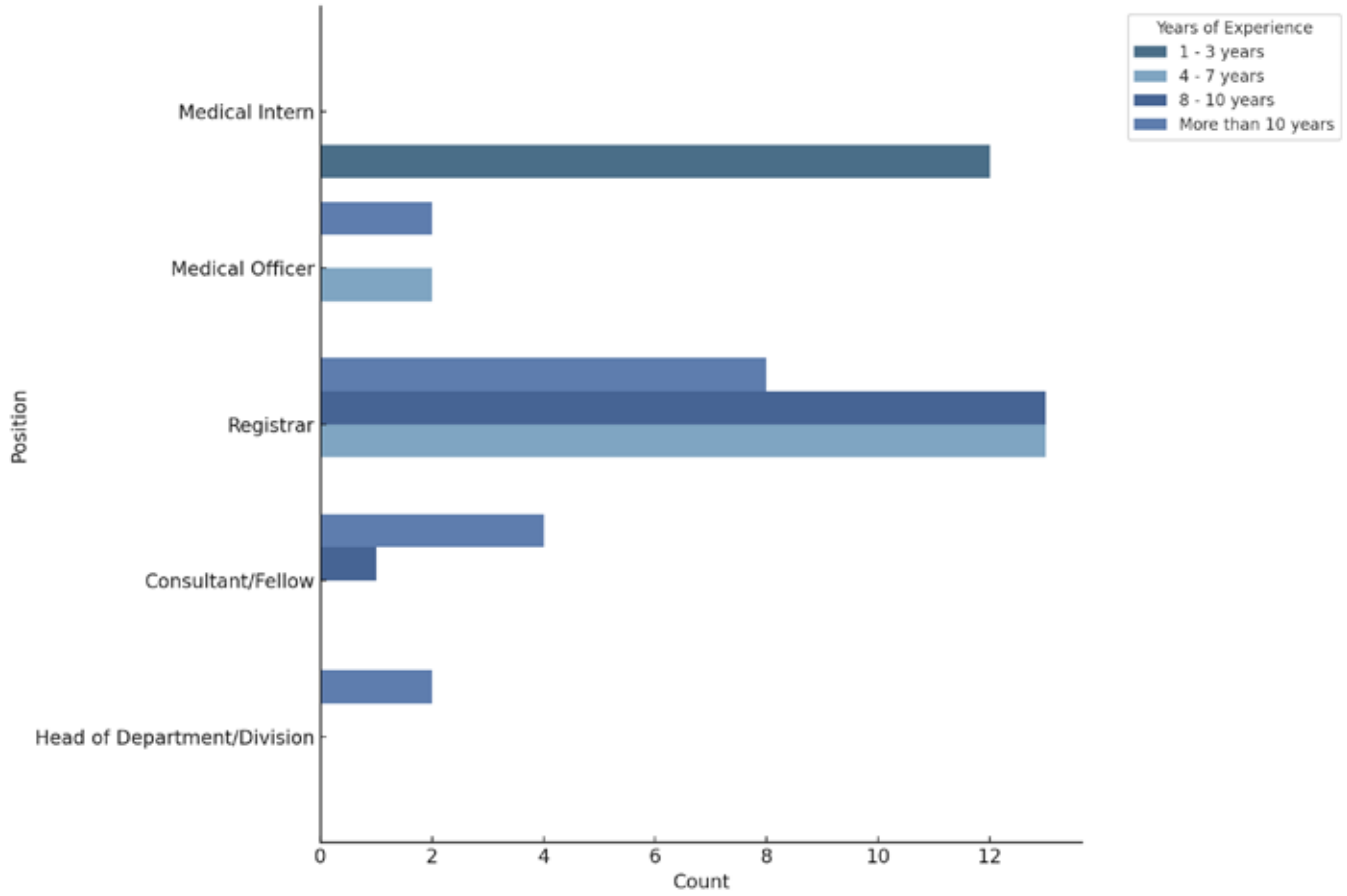
Results

A total of 63 responses were received, of which 58 were complete and included in the analysis. Five responses (7.94%) were excluded: one from a clinician who did not work at TBH and four because they were incomplete.

Demographics

Figure 1 depicts the demographics of the study respondents. Of the 58 clinicians who responded, most were registrars (59%, n=34), with a smaller representation of consultants and fellows (9%, n=5). Most participants reported having extensive clinical experience, with 28% (n=16) indicating more than 10 years, 24% (n=14) reporting 8–10 years, and 26% (n=15) reporting 4–7 years. The clinical settings of the respondents varied, with the majority working in inpatient care (40%, n=23) or a combination of in- and outpatient care (59%, n=34).

Figure 1: Distribution of years of experience among the 58 healthcare professional respondents by rank at Tygerberg Hospital, September 2024 to December 2024.



Summary of Laboratory Response to Downtime

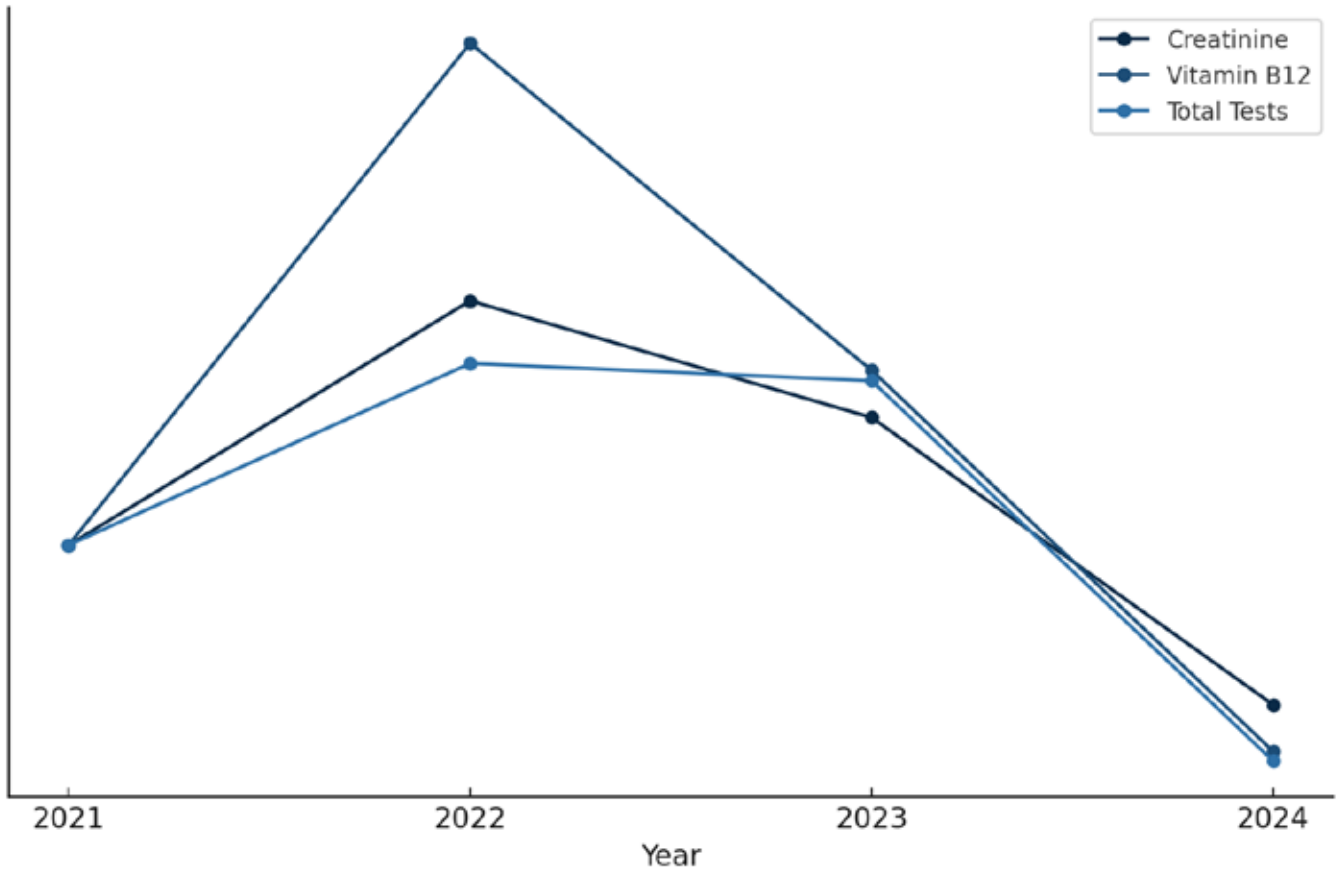
Clinicians acknowledged the efforts of the Chemical Pathology laboratory staff during the downtime, with 74% (n=43) agreeing that the staff were helpful and made efforts to assist clinicians. Moreover, 67% (n=39) of respondents believed the laboratory attempted to reduce delays in turnaround times, demonstrating a proactive approach to managing the situation. While 52% (n=30) agreed that communication of critical results was timely, 48% (n=28) reported issues with missing results, and 39% (n=23) noted difficulties interpreting results due to the lack of reference intervals, automatic calculations, or interpretive comments. Additionally, only 43% (n=25) of respondents felt the laboratory was adequately prepared for the unexpected downtime.

Clinicians' Use of the Critical Test List

While the majority (68.97%, n=40) reported scaling down testing using the published critical test list, 22.41% (n=13) indicated they were unaware of its availability, and 8.62% (n=5) reported not scaling down testing at all.

Test volumes for creatinine, a test included on the critical test list, showed a 26.8% reduction in July 2024 compared to previous years, while vitamin B12, a test not on the priority list, experienced a sharper decline at 34.1% (Figure 2). Total test volumes dropped by 34.3% overall.

Figure 2: Normalised test volumes for creatinine, vitamin B12, and total laboratory tests at Tygerberg Hospital during July months from 2021 – 2024, including the ransomware attack period of July 2024.



Clinician Response to Alternative Result Retrieval Methods

Collecting physical copies of results directly from the laboratory emerged as the most frequently used method, reported by 55% (n=32) of respondents. Additionally, 22% (n=13) relied on calling the laboratory for urgent results. Many clinicians noted that the ward-specific result filing system implemented during the downtime was inefficient, with 47% (n=27) disagreeing or strongly disagreeing that it facilitated timely access to results. On the other hand, direct communication with laboratory staff was highlighted as a helpful approach, with 71% (n=41) agreeing that laboratory personnel made efforts to assist during the downtime.

Operational Challenges

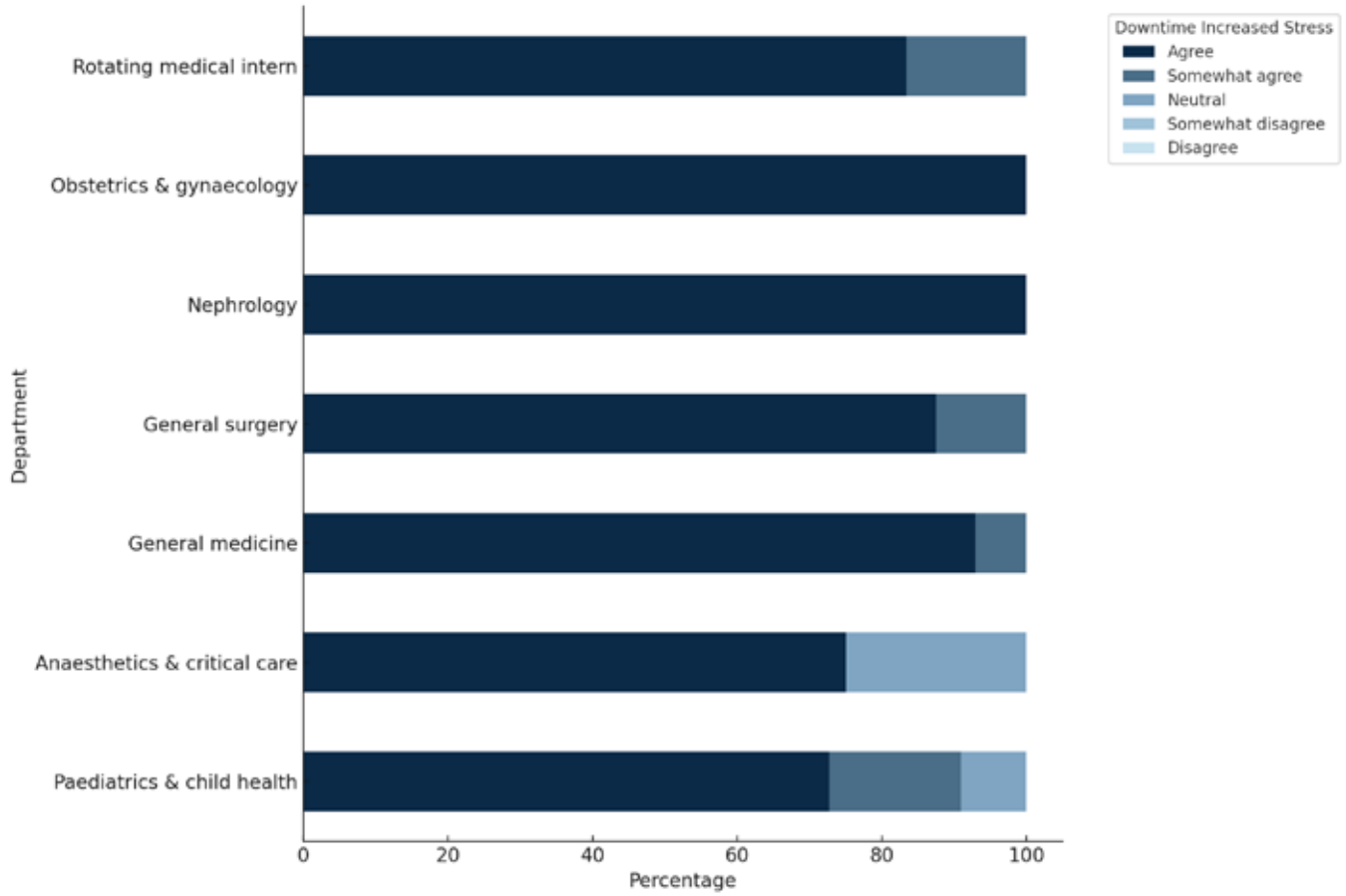
Delays in obtaining laboratory results were widely reported, affecting diagnostic timelines (78% agreed, n=45) and decisions regarding initiating or discontinuing patient treatment (73%, n=42). The nephrology department reported the highest percentage of delayed diagnoses (100% agreed), with general surgery and medical interns reporting similarly high levels (87.5% and 83.3% agreed, respectively).

Furthermore, 69% (n=40) of respondents indicated that the downtime impacted the scheduling and availability of special investigations, such as computed tomography scans, and 65% (n=38) reported delays in interdepartmental patient referrals. Surgical schedules were also disrupted, with 62% (n=36) noting delays in emergency surgery scheduling and 58% (n=34) reporting challenges with elective surgeries. Clinicians from Anaesthetics and Critical Care reported the highest rate of delays in emergency surgeries (100% agreed). Prolonged patient discharge and increased length of stay were significant issues for 60% (n=35) of respondents, reflecting the ripple effect of delayed laboratory results on hospital efficiency. The unavailability of specialised testing during downtime further complicated patient management for 68% (n=39) of clinicians.

Impact on Clinician Wellbeing

Most respondents (84%, n=49) reported that the downtime increased their stress levels, with 42% (n=24) strongly agreeing. Additionally, 71% (n=41) indicated that the downtime led to extended working hours. Stress levels were highest among the nephrology and obstetrics and gynaecology teams (100%), followed by general medicine (92%) and general surgery (87.5%) (Figure 3). Notably, none of the respondents disagreed with the statement that the downtime increased their stress levels.

Figure 3: Distribution of stress-related responses among healthcare professionals by department at Tygerberg Hospital, September 2024 to December 2024. Responses are presented as percentages on a five-point Likert scale.



Discussion

The study findings highlight the significant impact of the ransomware-induced downtime on clinical workflows, laboratory services, and clinician well-being. While the majority of clinicians acknowledged the efforts of the chemical pathology laboratory staff, many reported challenges with communication, missing results, and difficulties interpreting laboratory results.

In the chemical pathology laboratory, analyser-printed results were made available to clinicians once laboratory staff authorised them. However, these results lacked interpretive comments, reference intervals, and automated calculations. The impact of this was seen in the 39% (n=23) of respondents who noted challenges in report interpretation. This could have influenced the perception of more than half of the respondents (57%, n=33), who perceived the laboratory as unprepared for the unexpected downtime. In 2021, Duffy et al. described producing paper-based reports during downtime in Ireland following a cyberattack on their information technology infrastructure, where they similarly lost access to laboratory systems. They described a high level of scientific input and manual processes required for these reports [8]. Given our already resource-limited setting, these reports were not developed and would likely have increased the burden on laboratory staff [7]. As noted, the analyser-printed reports provided to clinicians lacked reference intervals. To address this, we provided reference intervals to hospital management for staff dissemination. However, it is unclear if the documentation was distributed to all clinicians, given the difficulty experienced by some respondents. This highlights the importance of establishing clear protocols with input from clinicians and management to ensure effective communication and dissemination of information between laboratory and clinical staff.

The European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) Task Force Preparation of Labs for Emergencies recommends the use of telephonic communication for critical laboratory results during downtime; however, they reported that there is still uncertainty regarding the preferred method of delivery of other test results [9]. Clinicians found direct communication with laboratory staff to be the most helpful approach to obtaining laboratory results compared to the other available methods described. Nearly half of the respondents found the box sorting system unhelpful, likely due to incomplete or illegible laboratory request forms that impeded accurate result sorting. As described by Cassim and Chapanduka, the distribution of results took the longest time during the NHLS ransomware attack; this likely greatly

impacted service delivery, especially before the introduction of SPV[7].

Stowman et al. described the reporting practices for anatomical pathology results during a systemwide cyberattack in the United States in 2020, which disrupted their LIS. They adopted a manual process where reports were typed by support staff, hand-signed by pathologists, and faxed to clients. They noted barriers to using fax included the lack of provider contact details or illegible information on laboratory request forms, an aspect also experienced by our laboratory staff when attempting to disseminate results. Additionally, digital faxes were rendered unusable, and only analogue faxes worked [10]. They further noted the challenges of managing phone calls about missing specimens or reports, which mirrored our laboratory's experience during the ransomware attack. This shows that even in centres with enhanced infrastructure for result dissemination, the distribution of results remains challenging in the absence of an LIS. Given the limited resources available during downtime, it was challenging to effectively distribute results telephonically. Goodwin et al. reported on a cyberattack which occurred in the United States in October 2020. They noted that results were also distributed by hospital runners who collected hard copies from a designated passage while delivering patient specimens to the laboratory [4]. Similarly, we implemented a practice where hard copies were placed in boxes organised by test request location for collection. Inefficiencies in this system may arise from missing ward location information or discrepancies between a patient's current admission location and their initial contact point, such as the emergency department, labour ward, outpatient clinic or an external referral hospital. One respondent stated that "the lab staff tried their best to try and help us as far as possible with new innovative ways to provide us with the results as timeously as possible. I do, however, believe that more should be done to have backup systems in place should something like this happen in future." This highlights the importance of establishing clear protocols with the involvement of clinicians and management to ensure that the systems developed ensure clinical utility and patient safety.

ISO 15189 is an international standard outlining requirements for quality and competence in medical laboratories. The updated ISO 15189: 2022 emphasises risk-based approaches and patient-centred practices while promoting continuous improvement within clinical laboratories[11]. Additionally, it expands accreditation requirements for information systems, mandating that they be "implemented with cybersecurity considerations to prevent unauthorised access and protect data from tampering or loss" [12]. As described by Cassim and Chapanduka, the NHLS had limited resources to deal with the incident and required a longer time to develop contingency plans for result dissemination [7]. As a result of the increased reliance on manual processes, the laboratory could not maintain the same test turnaround times (TAT) as before the ransomware incident, which invariably increased. The perceived impact of this by clinicians included delays to elective and emergency surgery and to patient discharge. Ghafur et al. performed a retrospective analysis of the 2017 global ransomware attack, WannaCry, and its impact on the National Health Service in England. They reported 9% fewer elective admissions during this time and 13,500 cancelled outpatient appointments across the affected facilities. They further estimated a total economic loss of £5.9 million due to the cyberattack resulting from the loss of hospital activity during this time [13]. To streamline operations and alleviate pressure from increased manual processes, NHLS expert committees compiled and disseminated essential test lists (7). The survey responses indicate that clinicians reduced non-priority testing during the period of the ransomware attack. The use of two contrasting sentinel analytes in this study was intended to provide a pragmatic illustration of prioritisation behaviour during downtime rather than a comprehensive assessment of laboratory utilisation patterns. The decline in creatinine testing compared to previous years highlights the decreased utilisation of laboratory services during this time. Additional research on the financial impact of the ransomware attack we experienced would be valuable for understanding its broader consequences and creating strategies to prevent future incidents. By examining these impacts, we can develop policies that reinforce operational stability, ensuring that patient outcomes remain a priority, even in unforeseen and challenging circumstances.

Seventy-three percent of respondents reported that the ransomware attack impacted decisions around initiating or discontinuing patient treatment. This highlights the disruption to clinical decision-making and patient care, with one respondent noting that "It was incredibly difficult to treat critically ill patients timeously". Flavin et al. highlighted the effects of a national cyberattack and its impact on radiation therapy services in the Republic of Ireland in 2021, noting that 513 patients nationwide experienced interruptions in their treatment [14]. This illustrates the impact that cyberattacks can have on critical healthcare services. Unfortunately, no responses were received from oncology services in our study, preventing the review of the perceived impact on their operations. A study by Neprash et al reviewed ransomware attacks on healthcare delivery organisations in the United States from 2016 to 2021. Of the 374 attacks during this period, 166 (44.4%) resulted in disrupted care delivery, with 38 (10.2%) specifically leading to delays or cancellations of scheduled care [15]. These findings further highlight the widespread and multifaceted challenges ransomware attacks pose to healthcare systems.

The ransomware attack caused disruptions by increasing TAT and the unavailability of results on electronic web portals forced clinicians to physically collect hard copies of laboratory reports if the laboratory was unavailable to relay the result telephonically due to staff constraints. This additional burden significantly increased stress levels among clinicians already facing extended working hours. In South Africa, burnout

among healthcare professionals is a well-documented issue. A study in 2010 by Rossouw et al. assessed burnout and depression among medical doctors in healthcare facilities in the Western Cape, South Africa. Of the 132 doctors surveyed, 76% experienced burnout. Long working hours were identified as a major contributing factor, emphasising how the ransomware attack potentially exacerbated an already critical issue in the South African healthcare sector [16]. Although our study did not directly measure burnout, increased stress levels and longer working hours were found, which are important factors that contribute to clinician burnout.

A key contribution of this study is its focus on clinicians' experiences during a laboratory ransomware incident in a resource-limited setting of Sub-Saharan Africa. While several studies discuss healthcare cyberattacks and laboratory responses, few have examined the unique challenges clinicians face during such incidents. This perspective enhances our understanding of the impact on patient care in contexts where backup systems and resources may be limited.

Several limitations of this study should be acknowledged. The exact response rate could not be determined due to the electronic distribution strategy, which made it difficult to establish how many clinicians were reached or viewed the survey. In addition, response rates were lower among medical interns, a group heavily involved in result retrieval during the downtime period, potentially resulting in an incomplete representation of certain operational challenges. The overall sample size was relatively small, which may limit the generalisability of the findings, although the responses obtained nonetheless provide valuable and diverse insights from clinicians working in this setting.

From a laboratory perspective, test volume analysis was limited to two analytes and was not intended to reflect overall laboratory utilisation. This approach was chosen to provide a focused illustration of prioritised versus non-prioritised test requesting behaviour during the ransomware-related downtime. Finally, the study did not include objective patient safety outcomes, an important area for future investigation to better quantify the downstream clinical impact of such incidents.

Conclusion

This study provides valuable insights into clinicians' perceptions of the impact of a laboratory ransomware attack, highlighting the critical role of information systems in healthcare and their particular vulnerabilities in resource-limited settings. As one respondent aptly observed, "This was a terrible time in NHLS history, and it showed how much we rely on PC systems." The findings emphasise the urgent need for investment in both robust cybersecurity infrastructure and comprehensive contingency planning to ensure patient safety and minimise disruptions during future incidents. This imperative is especially critical considering global trends that show increasing frequency and sophistication of ransomware attacks specifically targeting healthcare services. Healthcare institutions must prioritise developing resilient systems that can maintain essential laboratory services even when primary information systems are compromised, particularly in settings where resources for rapid response may be constrained.

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CRediT Author statements

All authors contributed to the conceptualisation, methodology, writing and review of the manuscript. AF performed the formal statistical analyses and generated the visualisations.

Data availability

Upon reasonable request, the data from the present study is available from the corresponding author.

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None.

Conflicts of interest

None declared by the authors.

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Supplementary file

Supplementary Table 1: Participant Information Leaflet and Consent Form.

Title of Research Project **"Clinician Experience on The Impact of a Ransomware Attack on a Chemical Pathology Laboratory in South Africa"**	
Details of Principal Investigator (PI) / Researcher(s)	
Title, first name, surname Dr Ameerah Davids Dr Aaqilah Fataar	**Ethics reference number** N24/08/095
Full postal address Division of Chemical Pathology, 9th Floor Tygerberg Hospital, Francie van Zijl Drive, Parow, Cape Town	**PI Contact number** 082 585 8656; 082 568 6098

We would like to invite you to take part in this research project. We are a group of researchers from the Division of Chemical Pathology at Stellenbosch University, including consultants and registrars.

Please take some time to read the information presented here, which will explain the aims of this project. It is especially important that you are completely satisfied and clearly understand what this research entails and what your involvement entails. Also, your participation is **entirely voluntary**, and you are free to decline to participate.

The Health Research Ethics Committee at Stellenbosch University has approved this study [study number to be added]. The study will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki (2013), the South African Guidelines for Good Clinical Practice (2006), the Medical Research Council (MRC) Ethical Guidelines for Research (2002), and the Department of Health Ethics in Health Research: Principles, Processes and Studies (2015).

What is this research study all about?

Ransomware is defined as a "type of malware attack in which the attacker locks and encrypts the victim's data, important files; this may be followed by demands of payment to unlock and decrypt the stolen data". Ransomware attacks in the healthcare sector, including clinical laboratories, seem to be on the increase. It is crucial to understand the impact of these attacks on clinical services by getting feedback directly from clinicians.

This study aims to get feedback on the experience of clinical staff on the recent Information System (IT) downtime at the National Health Laboratory Service (NHLS) due to a ransomware attack. We seek to understand how this impacted the clinicians in performing their daily duties.

Why are you invited to participate?

You are invited to participate because you are a doctor working at Tygerberg Hospital.

Will you benefit from taking part in this research?

You will not directly benefit from this study; however, the findings may be used to design and amend current operating procedures for unplanned future downtime at Tygerberg laboratories.

Are there any risks involved in your taking part in this research?
There is no risk associated with this study.

Are there any costs involved if I decide to participate/take part?
There is no cost to you for participating in this project.

You can contact the Principal Investigators of this study, Dr Ameerah Davids or Dr Aaqilah Fataar on the phone numbers above or via email at adavids@sun.ac.za or aaqilah.fataar@nhls.ac.za if you have any questions about this study or encounter any problems.
You can phone the Health Research Ethics Committee at 021 938 9677/9819 if there still is something that concerns you about how this study is being conducted, or if you have a complaint.
You can download a copy of this information and consent form for you to keep safe.

Declaration by participant
By electronically signing below, I agree to take part in a research study entitled **Clinicians' Perspectives on the Impact of a Ransomware Attack on the Chemical Pathology Laboratory at a Tertiary Hospital in South Africa**

I declare that:
I have read this information and consent form, and it is written in a language in which I am fluent and with which I am comfortable.
I understand that taking part in this study is voluntary, and I have not been pressurised to take part.
I may choose to withdraw from the study by discontinuing to complete this electronic form.
Once I have electronically signed and submitted this form, I will not be able to withdraw from the study
My personal identifying information is not included in this study and therefore my responses cannot be linked back to me.
By clicking SUBMIT you are confirming that you are over 18 years old and have read and understood the above explanation about the study, and that you agree to participate. You also understand that your participation in this study is strictly voluntary.

Survey
Demographic questions

Are you currently employed at Tygerberg Hospital?
- Yes
- No

Were you employed at Tygerberg Hospital after the 1st of April 2024?
- Yes
- No

What is your current job title?
- Medical Intern
- Medical Officer
- Registrar
- Consultant/Fellow
- Head of Department

How many years of clinical experience do you have?
- 0-3 years
- 4-7 years
- 8-10 years
- More than 10 years

In which clinical setting do you predominantly work?
- Inpatient care
- Outpatient care
- Inpatient and outpatient care

In which division do you work?
- Medical intern rotating through multiple disciplines
- Internal Medicine
- Surgery
- Obstetrics and gynaecology
- Orthopaedics
- Anaesthetics
- Psychiatry
- Family medicine
- Emergency medicine
- Trauma
- Nephrology
- Haematology
- Oncology
- Other
- Paediatric
- Critical Care (ICU)

Did you scale down lab testing using the critical test list that was made available?
- Yes
- No

What was the most effective way that you obtained patient results?
- Collecting physical copies
- Single patient viewer
- Calling for results
- Other (please state)

Inpatient and outpatient questions/Inpatient only questions

	Agree	Somewhat Agree	Neutral	Somewhat disagree	Disagree
Downtime resulted in delay to diagnosis					
Downtime affected patient treatment decisions (initiation/ discontinuation)					
Downtime affected special investigations (e.g. CT scan/ imaging)					
Downtime affected patient referral					
Downtime affected elective surgery time					
Downtime affected emergency surgery time					
Downtime resulted in more frequent patient testing/ sampling					
Downtime delayed patient discharge (length of stay)					
Downtime led to longer working hours					
Downtime increased my level of stress at work					
The lack of specialised testing affected patient testing					

Outpatients only

	Agree	Somewhat Agree	Neutral	Somewhat disagree	Disagree
Downtime resulted in delay to diagnosis					
Downtime affected patient treatment decisions (initiation/ discontinuation)					
Downtime affected special investigations (e.g. CT scan/imaging)					
Downtime affected patient referral					
Downtime affected elective surgery time					
Downtime resulted in more frequent patient testing/sampling					
Downtime led to longer working hours					
Downtime increased my level of stress at work					
The lack of specialised testing affected patient testing					
Downtime led to longer patient waiting times					

Lab experience

	Agree	Somewhat Agree	Neutral	Somewhat disagree	Disagree
The Chemical Pathology laboratory was helpful					
The Chemical Pathology laboratory was prepared for the unexpected downtime					
The Chemical Pathology laboratory communicated critical results timeously					
Communication from the Chemical Pathology laboratory was effective					
The ward box system was effective for obtaining results					
The Chemical Pathology laboratory attempted to mitigate delayed turnaround times					
Requested Chemical Pathology results were often lost					
Chemical Pathology laboratory results issued during downtime were user friendly					
Lack of auto calculations (e.g. eGFR, urine protein creatinine ratio, transferrin saturation etc.) made result interpretation more challenging					
Lack of reference intervals made result interpretation more challenging					
Lack of interpretive comments made result interpretation more challenging					
The Chemical Pathology patient results were trustworthy during downtime					

Research Article

Correlation of D-dimer Measurement Values Using Quantum Dots Fluorescence Immunochromatographic Assay and Latex-Enhanced Immunoturbidimetric Assay

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Keywords

D-dimer, fluorescence immunochromatography, latex-enhanced immunoturbidimetric assay

Abstract

Introduction: D-dimer, a fibrin degradation product, is widely used in the diagnosis of thrombotic disorders and in clinical decision-making. Quantum dots (QDs) fluorescence immunochromatography has emerged as a rapid method for detecting protein biomarkers; however, comparative data with the established latex-enhanced immunoturbidimetric assay remain limited.

Methods: A laboratory-based method comparison study was conducted using 80 consecutively collected plasma samples from patients at Dr. Soetomo General Academic Hospital, Surabaya. D-dimer levels were measured using a QDs fluorescence immunochromatography assay (QD-S2000, Vazyme, China) and a latex-enhanced immunoturbidimetric assay (Sysmex CS-2500, Sysmex Corporation, Japan). Correlation and agreement between methods were assessed using Spearman correlation analysis and Bland–Altman plots.

Results: Among the 80 samples (60% female, 40% male), a very strong correlation was observed between the two methods (Spearman $r = 0.951$, $p < 0.001$). Bland–Altman analysis demonstrated good agreement, with most data points falling within the 95% limits of agreement. Although the QDs fluorescence immunochromatography method consistently produced higher absolute D-dimer values, both assays showed comparable trends across the measurement range.

Conclusion: QDs fluorescence immunochromatography and the latex-enhanced immunoturbidimetric assay demonstrated strong correlation and acceptable agreement in D-dimer measurement. Despite yielding higher absolute values, the QDs-based method showed consistent performance, supporting its potential as a reliable and rapid alternative for clinical D-dimer assessment.

Introduction

During the hemostatic process, fibrin clots are formed through activation of the coagulation cascade in response to vascular injury, followed by clot degradation via the fibrinolytic system. D-dimer is a specific degradation product generated when cross-linked fibrin is cleaved by plasmin. Structurally, D-dimer consists of two fibrin D domains covalently linked by factor XIII during clot formation. This unique molecular structure forms a specific epitope that can be recognized by monoclonal antibodies, making D-dimer a useful biomarker for detecting activation of coagulation and fibrinolysis pathways [1–4].

For nearly three decades, D-dimer testing has been widely used in the initial evaluation of suspected venous thromboembolism, including deep vein thrombosis (DVT) and pulmonary embolism (PE), as well as in the assessment of disseminated intravascular coagulation (DIC) [5]. Due to its high analytical sensitivity, D-dimer testing is primarily applied as a rule-out tool in appropriate clinical settings [6,8].

Since the COVID-19 pandemic, the demand for D-dimer testing has increased substantially in many healthcare facilities. At Dr. Soetomo General Academic Hospital, Surabaya, the number of D-dimer examinations rose markedly during 2020–2021, highlighting the growing reliance on laboratory-based coagulation markers in routine clinical practice. This increase underscores the importance of understanding the analytical characteristics and comparability of different D-dimer assay methods.

Various methods are available for D-dimer measurement, including enzyme immunoassays, latex-enhanced immunoturbidimetric assays, and immunochromatographic techniques, which may be performed manually, semi-automatically, or on fully automated platforms. Enzyme-linked immunosorbent assay (ELISA) methods have historically been used as reference procedures in analytical evaluations of D-dimer assays [9]. Latex-enhanced immunoturbidimetric assays are widely implemented in clinical laboratories due to their automation compatibility and suitability for high-throughput testing [6,10]. These assays are based on the agglutination of antibody-coated particles, producing increased turbidity proportional to the D-dimer concentration, which is measured photometrically [6,8,11].

As a rapid and cost-effective alternative, immunochromatographic or lateral flow immunoassays (LFIA) have been developed [12,13]. Conventional LFIA methods, however, may suffer from limited signal intensity, particularly when using organic fluorescent dyes that are prone to photobleaching [12]. To address these limitations, quantum dot (QD)-based labeling technologies have been introduced. Quantum dots are semiconductor nanocrystals with favorable optical properties, including high fluorescence intensity, resistance to photobleaching, tunable emission spectra, and high absorption coefficients [14–16].

The Vazyme QD-S2000 is an automated immunoassay analyzer that applies quantum dots fluorescence immunochromatography for quantitative D-dimer measurement. This system employs a double-antibody sandwich principle, in which D-dimer in the sample binds to a quantum dot–conjugated anti–D-dimer antibody and is subsequently captured by an immobilized monoclonal antibody on a nitrocellulose membrane. The emitted fluorescence signal is then detected and quantified by the instrument [14,17].

The aim of this study was to evaluate the analytical agreement between D-dimer measurements obtained using a latex-enhanced immunoturbidimetric assay on the Sysmex CS-2500 analyzer and a quantum dots fluorescence immunochromatographic assay using the Vazyme QD-S2000 system.

Materials and methods

Study Population and Sampling

The study population comprised inpatients and outpatients at Dr. Soetomo General Academic Hospital, Surabaya, who underwent D-dimer testing between September and November 2022. Clinical suspicion was defined as physician-indicated testing based on symptoms suggestive of thromboembolic events, such as unexplained dyspnea, chest pain, or limb swelling. Risk factors included conditions associated with coagulation abnormalities, such as age >60 years, prolonged immobility, active malignancy, recent surgery, pregnancy, or prior venous thromboembolism.

Inclusion criteria consisted of patients for whom D-dimer testing was clinically indicated, with venous blood collected into 3 mL sodium citrate tubes and processed within 8 hours. Only plasma samples without visible hemolysis, icterus, or lipemia were included. Samples that did not meet these criteria were excluded. A consecutive sampling strategy was applied, enrolling all eligible specimens obtained during the study period. The source population included patients attending both inpatient and outpatient services at Dr. Soetomo General Academic Hospital.

The sample size was considered adequate for a preliminary method comparison study and is consistent with the Clinical and Laboratory Standards Institute (CLSI) EP09-A3 guideline, which recommends the use of 40–100 patient samples for method comparison and bias estimation.

Sample Collection and Handling

Venous blood samples (3 mL) were collected into sodium citrate tubes (BD Vacutainer®, Becton Dickinson, Franklin Lakes, NJ, USA) using standard aseptic technique, in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines for specimen collection and handling (CLSI GP41-A7). Samples were gently inverted immediately after collection to ensure proper anticoagulant mixing.

Citrate plasma was obtained by centrifugation at 3000 rpm for 10 minutes at room temperature within 2 hours of sample collection. Plasma aliquots were transferred into polypropylene cryovials and stored at - 80 °C until analysis.

Samples were stored for a maximum of three months before testing. Although repeated freeze–thaw cycles were avoided, minimal unavoidable freeze–thaw exposure related to sample handling cannot be entirely excluded and is acknowledged as a potential source of analytical variability.

Laboratory Methods

Plasma D-dimer concentrations were measured using two analytical platforms: a Quantum Dots Fluorescence Immunochromatography assay (Vazyme QD-S2000, Nanjing Vazyme Medical Technology Co., Ltd., China; QD-D-Dimer Kit, Cat. No. QD2001-DD) and a Latex-Enhanced Immunoturbidimetric Assay (Sysmex CS-2500, Siemens Healthcare Diagnostics Inc., USA; Innovance® D-dimer, Cat. No. OQGN14). Both assays were performed strictly according to the manufacturers' instructions.

For analytical classification purposes, a D-dimer concentration ≥0.5 mg/L FEU was defined as abnormal and <0.5 mg/L FEU as normal for both platforms. This fixed cut-off was applied to ensure methodological comparability between assays. Although age-adjusted thresholds have been proposed in clinical practice, they were not applied in this analytical comparison study.

Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics, version 26.0 (IBM Corp., Armonk, NY, USA), and a two-sided p-value <0.05 was considered statistically significant. The Kolmogorov–Smirnov test was applied to assess the normality of continuous D-dimer data. Since the values were not normally distributed, comparisons of paired results obtained from the two analytical platforms were conducted using the Wilcoxon signed-rank test, which is appropriate for related nonparametric data. To evaluate the strength and direction of association between the two methods, Spearman's rank correlation coefficient was employed, as it does not assume linearity or normal distribution. Agreement between assays was further explored using the Bland–Altman method, which allows identification of systematic bias across measurement ranges. For categorical comparisons, D-dimer results were classified as normal or abnormal based on the manufacturer-defined cut-off, and concordance was assessed using cross-tabulation and Cohen's kappa coefficient to quantify inter-method agreement beyond chance. Additionally, the Chi-square test was used to examine associations between categorical classifications; Fisher's exact test was substituted where expected frequencies were less than five. This combination of statistical approaches ensured robust evaluation of distribution, correlation, agreement, and categorical associations between the two D-dimer assays.

Laboratory Procedure

All plasma specimens were analyzed using both assay platforms. The Quantum Dots Fluorescence Immunochromatography assay (Vazyme QD-S2000) is based on a double-antibody sandwich immunoassay with quantum dot-conjugated antibodies for fluorescence detection. The Latex-Enhanced Immunoturbidimetric Assay (Sysmex CS-2500) measures photometric changes resulting from antigen-antibody-mediated latex particle agglutination.

Both analyzers were calibrated in accordance with the manufacturer's recommendations before analysis. Internal quality control was performed daily at two concentration levels using manufacturer-provided control materials. Analytical runs were accepted or rejected based on internal quality control performance; no individual patient results were selectively excluded.

All laboratory procedures were conducted in compliance with Clinical and Laboratory Standards Institute (CLSI) guidelines for method comparison and internal quality control (CLSI EP09-A3 and EP15-A3).

This study received ethical approval from the Health Research Ethics Committee of Dr. Soetomo General Academic Hospital, Surabaya, Indonesia (Reference No. 0513/KEPK/X/2022), and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants or their legal guardians before sample collection.

Results and discussion

Results

A total of 80 samples were analyzed, including 32 samples from male patients (40%) and 48 from female patients (60%). The mean age of participants was 41 years (range: 1–78 years) (Table 1).

Table 1: Patient Characteristics.

No.	Variable	Count
1	Age (years) Mean (Min-Max)	41 (1-78)
2	Gender Male Female	32 (40%) 48 (60%)

The Kolmogorov–Smirnov test indicated that D-dimer values obtained from both analytical platforms were not normally distributed ($p<0.05$). Therefore, paired comparisons were performed using the Wilcoxon signed-rank test. A statistically significant difference was observed between D-dimer concentrations measured by the two methods ($p < 0.001$), with higher median values obtained using the Vazyme QD-S2000 compared to the Sysmex CS-2500 (Table 2).

Table 2: Comparison of D-dimer levels between Vazyme QD-S2000 and Sysmex CS2500 (Wilcoxon signed-rank test).

	Median (Range) mg/L FEU	p-value
Vazyme QD-S2000	0.72 (0.17–13.94)	<0.001*
Sysmex CS-2500	0.49 (0.08–11.51)	

* $p < 0.001$ (Wilcoxon signed-rank test), significantly different p-value < 0.05

Table 2 summarizes the comparison of D-dimer values measured by Vazyme QD-S2000 and Sysmex CS2500. The Wilcoxon signed-rank test revealed a statistically significant difference between the two methods ($p < 0.001$). The median D-dimer level measured with Vazyme (0.72 mg/L FEU) was consistently higher than that measured with Sysmex (0.49 mg/L FEU). The Kolmogorov–Smirnov test yielded a p-value < 0.05 for the overall sample, indicating that D-dimer data from both methods did not follow a normal distribution. Subsequent comparison using the Wilcoxon signed-rank test across all samples and subgroups yielded a p-value < 0.001, confirming a significant difference between D-dimer values obtained from Vazyme and Sysmex.

Figure 1: Correlation of D-Dimer levels using Quantum Dots immunofluorescence chromatography and turbidimetric immunoassay (Spearman r = 0.951, $p<0.05$).

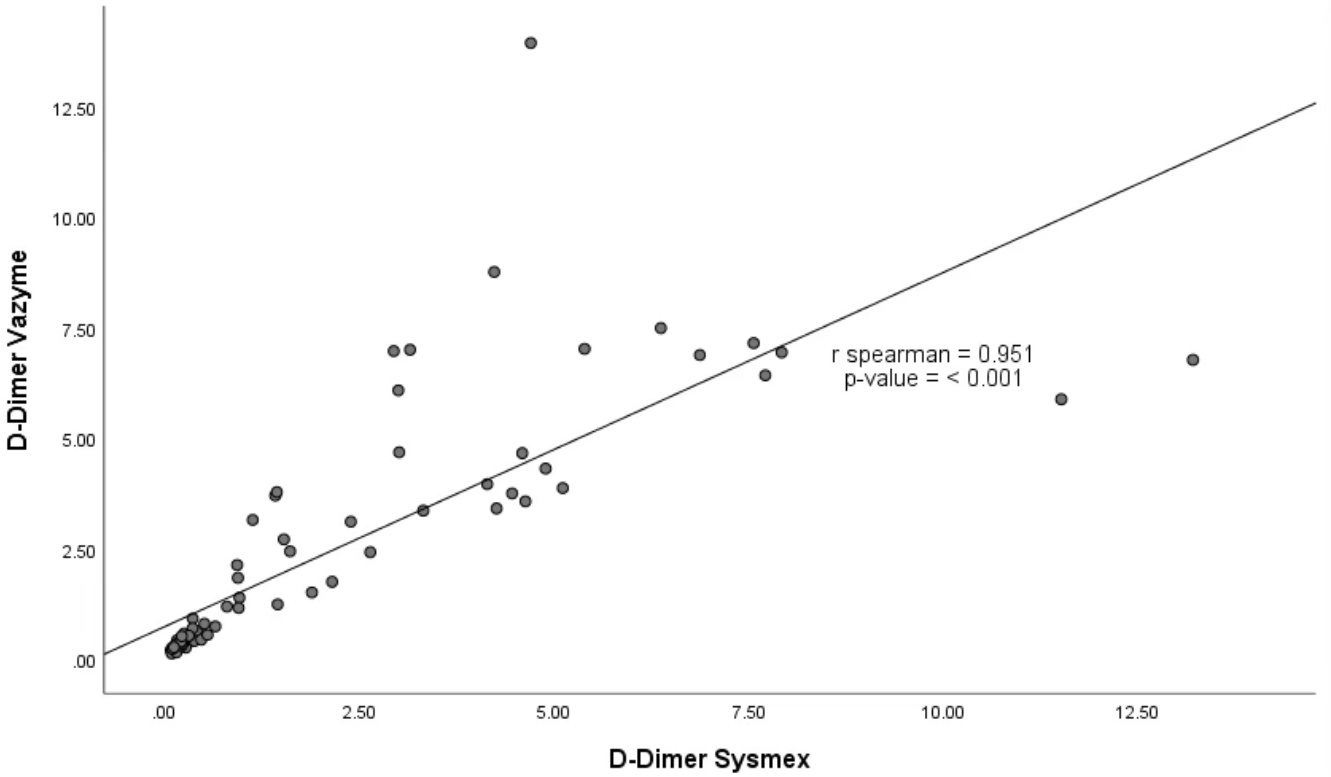
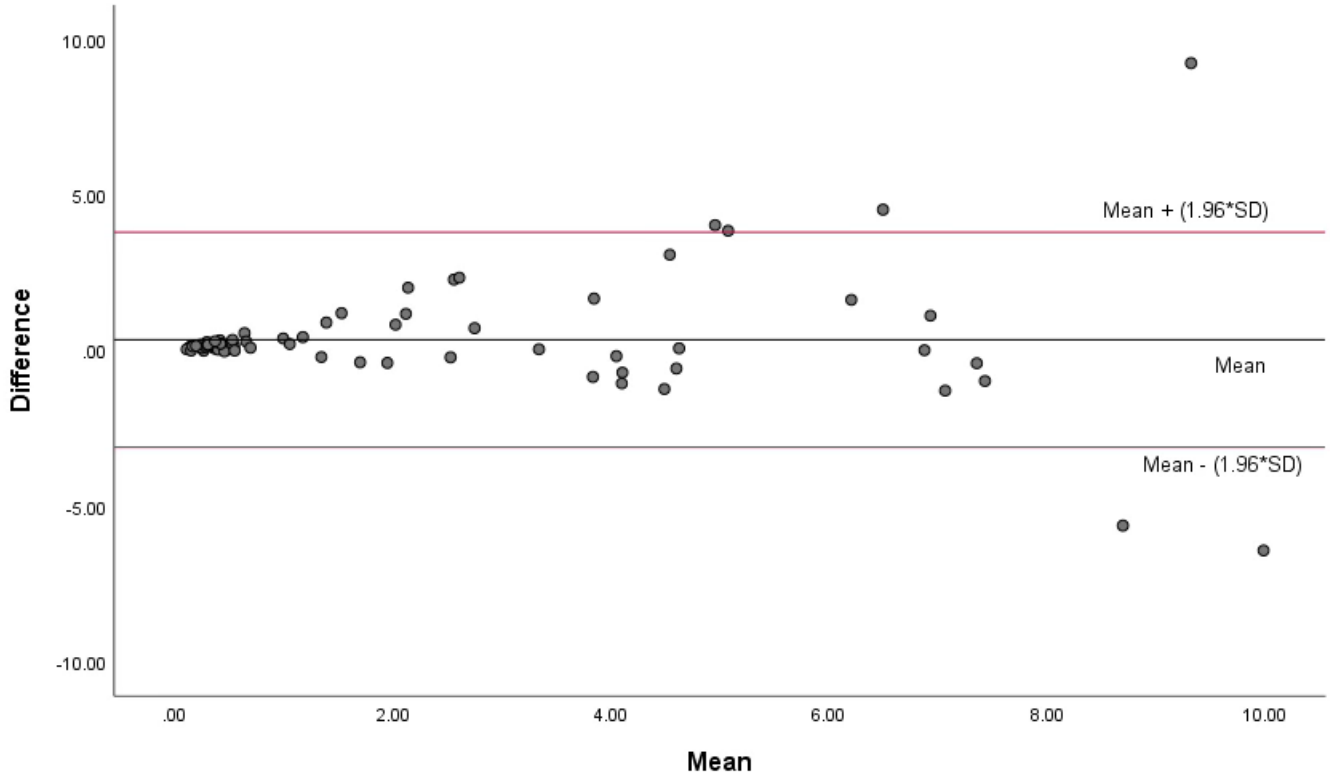


Figure 2: Bland-Altman Plot comparing Quantum Dots immunofluorescence chromatography and latex immunoturbidimetric assay.



Cross-tabulation analysis confirmed concordance between both instruments (Table 3), with a Kappa coefficient of 0.700 indicating good agreement in classifying results.

Table 3: Cross-tabulation of D-dimer results between Vazyme QD-S2000 and Sysmex CS2500.

D-Dimer Vazyme	D-Dimer Sysmex		Total n (%)
	Normal n (%)	Abnormal n (%)	
Normal	28 (35%)	0 (0%)	28 (35%)
Abnormal	12 (15%)	40 (50%)	52 (65%)
Kappa	0,7		
p-value	< 0.001		

Spearman correlation analysis demonstrated a strong positive association between D-dimer measurements obtained from the two platforms ($r = 0.951$, $p < 0.001$) (Figure 1).

Agreement between the two methods was further assessed using Bland–Altman analysis (Figure 2). The analysis demonstrated a positive mean bias of 2.44 mg/L FEU, indicating that the Vazyme QD-S2000 systematically yielded higher D-dimer values compared to the Sysmex CS-2500. The 95% limits of agreement ranged from –11.53 to 16.40 mg/L FEU, with most data points falling within these limits. Categorical agreement was evaluated by classifying results as normal or abnormal based on a cut-off value of 0.5 mg/L FEU. Cross-tabulation analysis showed concordance between the two methods in 68 of 80 samples (85%). Cohen's kappa coefficient was 0.700 ($p < 0.001$), indicating good categorical agreement (Table 3).

Discussion

This study demonstrated a statistically significant difference in D-dimer concentrations measured by the Vazyme QD-S2000 and Sysmex CS-2500 analyzers, with consistently higher values observed using the quantum dot–based immunochromatographic method. Despite this difference, Spearman correlation analysis showed a strong positive association between the two platforms ($r = 0.951$; $p < 0.001$),

indicating that relative changes in D-dimer levels were preserved across both methods.

Bland - Altman analysis revealed a systematic positive bias of 2.44 mg/L FEU, with relatively wide 95% limits of agreement ranging from –11.53 to 16.40 mg/L FEU. Although most measurements lay within the limits of agreement, the magnitude of dispersion, particularly at higher D-dimer concentrations, suggests that the two methods are not directly interchangeable without method-specific considerations.

Categorical agreement analysis based on a fixed cut-off of 0.5 mg/L FEU yielded a Cohen's kappa coefficient of 0.700, reflecting good agreement beyond chance. Importantly, all discordant results were attributable to samples classified as abnormal by the Vazyme assay but normal by the Sysmex assay. This pattern further supports the presence of a systematic positive bias rather than random misclassification [18–20].

Inter-assay variability in D-dimer measurement has been widely reported and is influenced by both biological and analytical factors. Biological variation includes intra-individual and inter-individual variability, while analytical variation arises from differences in assay design, antibody specificity, reagent composition, calibration traceability, and analytical principles employed by different platforms [6,18]. These factors contribute to the limited comparability of D-dimer results obtained using different immunoassays.

The lack of harmonization among D-dimer assays remains a major challenge in laboratory medicine. Differences in antibody specificity and analytical commutability mean that results generated by one platform cannot be directly extrapolated to another [19,20]. Consequently, D-dimer assays should be interpreted within the context of their respective analytical characteristics, and method-specific validation is required prior to clinical implementation [18-21].

Several limitations of this study should be acknowledged. First, this was a single-center study with a relatively limited sample size, which may restrict the generalizability of the findings to other clinical settings or populations. Second, a reference method such as enzyme-linked immunosorbent assay (ELISA) was not included; therefore, this study focused on inter-method comparison rather than analytical accuracy against a gold standard. Third, although samples were stored under controlled conditions and repeated freeze–thaw cycles were avoided, the potential impact of sample storage duration on D-dimer stability cannot be entirely excluded. Finally, clinical outcomes were not assessed, and thus the implications of inter-assay bias on diagnostic or prognostic decision-making were beyond the scope of this study

Overall, the findings of this study indicate that while the Vazyme QD-S2000 and Sysmex CS-2500 assays demonstrate strong correlation and acceptable categorical agreement, the presence of systematic bias precludes their interchangeable use. Awareness of assay-specific behavior is therefore essential when interpreting D-dimer results, particularly near clinical decision thresholds.

Conclusions

This study demonstrated a statistically significant difference in median D-dimer concentrations between the quantum dots fluorescence immunochromatography assay (Vazyme QD-S2000) and the latex-enhanced immunoturbidimetric assay (Sysmex CS-2500), with the Vazyme platform consistently yielding higher values. Despite this difference, a strong positive correlation was observed between the two methods (Spearman r = 0.951), indicating that relative changes in D-dimer levels were preserved across both analytical platforms.

Categorical analysis based on a fixed cut-off value of 0.5 mg/L FEU showed good agreement, with a Cohen's kappa coefficient of 0.700. However, all discordant classifications were attributable to higher D-dimer values measured by the Vazyme assay, reflecting a systematic positive bias rather than random disagreement.

Bland–Altman analysis further confirmed the presence of systematic bias, indicating that the two methods are not directly interchangeable. The observed mean bias of 2.44 mg/L FEU with wide limits of agreement further supports that these two assays should not be used interchangeably, particularly in samples with markedly elevated D-dimer levels. These findings highlight the importance of method-specific interpretation when comparing D-dimer results obtained from different analytical platforms, particularly near clinical decision thresholds.

In conclusion, while the Vazyme QD-S2000 and Sysmex CS-2500 assays demonstrate strong correlation and acceptable categorical agreement, the observed systematic bias precludes their interchangeable use. Awareness of assay-specific analytical characteristics is therefore essential for accurate interpretation of D-dimer measurements in laboratory practice.

Author contributions

Conceptualization, A.A. and P.B.N.; methodology, F.R.M.; validation, A.A.; formal analysis, A.A.; investigation, A.A. and F.R.M.; writing - original draft preparation, A.A.; writing - review and editing, A.A. and P.B.N.; supervision, P.B.N. All authors have read and agreed to the published version of the manuscript.

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Ethics statement

This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Dr. Soetomo General Academic Hospital, Surabaya, Indonesia (approval number: 0513/KEPK/X/2022).

Conflict of interest

The authors declare no conflict of interest.

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Research Article

Establishment of Trimester-Specific Reference Intervals for TSH and Thyroid Hormones in Pregnant Women living in Oran, Western Algeria

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Keywords

Reference interval, TSH, FT3, FT4, pregnancy, Oran

Abstract

Background and Aim: The thyroid gland undergoes physiological changes during pregnancy, leading to variations in reference intervals (RIs) for TSH and free thyroid hormones across the different trimesters. To ensure accurate interpretation of these tests, the American Thyroid Association (ATA) recommends the use of trimester-specific and population-based reference intervals. The aim of our study was: to establish trimester-specific reference intervals of TSH, FT3, and FT4 in pregnant women living in Oran western Algeria.

Materials and Methods: The reference intervals were established accordingly to the CLSI guideline (EP28-A3c). The study included 401 apparently healthy pregnant women, classified as follows: 120 in the first trimester, 154 in the second trimester, and 127 in the third trimester. Reference subjects were selected based on NACB exclusion criteria. Hormone assays were performed using the Roche Cobas e411 analyzer.

Results: The established RIs corresponding to the 2.5th and 97.5th percentiles were in the First, Second and Third trimester for TSH: 0.25–3.57 mIU/L, 0.15–3.43 mIU/L,0.57–4.23 mIU/L, FT4 : 11.41–20 pmol/L, 10.56–18 pmol/L, 9.43–17.54 pmol/L, and FT3 : 3.16–7.02 pmol/L, 3.02–7.54 pmol/L, 3.2–7.37 pmol/L.

Conclusion: In the absence of population-specific reference intervals for TSH and thyroid hormones in our country, establishing such values represents a significant advancement, enabling more accurate diagnosis and improved management of thyroid disorders.

Introduction

Thyroid hormones play a crucial role in maintaining pregnancy and ensuring proper fetal development [1,2]. However, the fetal thyroid gland does not reach functional maturity until approximately the 18th–20th week of gestation, making maternal thyroid hormones the primary fetal source during the first half of pregnancy and even in the second half of pregnancy, the fetus remains partially dependent on maternal thyroid hormones [3]. Therefore, requirements for thyroid hormone and iodine increase by approximately 50% during pregnancy [4-6]. To ensure adequate fetal Thyroid hormones supply, maternal thyroid gland undergoes several physiological changes including: a transient reduction in thyroid-stimulating hormone (TSH) levels due to the TSH-like stimulatory effect of human chorionic gonadotropin (hCG) [7], increased production of thyroid-binding globulin (TBG) [8], enhanced degradation of thyroid hormones by placental deiodinases, increased urinary iodide clearance [9]. These changes lead to dynamic variations in maternal serum TSH and thyroid hormone levels, particularly free thyroxine (FT4), across different trimesters of pregnancy [10].

Maternal thyroid dysfunction during pregnancy increases the risk of adverse maternal and child outcomes, such as pregnancy loss, miscarriage, premature delivery, low birth weight, impaired neuropsychological development, pre-eclampsia and gestational hypertension [11-14].

Considering the adverse outcomes, accurate assessment of thyroid function during pregnancy is essential to prevent both maternal and fetal complications. Due to the physiological alterations occurring during pregnancy, reference intervals (RIs) established in non-pregnant individuals are not applicable to pregnant women. Given the impact of thyroid dysfunction on both the mother and the fetus, the American Thyroid Association (ATA) [15], and other guidelines [16,17], recommend the use of population-based and trimester-specific reference ranges for TSH and thyroid hormones to ensure proper diagnosis and management. Several studies have established trimester-specific reference intervals in diverse populations, showing variability influenced by ethnicity, iodine status, assay methodology, and environmental factors [15,18,19]. However, there is a lack of specific data for Algerian pregnant women, which underscores the need to establish population- and trimester-specific reference intervals in order to improve clinical decision-making and pregnancy outcomes. This study aims to establish the reference intervals of TSH and free thyroid hormones in pregnant women living in Oran (western Algeria), thereby providing essential data to improve the screening and management of thyroid disorders during pregnancy.

Materials and Methods

In this study, we established reference intervals for TSH and free thyroid hormones following the EP28A3c protocol published by the Clinical and Laboratory Standards Institute (CLSI). This guideline provides standardized recommendations for defining, establishing, and verifying reference intervals in clinical laboratories to ensure reliability and clinical relevance [20]. A total of 401 apparently healthy pregnant women living in Oran with singleton pregnancies were included in the study. Only women who provided informed consent were enrolled. Participants were recruited between September 2022 and April 2024 from outpatient clinics in Oran where prenatal follow-up is performed. Pregnancy was confirmed by the presence of fetal cardiac activity on obstetric ultrasound. Gestational age was determined using early obstetric ultrasound.

Reference subjects were selected using an a priori direct sampling method, which is the CLSI recommended approach [20]. Population selection was based on exclusion criteria defined by the National Academy of Clinical Biochemistry (NACB) [21]: personal or family history of thyroid dysfunctions, positive thyroid peroxidase antibodies (TPO-Ab), goiter or thyroid nodules and medications that affect thyroid function. Additionally, the study excluded smoker women, multiple gestation, acute pathology within the past three months, chronic diseases, use of any medication, pregnancies resulting from assisted reproductive technologies, complicated pregnancies (gestational diabetes, preeclampsia, gestational hypertension, retroplacental hematoma, threatened miscarriage or preterm labor, preterm premature rupture of membranes, genital bleeding, severe anemia, Hemolysis Elevated Liver enzymes Low Platelets “HELLP” syndrome), hyperemesis gravidarum, intrauterine growth retardation and women in active labor.

Venous bloods were collected between 8:00 and 10:00 AM in a non-fasting state and serum stored at -20°c for further analysis. TSH and thyroid hormone tests were performed on the Cobas e411 immunoassay analyzer (Roche Diagnostics), a widely used platform known for its precision and accuracy in hormonal assays which use the Electrochemiluminescence Immunoassay.

Results were validated through internal quality control. The precision of the assays was assessed through intra- and inter-assay imprecision of TSH, free triiodothyronine (FT3), and FT4.

As our population lacks specific reference intervals for TPO-Ab, we performed a study to verify the transference of the manufacturer cutoff according to CLSI (EP28-A3c) guideline. We carried out this verification on 20 men. Subject selection was based on NACB criteria [21]: we included healthy men younger than 30 years, with TSH levels between 0.5 and 2 mIU/L, and excluded smokers, men with a personal or family history of thyroid disease, or those with goiter and thyroid nodules. The measurement of TPO-Ab was also performed using the Cobas e411 analyzer.

In our study, since urinary iodine measurement was not feasible, we subjectively assessed the iodine status of the participants by considering their intake of iodine-rich nutrients. The main dietary sources considered were fish, milk, and salt.

Notably, the salt available on the Algerian market is iodized. As recommended by EP28A3c CLSI guideline [20], we eliminated outliers using Tukey method.
Reference intervals covering 95% of the population were determined using the non-parametric method. The lower and upper reference limits were defined as the 2.5th and 97.5th percentiles, respectively.
After outliers removing, a total of 401 pregnant women were included in the study: 120 in the first trimester (5 - 13 weeks (w)), 154 in the second trimester (13w+ 1day - 28 w), and 127 in the third trimester (28 w+1 day - 40 w).

Statistical analysis

Statistical analysis was performed using MedCalc software. The normality of data distribution was assessed using the Shapiro–Wilk test. Results are expressed as mean ± standard deviation (SD) for normally distributed variables, and as median for non-normally distributed variables. Outliers were identified and excluded using Tukey's method. Reference intervals were determined using the non-parametric approach, and 90% confidence intervals (CI) were computed for the upper and lower limits of the reference range.

Results

The median maternal age was 29 years in the first trimester (range: 18–43 years), and 30 years in both the second trimester (range: 19–43 years) and the third trimester (range: 18–47 years). The median gestational age was 8 weeks in the first trimester, 19.8 weeks in the second trimester, and 36.2 weeks in the third trimester. Median parity was 1 in all three trimesters. The consumption of salt and milk, the main sources of iodine, was satisfactory with a reported daily intake among the participants. However, fish consumption was infrequent. Verification of TPO-Ab cut-off showed that all values were below the manufacturer's threshold of 34 IU/mL. Summary statistics and reference intervals of TSH, FT4, FT3 are shown in Table 1.

Table 1: Trimester specific Reference intervals of TSH, FT3 and FT4.

	1st trimester	2nd trimester	3rd trimester
TSH			
Median	1.28	1.53	2.17
RI	0.25 – 3.57	0.15 – 3.43	0.57 – 4.08
LL 90% CI	0.019 – 0.39	0.013 – 0.41	0.011 – 0.75
UL 90% CI	3.29 – 4.03	3.22 – 3.84	3.87 – 4.57
FT4			
Median	15.20	13.24	13.63
RI	11.41 - 20	10.56 - 18	9.43 – 17.54
LL 90% CI	10.68 – 11.93	10.32 – 11.13	8.8 – 11
UL 90% CI	19.67- 20.33	17 - 18	16.86 – 18
FT3			
Median	4.93	5.49	5.47
RI	3.65 – 7.33	3.23 – 7.72	3.2 – 7.37
LL 90% CI	3.30 – 3.88	2.97 – 3.58	2.97 – 3.37
UL 90% CI	6.71 – 7.55	7.41 – 7.86	– 8.21

RI: reference interval, LL: lower limit, UL: upper limit, CI: confidence interval

Reference intervals estimated were in the First, Second and Third trimester respectively, for:
TSH: 0.25–3.57 mIU/L, 0.15–3.43 mIU/L, 0.57–4.23 mIU/L (Figure 1),
FT4 : 11.41–20 pmol/L, 10.56–18 pmol/L, 9.43–17.54 pmol/L (Figure 2),
FT3 : 3.16–7.02 pmol/L, 3.02–7.54 pmol/L, 3.2–7.37 pmol/L (Figure 3).

Figure 1: Trimester-specific reference intervals of TSH during pregnancy.

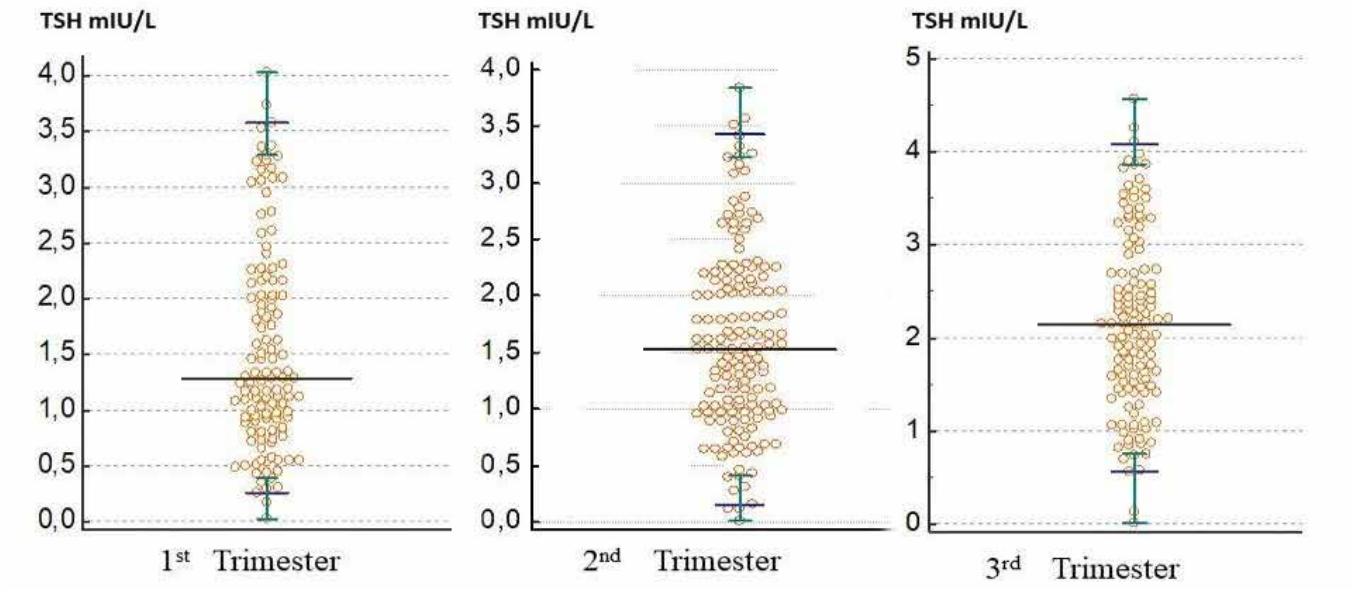


Figure 2: Trimester-specific reference intervals of FT4 during pregnancy.

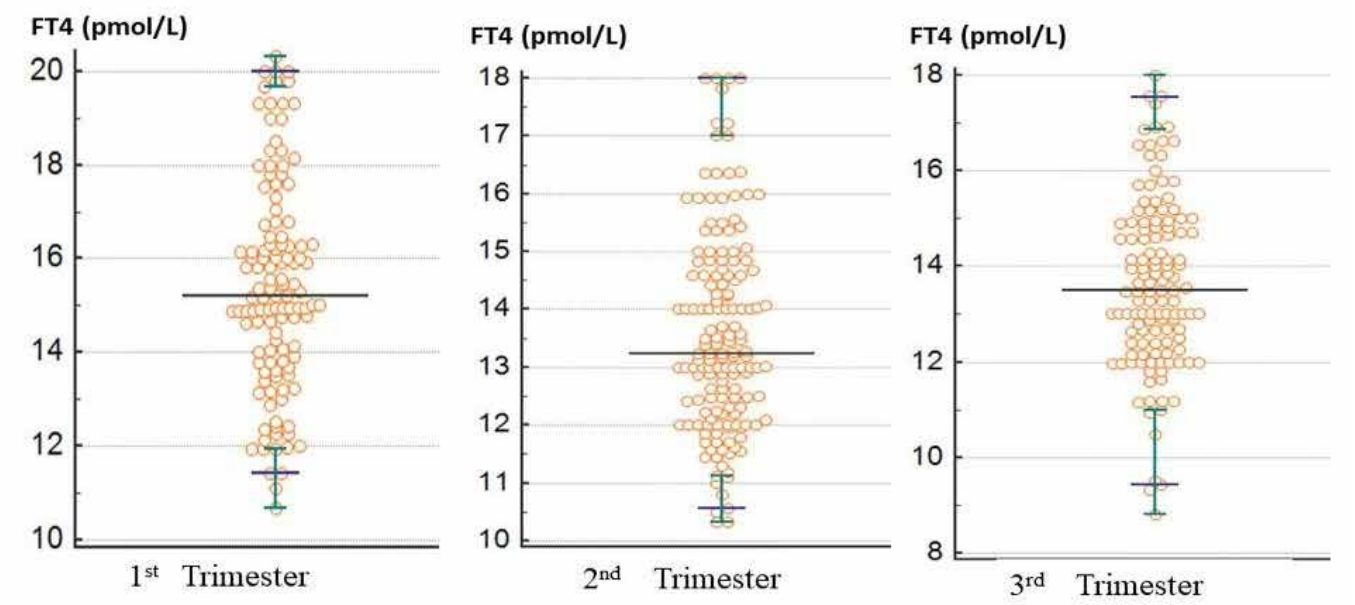
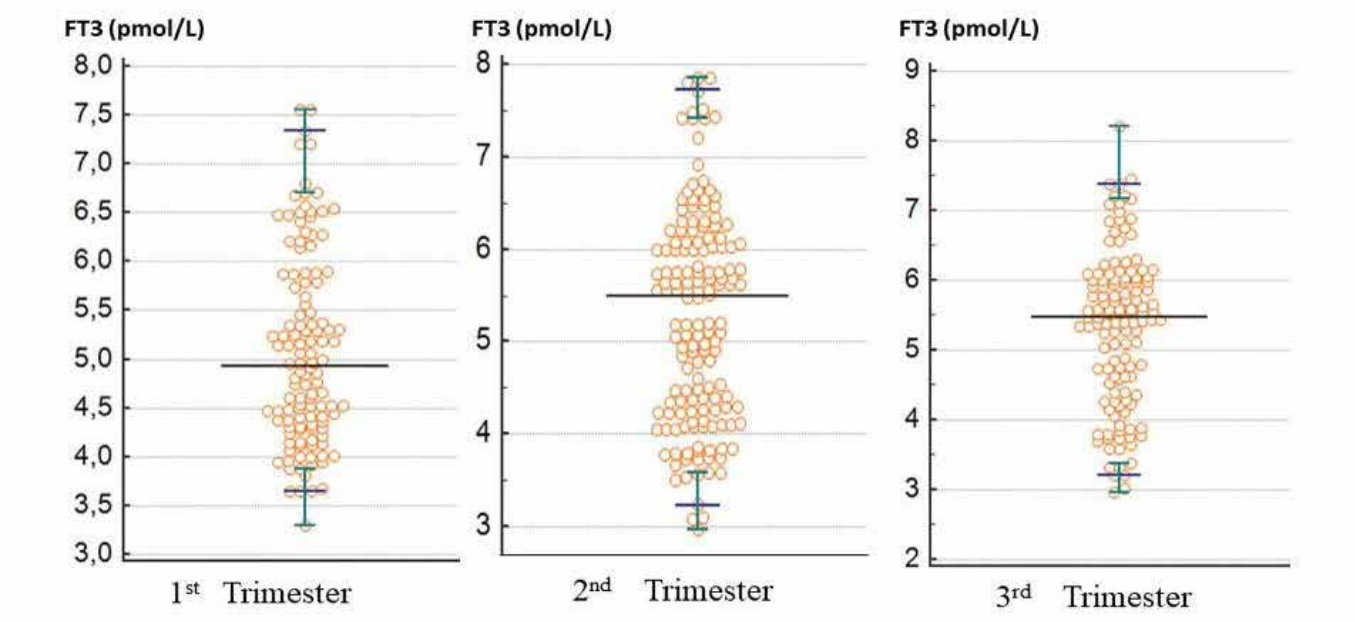


Figure 3: Trimester-specific reference intervals of FT3 during pregnancy.



Discussion

TSH is considered the primary and most sensitive biomarker for assessing thyroid function [15]. During pregnancy, early detection of thyroid dysfunction is crucial to prevent adverse outcomes affecting both fetal development and postnatal neurocognitive growth. Accordingly, the ATA guideline recommend applying trimester-specific and population-based reference intervals to ensure accurate interpretation [15].

In this work, we established population- and trimester-specific RIs of TSH, FT3, and FT4 in a cohort of pregnant women living in Oran, western Algeria. To the best of our knowledge, this is the first nationwide study in Algeria to establish pregnancy-specific reference intervals for TSH and thyroid hormones. For this purpose, healthy participants were carefully selected according to NACB exclusion criteria. Moreover, additional stringent exclusions were applied (see above).

The manufacturer-provided TPO-Ab cut-off was verified as appropriate for our population, with all measured concentrations falling below the specified threshold. This allowed us to use the manufacturer's cut-off to exclude women with positive TPO-Ab from the sample.

Repeatability and reproducibility tests confirmed excellent analytical precision, with coefficients of variation less than 5% for TSH, FT3, and FT4.

In our study, outliers accounted for less than 5% of the total population, underscoring both the rigorous selection of reference subject and the robustness of the analytical system.

Regarding the iodine status of the population, the ATA 2017 guidelines recommended establishing TSH reference intervals in subjects with adequate iodine intake [15]. Since direct urinary iodine assessment was not feasible, iodine status was evaluated based on dietary intake, particularly iodized salt and milk, both of which were consumed daily by participants. The overall pattern suggested no severe iodine deficiency, a finding further supported by a recent study in Oran reporting a mean urinary iodine concentration of 131.4 µg/L [22], consistent with adequate iodine status in the population.

The median TSH concentration showed a gradual increase throughout the three trimesters. Other studies [23–25], performed using Roche analyzers, have reported upper reference limit values for TSH across all trimesters, similar to those observed in our study, as shown in Table 2. Although these results show similarities with ours, variations persist across populations, emphasizing the need to establish population-specific RIs for reliable interpretation of TSH values. The lower-limit of RIs reported in other studies [23-29], summarized in Table 2, whether performed on Roche analyzers or other analytical platforms, reported lower values for TSH compared with our findings. This discrepancy may be explained by differences in gestational age at the time of sampling. In our study, at first trimester recruitment began as early as the 5th week, whereas in those studies, participants were enrolled later, typically from the 9th, 12th, or even 14th week of gestation. These findings may be explained by the progressive influence of hCG on TSH, which becomes more evident on the reference interval as gestational age increases and hCG concentrations rise. At second trimester, we note that the lower limit of our RI is similar to that observed in the study of Yuen LY [25], where recruitment began at 16th week. By contrast, the study of Joosen AM [27], which recruited later (27 weeks), reported a slightly higher lower limit. At the third trimester, we started recruitment at 28th week and we notice that the lower limit of our RI is comparable to that reported in other studies where recruitment began at 30, 32 or even 36 weeks. We observe that in the third trimester, the lower limit of the TSH reference interval is less influenced by gestational

age at recruitment than in the earlier trimesters. This may be explained by the significant decline in hCG levels at this stage of pregnancy, stabilization of thyroid function, and reduction in TSH fluctuations.

Table 2: Trimester specific Reference intervals of TSH, FT3 and FT4 in other studies.

Study	Analysing platform	Gestation week	TSH (mIU/L)	FT4 (pmol/L)	FT3 (pmol/L)
Dorizzi et al. 2023 Italy (23)	Roche Elecsys	14–16 24–26 30–32	0.34-3.81 0.68 - 4.07 0.63 - 4	11-17 9.98-15.25 9.49 -15.1	3.81 - 6.05 3.61-5.38 3.99-5.37
Sekhri et al. 2016 India (29)	Roche Elecsys	10.8 2nd T 3rd T	0.09-6.65 0.51-6.66 0.91-4.86	9.81-18.53 8.52-19.52 7.39-18.28	3.1-6.35 2.39-5.12 2.57-5.68
Joosen et al. 2016 Spain (27)	Roche Elecsys	9–13 27–29 36–39	0.11-3.39 0.25-3.38 0.51-3.85	11.70-20 9.3-14.2 8.1-14.9	/ / /
Ortega Carpio 2018 Spain (26)	Roche Elecsys	9–11 26–28 34–36	0.25-4.68 0.62-4.83 0.76-4.57	12.30-20.2 12-16.9 9-16.3	/ / /
Kostecka-Matyja 2017 Poland (24)	Roche Elecsys	1st T 2nd T 3rd T	0.01-3.18 0.05-3.44 0.11-3.53	11.99-21.9 10.46- 16.6 8.96-17.23	3.63-6.55 3.29-5.45 3.1-5.37
Yuen 2020 China (25)	Roche Elecsys	12–13 16–20 32–36	0.06-3.14 0.15-3.78 0.31-4.54	11.2-22.2 10.1-19.4 9-17	3.47-5.06 3.25-5.2 2.94-4.56
Yuen 2020 China (25)	Siemens Centaure	12–13 16–20 32–36	0.03-2.5 0.08-2.95 0.25-3.9	11.9-20.2 11.3-18.7 10.1-16	3.63-5.73 3.99-5.26 3-4.52
Yuen 2020 China (25)	Beckman DXI	12–13 16–20 32–36	0.11-2.71 0.19-3.25 0.3-3.87	8.3-14.4 7.4-12.6 6.7-11	4.04-6.14 3.85-5.75 3.57-5.18
Yuen 2020 China (25)	Abbott architect	12–13 16–20 32–36	0.04-2.11 0.1-2.51 0.19-2.99	11.2-19 10.5-17.1 9.5-14.7	3.45-5.52 3.39-5.52 3.22-5.12
Ollero et al. 2019 Spain (28)	Abbott architect	9 15 36	0.13-4.16 0.31-3.73 0.58-4.36	10.94-15.9 10.55-15.4 8.62-13.64	/ / /

The analysis of our reference intervals shows partial concordance with the ATA recommended upper limit of 4 mIU/L, as our TSH upper limit was close to this threshold. Although some similarities are observed with ATA recommendations, discrepancies remain, highlighting the importance of population-specific RIs as the optimal reference standard.

For FT4 reference interval, analytical limitations significantly contribute to the wide variability in FT4 RIs reported across studies in pregnant populations. Given this heterogeneity, the ATA recommends establishing FT4 reference intervals that are both population and method-specific in order to ensure accurate assessment of thyroid function during pregnancy.

In our study, we observed a continuous decline in FT4 reference interval values across pregnancy, a trend that has been consistently documented in the literature. Measurements performed with reference methods, such as direct equilibrium dialysis and Liquid Chromatography–Tandem Mass Spectrometry (LC/MS/MS), also confirm this progressive decline with advancing gestational age [30]. This reduction is explained by physiological adaptations of the thyroid gland during pregnancy, particularly increased demand for thyroid hormones and iodine, as well as enhanced renal clearance, among other contributing factors.

Regarding FT3, RIs remained relatively stable throughout pregnancy, which is consistent with several previously published studies listed in Table 2. However, the FT3 upper reference limit of our population was slightly higher compared

to that reported in other studies performed on Roche analyzers. This discrepancy may be attributed to inter-population differences.

Strengths of the Study

The main challenge in our work was the selection of apparently healthy pregnant women. This issue represents a major difficulty for clinical biologists worldwide when establishing biological reference intervals and explains the on-going efforts of scientific societies to develop alternative approaches. The principal strength of our study lies in the rigorous selection of participants, as demonstrated by the very low proportion of outliers, which did not exceed 5% of the study population. In addition, we applied additional criteria beyond those defined by the NACB, ensuring a more accurate and reliable selection of reference subjects.

Limitations of the Study

The limitations of our study regarding the establishment of RIs include, first, the absence of iodine status assessment among participants, which would have allowed us to exclude women with severe iodine deficiency. However, it is recognized that the Oran region is not considered an iodine-deficient area. Another limitation is the exclusive use of a Roche analyzer for measurements, whereas variations across different analytical platforms are well documented. Ideally, assessments should have been conducted across multiple analyzers to establish method-specific RIs. Nevertheless, our study provides valuable data for populations assessed on Roche platforms.

Conclusion

Thyroid dysfunctions during pregnancy constitute a major maternal–fetal health concern, requiring careful evaluation and tailored clinical management. In the absence of population-specific reference intervals for TSH and thyroid hormones in our country, establishing such values represents a significant advancement, enabling more accurate diagnosis and improved management of thyroid disorders. Our work therefore provides a basis for revising screening and monitoring protocols for thyroid disorders in Algeria, with the ultimate aim of promoting a more personalized and effective approach to care that reflects the specific characteristics of our population. This study represents a first step, and we intend to extend this work to include the entire country in the future.

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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CRediT Author Statement

All authors contributed substantially to the development of this work: Conceptualization, Methodology, Investigation, Data Curation, Formal Analysis, Writing – Original Draft, Supervision.

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Data Availability Statement

The data supporting the findings of this study are available from the corresponding author.

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Research Article

Plasma Gasdermin D as a Biomarker for Pyroptosis in Early Detection of Newly Diagnosed Type 2 Diabetes Mellitus

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Keywords

Gasdermin D, Pyroptosis, Type 2 diabetes mellitus, Inflammation, Biomarker, IL-18, IL-1β, GSDMD, HOMA-β

Abstract

Pyroptosis, a caspase-mediated inflammatory cell death pathway driven by Gasdermin D (GSDMD), has emerged as a potential mechanistic link between metabolic stress, β-cell injury, and chronic inflammation in Type 2 diabetes mellitus (T2DM). This study evaluated plasma GSDMD as a biomarker for newly diagnosed T2DM and explored its metabolic and inflammatory correlates. In a hospital-based case–control study, 130 newly diagnosed T2DM patients and 130 age- and sex-matched normoglycemic controls were recruited. Anthropometric indices, glycemic parameters, βFins, HOMA-IR, HOMA-β, lipid profile, and hs-CRP were measured using standard methods. Plasma GSDMD, IL-18, and IL-1β were quantified by ELISA. Group differences, correlations, multivariable logistic regression, and receiver operating characteristic (ROC) analyses were performed. T2DM patients exhibited higher BMI, adverse lipid profile, increased hs-CRP, and marked elevation of GSDMD, IL-18, and IL-1β compared with controls (all $p<0.0001$). Plasma GSDMD correlated positively with FBS, HbA1c, HOMA-IR, IL-18, IL-1β, and hs-CRP, and negatively with HOMA-β, indicating close links to hyperglycemia, insulin resistance, β-cell dysfunction, and systemic inflammation. In adjusted models, GSDMD remained an independent predictor of T2DM (OR 1.18 per 10 pg/mL, 95% CI 1.09–1.29), alongside IL-18, hs-CRP, higher BMI, and lower HDL-C. ROC analysis showed excellent diagnostic performance for GSDMD (AUC 0.98, 95% CI 0.96–0.99; cutoff 17.5 pg/mL; sensitivity 93.0%; specificity 97.0), superior to IL-18, IL-1β, and hs-CRP. Plasma GSDMD is markedly elevated in T2DM and integrates metabolic and inflammatory information, functioning as an independent risk factor and highly accurate diagnostic biomarker. These findings support GSDMD-mediated pyroptosis as a promising target for early diagnosis and risk stratification in T2DM.

Introduction

The International Diabetes Federation projects 693 million diabetes cases among adults aged 18 and older by 2045 [1]. This chronic metabolic-inflammatory disease arises from deficient insulin secretion or resistance, disrupting anabolic-catabolic equilibrium and elevating blood glucose [2]. Type 2 diabetes mellitus (T2DM) features insulin shortfall and target-cell resistance alongside β-cell impairment, predisposing to complications like cardiomyopathy, nephropathy, and atherosclerosis [3].

Microvascular progression involves interconnected pathways including amplified polyol/hexosamine activity, advanced glycation end-products (AGEs), protein kinase C isoforms, oxidative stress, and diminished antioxidant defences [4]. T2DM commonly presents mixed dyslipidemia with raised triglycerides, lowered HDL-C, and small dense atherogenic LDL-C particles. Chronic sterile inflammation - elevated IL-6 and IL-1β - critically drives disease onset and advancement [5].

Pyroptosis, an inflammatory programmed cell death first identified in Salmonella-infected macrophages, activates via caspase-1/4/5/11 responding to damage- or pathogen-associated molecular patterns, triggering IL-1β/IL-18 release. Gasdermin D (GSDMD), cleaved by these caspases, produces an N-terminal fragment that forms plasma membrane pores, facilitating cytokine efflux and pyroptotic lysis. GSDMD regulates immune cell death and proinflammatory mediator release, accelerating conditions like T2DM [6,7].

Chronic hyperglycemia and inflammation induce insulin resistance in T2DM. High-glucose/fat milieus elevate ROS, activating NLRP3 inflammasomes and caspase-1 to cleave GSDMD, promoting pyroptosis. This intensifies islet inflammation, β-cell loss, and structural damage through cytokine amplification [8].

As per the above content, this study evaluates plasma GSDMD differences across glucose metabolism states, its correlations with disease severity, independent risk for T2DM onset, and diagnostic value via ROC analysis to enable early screening.

Methdology

Study Setting and Design

This hospital-based case–control study was conducted in the Department of Biochemistry in collaboration with the Department of General Medicine at Integral Institute of Medical Sciences & Research, Integral University, Lucknow, Uttar Pradesh, India, a tertiary care teaching hospital. A total of 260 subjects were enrolled, including 130 newly diagnosed Type 2 diabetes mellitus (T2DM) patients and 130 age- and gender-matched normoglycemic controls with no history of T2DM diagnosed as T2DM as per ADA guideline considered as cases and Non-T2DM patients were considered as control.

Diagnostic Criteria

T2DM was diagnosed according to the American Diabetes Association (ADA) criteria: fasting blood glucose (FBG) ≥7.0 mmol/L (126 mg/dL) and/or glycated hemoglobin (HbA1c) ≥6.5%. Normoglycemic controls had FBG <5.6 mmol/L and HbA1c <5.7% and didn't meet any diagnostic criteria for T2DM [9,10].

Inclusion and Exclusion Criteria

The study enrolled participants aged 18–55 years who were either newly diagnosed with Type 2 Diabetes Mellitus (T2DM) according to ADA 2020 criteria or were normoglycemic.

To ensure a controlled cohort, individuals were excluded if they had other forms of diabetes (Type 1, gestational, or secondary), active infections, or recent acute cardiovascular or cerebrovascular events. Furthermore, the study barred those with malignant tumors, heart failure, or severe hepatic or renal dysfunction, as well as anyone with a history of diabetic ketoacidosis or hyperosmolar hyperglycemic states. Finally, patients currently using systemic corticosteroids, immunosuppressants, or lipid-lowering medications were disqualified from participation.

Ethical consideration

Ethics approval was granted by the Institutional Ethics Committee of Integral Institute of Medical Sciences & Research, Integral University, Lucknow approval number: IEC/IIMSR/2025/79, dated 11/07/2025.

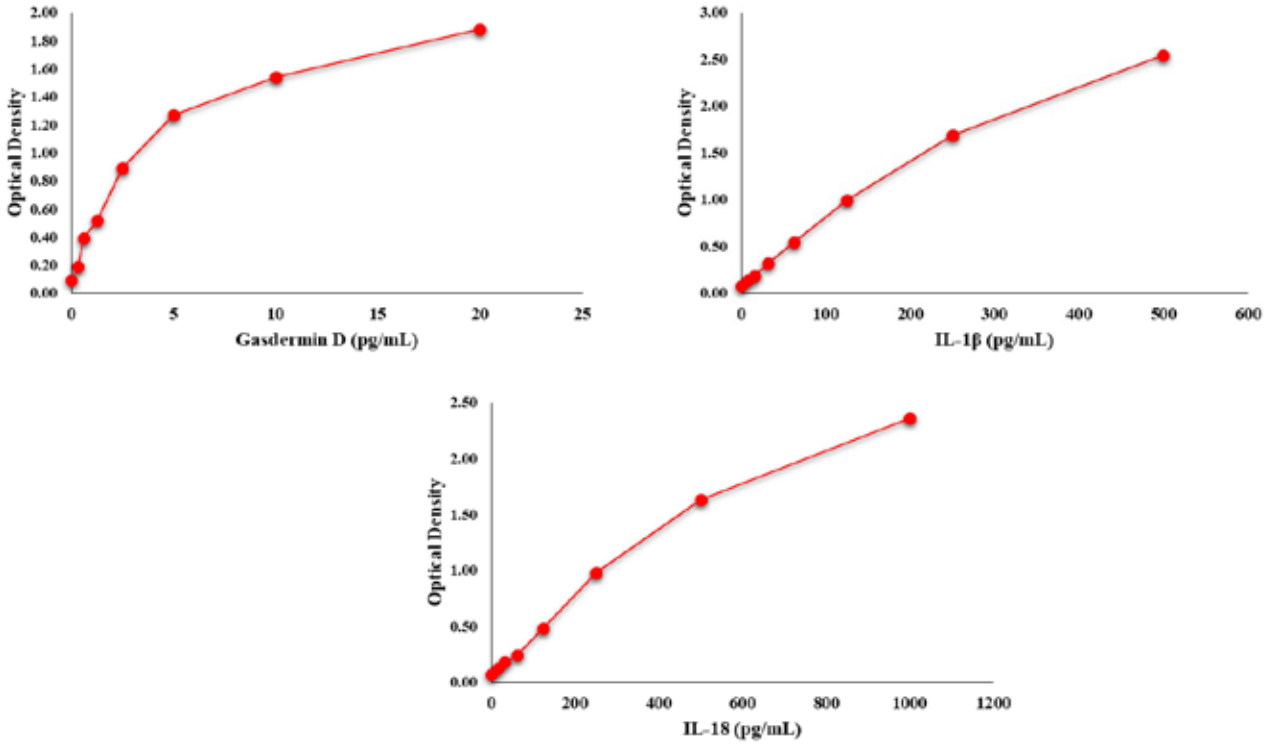
Clinical Data Collection

Demographic, anthropometric, Fasting Insulin (Fins), FBS, HbA1c, high-sensitivity C-reactive protein (hs-CRP), and lipid profile were recorded from patient sheet were recorded. The homeostasis model assessment indices were calculated as follows: HOMA-β = 20 × Fins (μIU/mL) / [FBG (mmol/L) − 3.5] and HOMA-IR = FBG (mmol/L) × Fins (μIU/mL) / 22.5.

Sample Collection and Biomarker Assays

All participants had an overnight fast of at least 8 hours. On the following morning, 5 mL of venous blood was collected in EDTA vial and centrifuged at 3500 rpm for 15 minutes. Plasma was used to do ELISA experiment. Plasma Gasdermin D (GSDMD) (ELK Biotechnology; Catalog no. ELK-5357), interleukin-18 (IL-18) (Elabscience ; Catalog no. E-EL-H0253), and interleukin-1β (IL-1β) (Elabscience ; Catalog no. E-EL-H0149) were quantified using an ELISA-based platform , with all calibration, quality-control, and validation procedures performed as per the kit manuals (Figure 1).

Figure 1: Calibration Graphs on ELISA.



Statistical Analysis

Data is presented as mean ± standard deviation. Between-group comparisons (T2DM vs. controls) was performed using the independent student t-test for distributed variables and categorical variables were compared using the chi-square test. Pearson correlation analysis was applied to assess associations between plasma GSDMD levels and clinical parameters such as FBG, HbA1c, HOMA-IR, HOMA-β, IL-18, IL-1β, lipids, and hs-CRP. Multiple logistic regression was used to evaluate GSDMD and other variables as independent risk factors for the presence of T2DM. The diagnostic performance of plasma GSDMD was determined using receiver operating characteristic (ROC) curve analysis, with calculation of the area under the curve (AUC), optimal cutoff value, sensitivity, and specificity. All statistical tests were two-tailed, with a significance threshold of $p< 0.05$, and analyses was carried out using SPSS version 24.0 (IBM Corp., Armonk, NY, USA).

Result

Table 1 shows that, compared with normoglycemic controls, patients with T2DM had significantly higher BMI, fasting blood glucose, HbA1c, fasting insulin, HOMA-IR, total cholesterol, triglycerides, LDL-C, and hs-CRP, and significantly lower HOMA-β and HDL-C (all $p< 0.001$). Age and sex distribution did not differ between groups ($p>0.05$), indicating that the two groups were well matched demographically. These findings suggest that the T2DM group exhibited a more adverse metabolic and inflammatory profile, with marked insulin resistance, β-cell dysfunction, atherogenic dyslipidemia, and systemic low-grade inflammation compared with controls.

Table 1: Baseline clinical and biochemical characteristics of study participants.

Variable	T2DM (n=130) mean ± SD	Control (n=130) mean ± SD	p-value
Age (years)	48.5 ± 7.8	47.9 ± 8.1	0.52
Male/Female n (%)	66/64	64/66	0.79
BMI (kg/m²)	26.2 ± 3.4	24.8 ± 3.1	0.001
FBG (mmol/L)	8.7 ± 7.9	5.1 ± 4.8	<0.001
HbA1c (%)	8.4 ± 5.3	5.6 ± 2.4	<0.001
Fins (μIU/mL)	16.8 ± 14.5	9.6 ± 7.8	<0.001
HOMA-IR	6.4 ± 5.3	2.1 ± 1.7	<0.001
HOMA-β	68.0 ± 22.0	132.0 ± 45.0	<0.001
TC (mmol/L)	5.3 ± 0.8	4.5 ± 0.7	<0.001
TG (mmol/L)	1.7± 1.2	1.0 ± 0.8	<0.001
LDL-C (mmol/L)	3.3 ± 0.7	2.7 ± 0.6	<0.001
HDL-C (mmol/L)	1.02 ± 0.21	1.30 ± 0.24	<0.001
hs-CRP (mg/L)	4.8 ± 3.2	1.9 ± 1.0	<0.001

Values are presented as mean ± standard deviation (SD) for normally distributed. BMI, body mass index; FBG, fasting blood glucose; HbA1c, glycated hemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-β, homeostasis model assessment of β-cell function; TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; Fins: Fasting Insulin; hs-CRP, high-sensitivity C-reactive protein. Student t-test and chi-square test were used to calculate the p-value. $p<0.05$ considered significant.

Plasma levels of GSDMD and inflammatory cytokines were markedly higher in T2DM patients than in controls. Median GSDMD was 70.5 (40–110) pg/mL in the T2DM group versus 6.0 (3.5–8.5) pg/mL in controls ($p< 0.001$), indicating a pronounced activation of pyroptosis in diabetes. Similarly, IL-18 and IL-1β were significantly elevated in T2DM [12.0 (9.5–15.2) vs 3.5 (2.5–4.8) pg/mL and 2.4 (1.6–3.8) vs 0.9 (0.5–1.4) pg/mL, respectively; both $p< 0.001$], reflecting an enhanced systemic inflammatory state in diabetic subjects compared with normoglycemic individuals (Table 2).

Table 2: Comparison of plasma GSDMD and inflammatory markers between T2DM patients and controls.

Marker	T2DM (n=130) median (IQR)	Control (n=130) median (IQR)	p-value
GSDMD (pg/mL)	70.5 (40–110)	6.0 (3.5–8.5)	<0.001
IL-18 (pg/mL)	12.0 (9.5–15.2)	3.5 (2.5–4.8)	<0.001
IL-1β (pg/mL)	2.4 (1.6–3.8)	0.9 (0.5–1.4)	<0.001

Abbreviation: GSDMD: Gasdermin D, IL-18: Interleukin-18, IL-1β: Interleukin 1 beta. Mann-Whitney test used to calculate the p-value. $p<0.05$ considered as statistical significance.

Plasma GSDMD levels showed significant correlations with both glycemic control and inflammatory status. GSDMD was strongly and positively correlated with fasting blood glucose ($r = 0.62$, $p< 0.001$) and HbA1c ($r = 0.58$, $p< 0.001$), indicating that higher GSDMD concentrations are associated with poorer glycemic control. A moderate positive correlation with HOMA-IR ($r = 0.40$, $p<0.001$) and a moderate negative correlation with HOMA-β ($r = −0.36$, $p<0.001$) suggest that elevated GSDMD is linked to greater insulin resistance and impaired β-cell function.

In addition, GSDMD demonstrated significant positive correlations with inflammatory markers IL-18 ($r = 0.46$, $p<0.001$), IL-1β ($r = 0.35$, $p<0.001$), and hs-CRP ($r = 0.24$, $p=0.002$), reflecting that higher pyroptosis activity parallels heightened systemic inflammation. Collectively, these findings imply that plasma GSDMD integrates information on metabolic dysregulation and inflammation and may serve as a composite indicator of T2DM severity.

Table 3: Correlation between plasma GSDMD levels and metabolic/inflammatory parameters in all participants.

Variable vs GSDMD	r value	p-value
FBG	0.62	<0.001
HbA1c	0.58	<0.001
HOMA-IR	0.4	<0.001
HOMA-β	-0.36	<0.001
IL-18	0.46	<0.001
IL-1β	0.35	<0.001
hs-CRP	0.24	0.002

Correlation coefficients (r) were calculated using Pearson's rank correlation. GSDMD, Gasdermin D; FBG, fasting blood glucose; HbA1c, glycated hemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-β, homeostasis model assessment of β-cell function; IL-18, interleukin-18; IL-1β, interleukin-1 beta; hs-CRP, high-sensitivity C-reactive protein. $p<0.05$ considered significant.

Multivariable logistic regression analysis identified several variables as independent predictors of T2DM after mutual adjustment. For every 10 pg/mL increase in plasma GSDMD, the odds of having T2DM increased by 18% (adjusted OR 1.18, 95% CI 1.09–1.29, $p<0.001$), indicating a strong association between higher GSDMD levels and diabetes status. IL-18 and hs-CRP were also independently related to T2DM, with each 1 pg/mL rise in IL-18 (OR 1.10, 95% CI 1.03–1.18, $p= 0.004$) and each 1 mg/L rise in hs-CRP (OR 1.12, 95% CI 1.04–1.20, $p=0.002$) conferring higher odds of disease.

Among traditional risk factors, each 1 kg/m² increase in BMI was associated with a 9% increase in T2DM odds (OR 1.09, 95% CI 1.01–1.18, $p=0.03$), whereas higher HDL-C showed a protective effect, with an OR of 0.35 (95% CI 0.18–0.68, $p=0.002$), meaning greater HDL-C levels substantially reduced the likelihood of T2DM. These results suggest that GSDMD, alongside IL-18, hs-CRP, adiposity, and low HDL-C, independently contributes to T2DM risk, highlighting the importance of pyroptosis-related and inflammatory pathways beyond conventional metabolic factors.

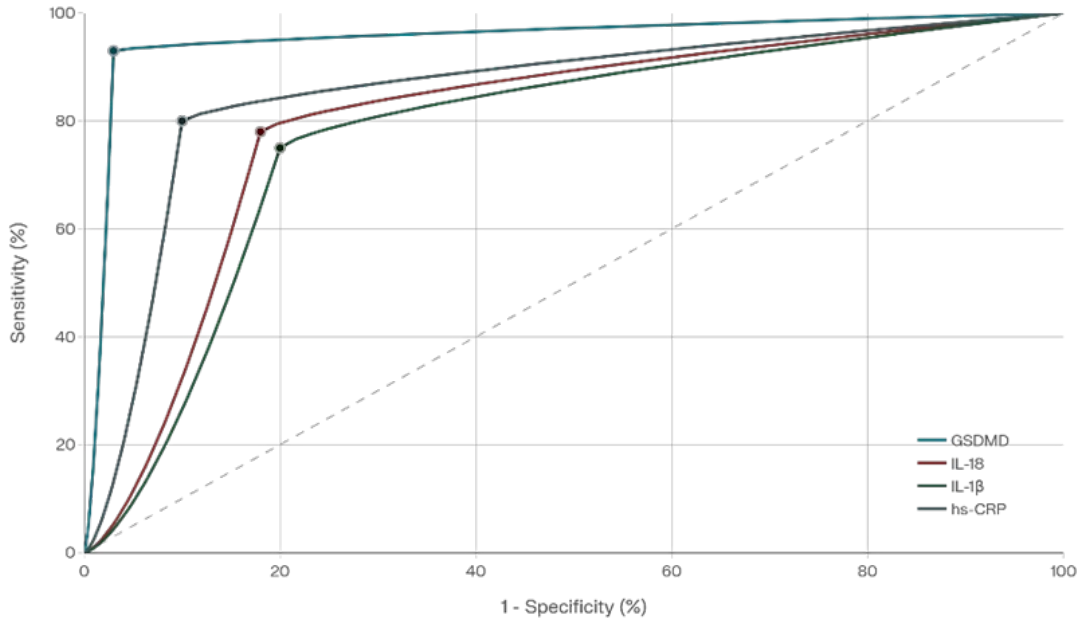
Table 4: Multivariable logistic regression analysis of factors independently associated with T2DM.

Variable	Adjusted OR	95% CI	p-value
GSDMD (per 10 pg/mL)	1.18	1.09–1.29	<0.001
IL-18 (pg/mL)	1.1	1.03–1.18	0.004
hs-CRP (mg/L)	1.12	1.04–1.20	0.002
BMI (kg/m²)	1.09	1.01–1.18	0.03
HDL-C (mmol/L)	0.35	0.18–0.68	0.002

Adjusted odds ratios (ORs) with 95% confidence intervals (CI) are presented for the presence of T2DM (1) vs normoglycemic controls (0). GSDMD, Gasdermin D; IL-18, interleukin-18; hs-CRP, high-sensitivity C-reactive protein; BMI, body mass index; HDL-C, high-density lipoprotein cholesterol.

ROC analysis showed that plasma GSDMD had the highest diagnostic accuracy for distinguishing T2DM patients from controls. With an AUC of 0.98 (95% CI 0.96–0.99), a cutoff value of 17.5 pg/mL provided 93.0% sensitivity and 97.0% specificity, indicating excellent discrimination. IL-18 and IL-1β demonstrated good but lower performance (AUC 0.88 and 0.86, respectively), with optimal cutoffs of 5.0 pg/mL and 1.5 pg/mL yielding sensitivities of 78.0% and 75.0% and specificities of 82.0% and 80.0%. hs-CRP showed an AUC of 0.90 (95% CI 0.86–0.94), with a cutoff of 3.5 mg/L achieving 80.0% sensitivity and 90.0% specificity, but still inferior to GSDMD, underscoring GSDMD as the most powerful single biomarker for T2DM in this cohort (Figure 2).

Figure 2: Receiver operating characteristic (ROC) curves of plasma GSDMD, IL-18, IL-1β, and hs-CRP for discrimination of patients with type 2 diabetes mellitus from controls.



The ROC analysis shows excellent diagnostic performance of GSDMD (AUC 0.98, 95% CI 0.96–0.99; cutoff 17.5 pg/mL; sensitivity 93.0%; specificity 97.0%), with lower but still good accuracy for IL-18 (AUC 0.88, 95% CI 0.83–0.92; cutoff 5.0 pg/mL; sensitivity 78.0%; specificity 82.0%), IL-1β (AUC 0.86, 95% CI 0.81–0.91; cutoff 1.5 pg/mL; sensitivity 75.0%; specificity 80.0%), and hs-CRP (AUC 0.90, 95% CI 0.86–0.94; cutoff 3.5 mg/L; sensitivity 80.0%; specificity 90.0%), highlighting GSDMD as the most accurate biomarker among the tested inflammatory markers for early detection of type 2 diabetes mellitus.

Table 5: Diagnostic performance of plasma GSDMD and related inflammatory markers for T2DM based on ROC curve analysis.

Marker	AUC	95% CI	Cutoff (pg/mL)	Sensitivity (%)	Specificity (%)
GSDMD	0.98	0.96–0.99	17.5	93	97
IL-18	0.88	0.83–0.92	5	78	82
IL-1β	0.86	0.81–0.91	1.5	75	80
hs-CRP	0.9	0.86–0.94	3.5	80	90

AUC, area under the receiver operating characteristic (ROC) curve; CI, confidence interval. Sensitivity and specificity are reported at the optimal cutoff (Youden index) for each marker. GSDMD, Gasdermin D; IL-18, interleukin-18; IL-1β, interleukin-1 beta; hs-CRP, high-sensitivity C-reactive protein.

Discussion

The present study demonstrates that plasma GSDMD is markedly elevated in newly diagnosed T2DM patients compared with age- and sex-matched normoglycemic controls and is strongly linked to both metabolic derangement and systemic inflammation. The two groups were demographically comparable in terms of age and sex, which minimizes confounding from these factors, but the T2DM group exhibited significantly higher BMI, fasting glucose, HbA1c, Fins, HOMA-IR, lipid profile, and hs-CRP, along with lower HOMA-β and HDL-C, confirming a classic picture of insulin resistance, β-cell dysfunction, dyslipidemia, and low-grade inflammation. Against this background, plasma GSDMD, IL-18, and IL-1β were all substantially raised in T2DM, indicating activation of pyroptosis-related inflammatory pathways in diabetic subjects [12-14].

The correlation analysis provides important mechanistic insight into the role of GSDMD in T2DM. GSDMD showed strong positive correlations with FBG and HbA1c, suggesting that higher GSDMD levels parallel worsening short- and long-term glycemic control. Its positive association with HOMA-IR and negative association with HOMA-β indicate that GSDMD tracks both peripheral insulin resistance and impaired β-cell function, consistent with experimental data implicating inflammasome-mediated pyroptosis in β-cell injury and insulin signaling defects. Moreover, GSDMD correlated moderately with IL-18, IL-1β, and hs-CRP, supporting the concept that it integrates information on inflammasome activation and systemic inflammation. Together, these relationships reinforce GSDMD as a central node connecting metabolic stress, innate immune activation, and β-cell failure in T2DM [15,16].

Multivariate logistic regression further highlights the independent contribution of GSDMD to T2DM risk. Even after adjusting for BMI, HDL-C, and other inflammatory markers, GSDMD remained a significant predictor, with an 18% increase in the odds of T2DM for each 10 pg/mL rise in its plasma concentration. IL-18 and hs-CRP were also independently associated with T2DM, reflecting the importance of chronic inflammation; however, the magnitude and robustness of the GSDMD association suggest that pyroptosis may play a more proximate role in disease pathogenesis than generalized inflammation alone. The inverse association of HDL-C and positive association of BMI with T2DM are in line with established cardiometabolic risk patterns, but their coexistence with a strong GSDMD signal indicates that pyroptosis adds explanatory value beyond traditional risk factors [17-19].

From a diagnostic standpoint, GSDMD clearly outperformed other evaluated markers. The ROC analysis showed an AUC of 0.98 for GSDMD, with an optimal cut-off of 17.5 pg/mL achieving 93% sensitivity and 97% specificity for identifying T2DM, which falls in the "excellent" range for biomarker performance. In comparison, IL-18, IL-1β, and hs-CRP yielded lower AUCs (0.88, 0.86, and 0.90, respectively) and less favorable sensitivity–specificity profiles, indicating that they are less accurate if used alone. These findings suggest that plasma GSDMD could serve as a superior diagnostic tool, particularly in settings where early identification of high-risk individuals is critical. Because traditional markers like fasting glucose and HbA1c often fail to capture early-stage or intermittent dysglycemia, integrating GSDMD into diagnostic protocols could improve the identification of metabolically unstable patients. This approach may allow for clinical intervention before these individuals reach a state of overt metabolic decompensation. [19,20]. The primary strengths of this investigation reside in its rigorous case-control design, characterized by stringent age- and sex-matching to minimize potential confounding. Furthermore, the simultaneous quantification of GSDMD alongside a comprehensive panel of metabolic and inflammatory biomarkers allows for a high-resolution analysis of their pathophysiological associations.

Notwithstanding these strengths, several methodological limitations merit further consideration. First, the cross-sectional nature of the data precludes causal inference; it cannot be definitively concluded whether elevated GSDMD precedes the onset of T2DM or mainly reflects established disease. Longitudinal studies following normoglycemic or prediabetic individuals over time was necessary to clarify temporal relationships and assess predictive value for incident diabetes. Second, the sample was drawn from a single tertiary care center, which may limit generalizability to community settings or other ethnic groups. Third, although multiple confounders were adjusted for, residual confounding by unmeasured factors such as diet, physical activity, or subclinical infections cannot be entirely excluded.

Despite these limitations, the present results align closely with emerging international data showing that GSDMD-mediated pyroptosis is central to the inflammatory–metabolic axis in T2DM and extend this evidence to an Indian hospital-based population. The strong independent association with T2DM, robust correlations with glycemic and inflammatory parameters, and excellent diagnostic performance collectively support plasma GSDMD as a promising biomarker and a potential therapeutic target. Future work should focus on validating these

findings in larger, multi-center cohorts, exploring the utility of GSDMD in prediabetes and complication risk stratification, and assessing whether interventions that attenuate inflammasome activation and GSDMD cleavage can improve metabolic outcomes.

Conclusion

Plasma GSDMD levels were markedly elevated in newly diagnosed T2DM patients compared with age- and sex-matched normoglycemic controls and showed strong associations with hyperglycemia, insulin resistance, β-cell dysfunction, and systemic inflammation. In multivariable analysis, GSDMD remained an independent predictor of T2DM, and ROC analysis demonstrated excellent diagnostic accuracy (AUC 0.98) with high sensitivity and specificity at a cut-off of 17.5 pg/mL, outperforming IL-18, IL-1β, and hs-CRP. These findings indicate that plasma GSDMD is a robust, clinically promising biomarker that integrates metabolic and inflammatory information and may be useful for early diagnosis and risk stratification in T2DM, warranting validation in larger, longitudinal and multi-center studies.

Author Contribution

MS: Proposal concept, writing of manuscript, experimental work, Data analysis.
AK: Formal analysis, Manuscript editing.
AG: Clinical sample, Resources.

Declaration of Conflict of interests

The authors of this article declare that there is no conflict of interest with regard to the content of this manuscript.

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Data Availability

The datasets used and/or analysed during the current study are not available because of the Institutional policy.

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Research Article

An optimized method for setting Internal Quality Control targets (mean and limits) for multi-instrument Internal Quality Control strategies in hematology?

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Keywords

components of random error in multi-instrument sites, peer group analyzers statistics, IQC of multiple analyzers

Abstract

Objectives: Internal Quality Control (IQC) procedures must evolve as laboratory practices change to meet the requirements of higher patient volumes and the greater need for comparability of results across laboratories. This study developed a strategy to detect significant errors in routine hematology analytical processes using a novel approach to setting IQC material targets (mean and limits) across multiple instruments. This has not been described for hematology laboratories before.

Content: The approach described used a common IQC sample mean and limits determined from a manufacturer-based peer group using the same IQC material. The limits involved a new parameter, the random error of the peer group analyzers around their own mean ($SD_{intra}90$), to detect bias or imprecision. The model was assessed over five months using 17 analyzers that measured hemoglobin and red cell count. Using a common mean requires that the different analyzers exhibit no significant bias.

Summary: The model effectively controls analytical error in a network of laboratories using the same measurement system. This model aligns with the best theoretical principles for patient risk reduction and harmonizes practices for acceptance and rejection across the network.

Outlook: The model presents an approach to IQC that meets the demands of real-world practice.

Introduction

Clinical hematology laboratories employ statistical methods and tools, primarily control charts, to monitor and control analytical processes, ensuring they operate efficiently and produce reliable results for diagnosing and monitoring patient diseases [1–3].

The nature of the cellular measurands in hematology presents special challenges in providing suitable internal quality control (IQC) samples, leading to the use of preserved human or animal blood materials to ensure pre-analytical stability [4]. Consequently, commercial controls from manufacturers and independent providers are widely used. These samples are stabilised to extend their shelf life but are often very sensitive to temperature and storage conditions [5]. Using the manufacturer-provided limits of acceptability in the insert is common [6], but not considered best practice as they are usually broader than the laboratory's self-determined limits [6,7].

In 2024, the International Council for Standardization in Haematology (ICSH) published the results of an international survey into IQC practices.[6]. They found diversity in both guidance and practice worldwide. There were two primary approaches to setting IQC targets: either use the IQC limits supplied by the manufacturer in the insert or calculate the targets using the IQC values. For hemoglobin (Hb), the limits used ranged from mean ± 1.5 to 6 SD (or 11.5S to 16S as Westgard rules [3]), with a median of 4 SD. These limits were significantly broader for manufacturer-provided limits. Responding to this variation, the ICSH produced guidelines to promote best practices [8].

However, they neglect the issue of the IQC situation, where multiple instruments measure the same analyte, a very real problem in modern practice, as all instruments will have a slight difference in bias and imprecision when compared to each other. There is little guidance in the literature on this situation, but what is suggested is individual instrument targets, which we will show is not theoretically optimal [9].

Suppose two or more different analyzers are measuring the same analyte. In that case, two alternative IQC strategies can be employed [10]; the first is to manage each instrument separately, that is, set an instrument-specific mean and SD. The other option is to have a common mean and SD (and IQC batch) for all instruments. Either option has advantages and disadvantages depending on what the laboratory aims to achieve. If the goal is to reduce unreliable patient results during an out-of-control situation, then Parvin et al [10] showed a need to centre the IQC rejection limits on a common mean. It has been demonstrated in simulation studies that entering the control rule on the common mean is an optimal strategy for reducing patient risk with analyzers that have significant and stable uncorrected bias [10,11]. Not surprisingly, Yago and Pla found that IQC procedures are generally not optimal in the presence of significant bias [11]. This must be assessed and monitored.

Figure 1 illustrates an example of an analytical site with a clustering of four analyzers where the laboratory uses individual instrument targets to develop its control charts. Each analyser has its own targets (mean/SD), and an identical IQC value can result in rejecting one analyzer run and accepting another.

Figure 1: The data from multiple instruments measuring the same IQC sample. The coloured plots represent separate analyzers' mean(m) and error distributions.

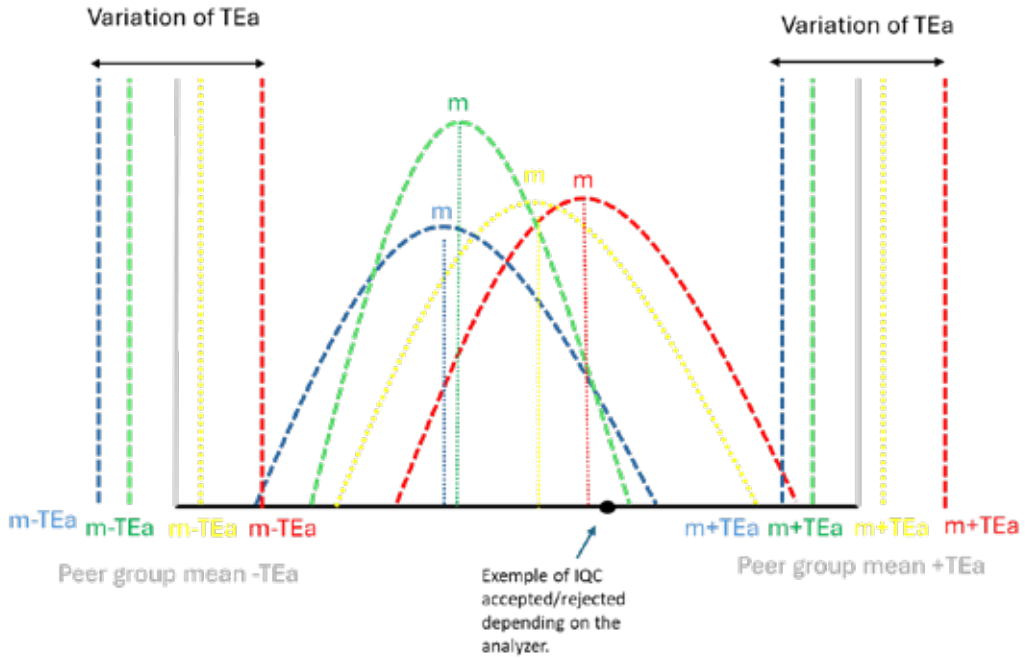


Figure 2: An example of the data from multiple instruments measuring the same IQC sample. The coloured plots show the error distribution of separate analyzers.

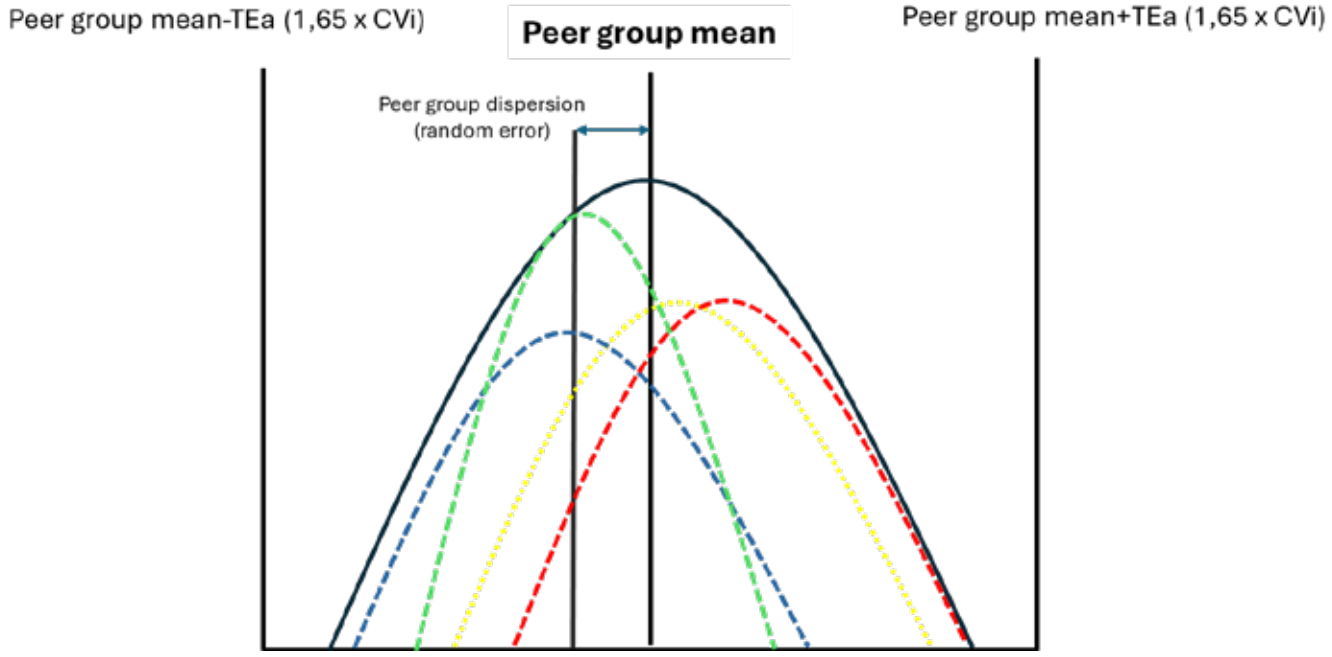


Figure 2 shows the effect of using a common CV and mean for error detection.

All IQC strategies rely on accurate IQC targets of mean and standard deviation (SD) to set rules for the acceptance/rejection of an IQC sample result. The ICSH Guideline recommended that "Laboratories should choose whether to apply their own calculated action limits as prescribed by CLSI H26-A2 [12], or to tighten the limits provided by the manufacturer. For example, suppose a cell counter manufacturer does not recommend that the target and limit values supplied with the assay sheet be used routinely, or whether they should be verified before use by the diagnostic laboratory. This should be clearly stated in the product insert" [6].

Deciding to use common IQC targets raises the question of how to derive them. The obvious and most optimal option for a laboratory is to use its own IQC data. However, this approach has disadvantages, including small sample sizes and the risk that small biases will accumulate over time, potentially leading to a significant bias that may not be detected [13]. External Quality Assurance schemes are designed to detect these trends but may be slow. Some manufacturers calculate peer-group means and SDs for the provided IQC material, based on all instruments supported by the supplier. A peer group is defined as a group of analyzers from the same manufacturer's analyzer series using the same lot number of QC material and measurement method. These are provided regularly and automatically calculated from results downloaded from each analyser. The advantage of this approach is the number of data points used to determine the IQC targets.

Different ways exist to set control rules (reject/accept) based on the mean and SD. Usually, the rule is to reject the batch of results if the IQC sample value lies outside the mean ± 2 or ± 3 SD. Based on the Gaussian distribution, these rules will only flag if the IQC result is outside the 95th or 99th percent of expected values. However, there are other options for the percentile to choose for rejection in the IQC rule.

This paper describes an IQC model for managing multiple hematology analyzers that measure the same analytes at the same site or within a network. The approach will utilize common, manufacturer-provided IQC targets and a novel method, the peer group rejection limit, specifically the 90th percentile ($CV_{intra}90$), as the limit of acceptability. The red cell count (RBC, measured using the impedance principle of measurement) and Hb (measured using the spectrophotometric principle of measurement) were used as examples in this study.

Methods

There were two arms to this study. A retrospective arm that collected long-term data to analyze long-term data to determine the IQC parameters that would be used for the construction of the IQC control charts, specifically the target mean and dispersion limit.

The second arm involved a prospective study using these targets and assessing their reliability.

Sources of retrospective peer group data

Sysmex provides data by instrument peer group, all instruments of the same model. These Sysmex instrument peer groups consisted of between 1,269 to 1,786 analyzers distributed

throughout Europe. The first step of the study consists of retrospectively analyzing Sysmex peer group data from previous IQC batches (years 2021/2022) to determine the parameters necessary for the construction of common control charts.

Target

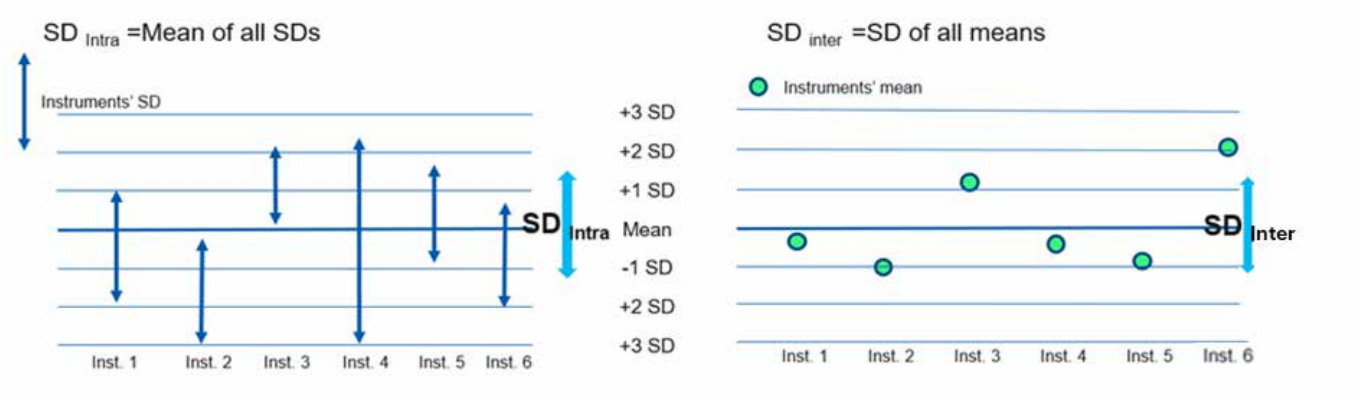
The robustness of the peer group averages, which will serve as target averages for each batch, is checked by recording the number of participants (N). The reliability of this target average is evaluated by assessing the distribution of these averages. The stability of the group average is crucial.

Determining the dispersion target

Sysmex outsourced IQC reports offer two peer group result variation points, the SD_{inter} and the SD_{intra}. The SD_{intra} is the random error of the peer group analyzers around their own mean (the mean of all the SDs), whereas the SD_{inter} is the dispersion of the analyzer means about the consensus mean (the SD of all the means). The total SD, SDtotal, is the sum of these two components and is a form of measurement uncertainty of the peer group (see Figure 3).

$$SDtotal = \sqrt{\{(SD_{intra})2 + (SD_{inter})2\}}$$

Figure 3: Components of the imprecision of separate analyzers.



Sysmex provided the authors with SD_{intra} percentile data from fifteen batches of IQC material (30 months) that had been used in Europe between 2021 and 2022. These SD_{intra} percentiles were converted to CV_{intra} with the mean of the peer group corresponding to each batch to obtain $CV_{intra}50$ / $CV_{intra}90$ / $CV_{intra}95$. After checking the normality of distribution of the percentile CVs of the fifteen batches (Shapiro-Wilk test), an average of the CVs weighted with the number of analyzers included in each batch was calculated.

The CV_{intra} chosen to set the limits is the average CV_{intra} 90, the state-of-the-art imprecision for specific analyzers and IQC level. The selection of the $CV_{intra}90$ over the $CV_{intra}95$ or $CV_{intra}50$ was empirical. The relevance of this choice was verified by comparing the $CV_{intra}50/90/95$ with the pooled CVs data from the analyzers used in the following part. The pooled CV was calculated as the total CV for an IQC sample batch from the seventeen analyzers and compared to the percentile data from the peer group provided by Sysmex.

The prospective study - using the IQC targets on real data

The prospective study was conducted over five months, monitoring the analytical process from December 1, 2023, to April 30, 2024. This period is covered by batch numbers 3339 and 4023. It was carried out with seventeen Sysmex XN analyzers (Sysmex Europe, Hamburg, Germany) distributed across four different sites covering the Lyon region (France). Five analyzers on a site that we will call "D", six analyzers on a site named "S", three analyzers on a site named "R" and three analyzers on a site named "F". These seventeen analyzers share the same reference intervals on the reports. Whether in a diagnostic context or during monitoring or screening, one of these seventeen instruments can analyze patient samples indifferently.

The total acceptable error is defined with the biological variations of intra-individual CV data obtained from the EFLM database [14,15]: 1.65 x CVi (intraindividual biological variation) for the IQC Hb and RBC level 2 (normal level). This approach comes from the notion of MAU EFLM (allowable measurement uncertainty) [16].

IQC rules

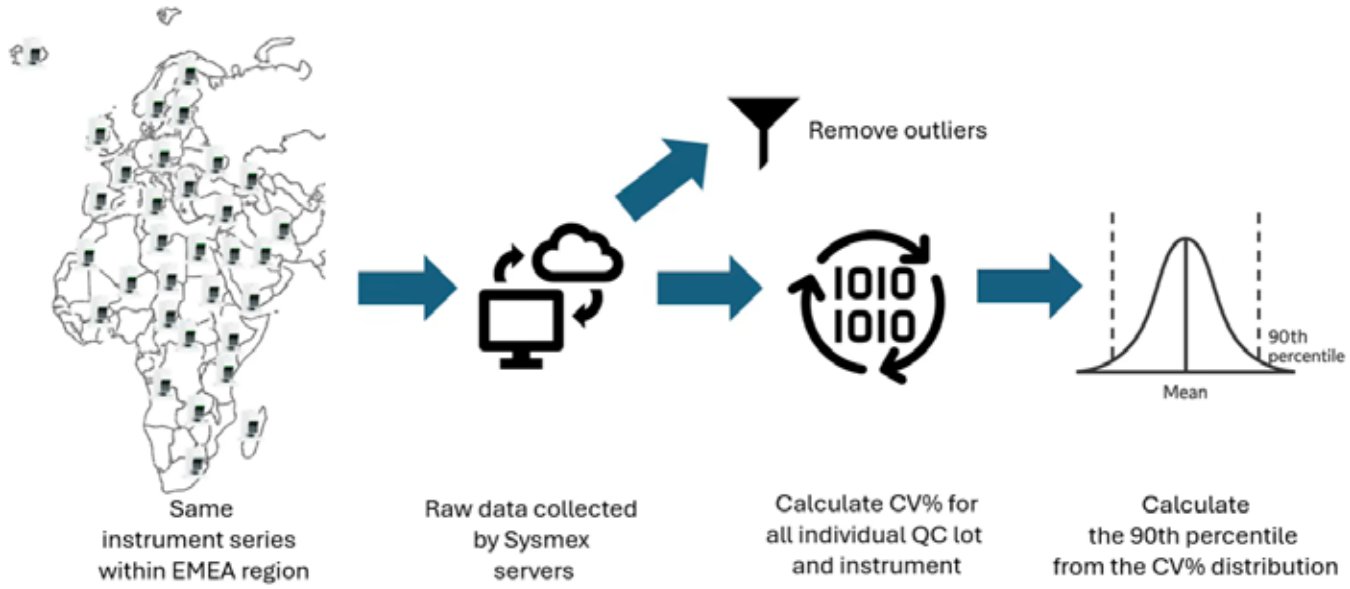
Depending on the number of samples, which routinely range between 300 and 500 per instrument per day, two or three levels of IQC are used at the start of the analysis series: one level in the middle of the series, and a third IQC event of one or two levels at the end. At the start or middle of the series, the Westgard 22s rule was used as an alarm rule, while the 13s and 2/32s rules (two levels of out-of-bounds controls in the same direction) were used to signal a rejection.

Use of patient-based moving medians.
Patient-based quality control is well established in hematology with Bull's algorithm [17] however, other models are available with better detection rates [18]. The ISLH suggested that "haematology analyzers may be able to provide regular moving averages for other parameters and programmable for adjusting outlier limits and the number of patient results averaged" [6]. Patient measurement data for Hb and RBC were extracted retrospectively. The moving median of block 50 for each analyzer was calculated after Winzorization from the usual values of each parameter. From the 15th day, the median ± 2 SD of each parameter was calculated and fixed for the remainder of the study to detect any retrospective drift or shift during the analyzed period. This analysis, presented in Figure 7, enables us to superimpose the variations observed in the IQC with those observed in the patient data.

Results

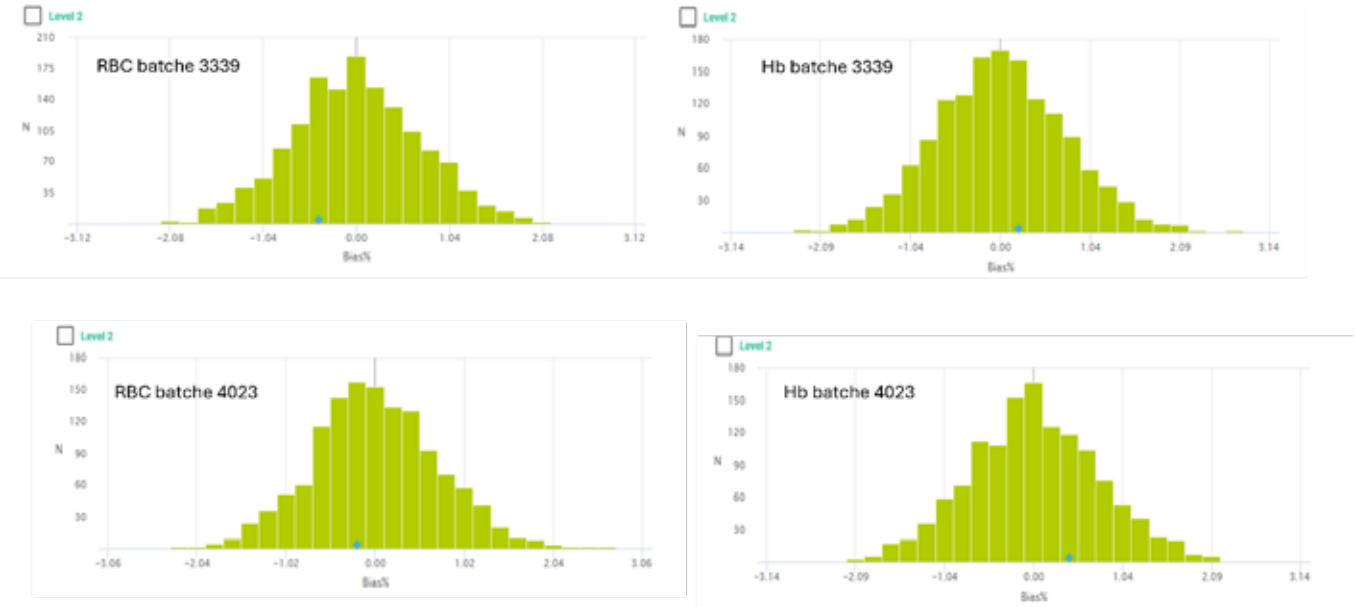
Prospective study IQC and patient data
Each new batch of manufacturer IQC samples (Sysmex Europe, Hamburg, Germany) was received the week before implementation. During the overlap period, samples from the latest batch of material are measured four times on the seventeen instruments. Then, two to three days before the routine implementation of this IQC batch, the control chart target is determined using the Caresphere data hub (https://www.sysmex-europe.com/services/qc-online-services/caresphere-xqc/). This is calculated after all values outside the group mean ± 5 SD_{inter} are excluded using the European reference group. This group average is available to all users in real-time and is updated daily. The group averages nevertheless remained stable throughout the validity period of the batches studied here. The concentration levels between IQC batches are always in the same part of the method accuracy profile. Thus, the SD used as limits for the seventeen analyzers is the $CV_{intra}90$ described above, multiplied by the peer group average corresponding to each of the two batches studied. Caresphere XQC is an EQA provider (ISO/IEC 17043) accredited by the Japanese Accreditation Body (JAB) (https://www.sysmex-europe.com/services/qc-online-services/caresphere-xqc/) (Figure 4).

Figure 4: The Caresphere process for determining IQC material targets (mean and CV) is provided to laboratories daily.



Target
The peer groups of batches 3339 and 4023 comprised 1,249 and 1,294 analyzers, respectively, on the first startup day and 1,474 and 1,313 on the last day. The distribution of the analyzer averages around the consensus average was normal (Figure 5).

Figure 5: The peer group data of IQC batches 3339 and 4023.



The group average on day one for lot 3339 was 4.310 T/L for RBC and 11.29 g/dl for Hb. The end-of-use group average for this lot was 4.306 T/L for RBC and 11.28 g/dl for Hb. Regarding batch 4023, the first and last day averages were 4,314 T/L and 4,310 T/L for RBC and 11.37 g/dl and 11.38 g/dl for Hb.

Verification of assumptions

It is essential to verify that all seventeen instruments have minimal differences in bias or imprecision. The absence of significant bias was assessed by constructing a figure showing the daily biases (%) of each analyzer relative to the peer-group average (Figure 6). The total error (TE) is defined as the bias plus 45% of the dispersion due to random error: bias + 1.65 CV. The system is in control if TE < TEa or bias + 1.65 x CV < TEa. The upper limit before significance of the positive bias at the 5% risk level can then be calculated as follows: upper limit of bias = TEa - 1.65 x actual CV. And the lower limit before significance of a negative bias is calculated as follows: lower limit of bias = -TEa + 1.65 x CV. The CV used corresponds to the actual mean CV of the analyzers over the studied period. In other words, if the bias of an analyzer plus the dispersion due to random error does not exceed the total error, we cannot conclude that it is significant. The significance of the bias between analyzers was estimated using the same principle, but this time considering the analyzer with the lowest average (MINa) and the one with the highest average (MAXa) for each batch of IQC analyzed. If the delta% between the MINa and MAXa plus the random error coefficient (CV) of the analyzer with the greatest dispersion is less than the TEa, we cannot conclude that the difference between the two most deviant analyzers is biologically significant.

The SD_{intra}90 used as the dispersion limit for the seventeen analyzers must represent their overall performance. The comparison of the overall actual dispersion of the method with the theoretical dispersions (SD_{intra}50/90/95) is carried out by calculating the pooled variance (S2pooled) converted to pooled CV from the SD, means and number of points of each IQC batch for the seventeen analyzers [19].

$$s^2_{pooled} = \frac{1}{N-1}\left(\sum_{k=1}^{m} s_k^2(n_k-1) + \sum_{k=1}^{m} n_k\bar{x}_k^2 - \frac{1}{N}\left[\sum_{k=1}^{m} n_k\bar{x}_k\right]^2\right)$$

Bias and comparability

The acceptable bias between analyzers and the group average over five months was verified through a daily visual review, which confirmed that the bias of the analyzers did not exceed the defined bias objective. The difference of the average MAX and average MIN of hemoglobin and RBC IQC values for each batch was compared.

For batch 3339, the MIN and MAX averages corresponded to analyzers D4 and D2 for Hb and D4 and to D1 for RBC. The deltas plus 1.65 x CV were 3.29% for Hb and 4.43% for RBC, with a TEa of 4.5% for Hb, and 4.6% for RBC. For batch 4023, the MIN and MAX averages corresponded to analyzers R3 and D2 for Hb, and to analyzers R1 and D1 for RBC. The deltas plus 1.65 x CV were 3.92% for Hb, and 4.43% for RBC, with a TEa of 4.5% for Hb and 4.6% for RBC. It cannot be concluded that the analyzers are not comparable.

Figure 6: Daily IQC bias for all analyzers over the assessment period.

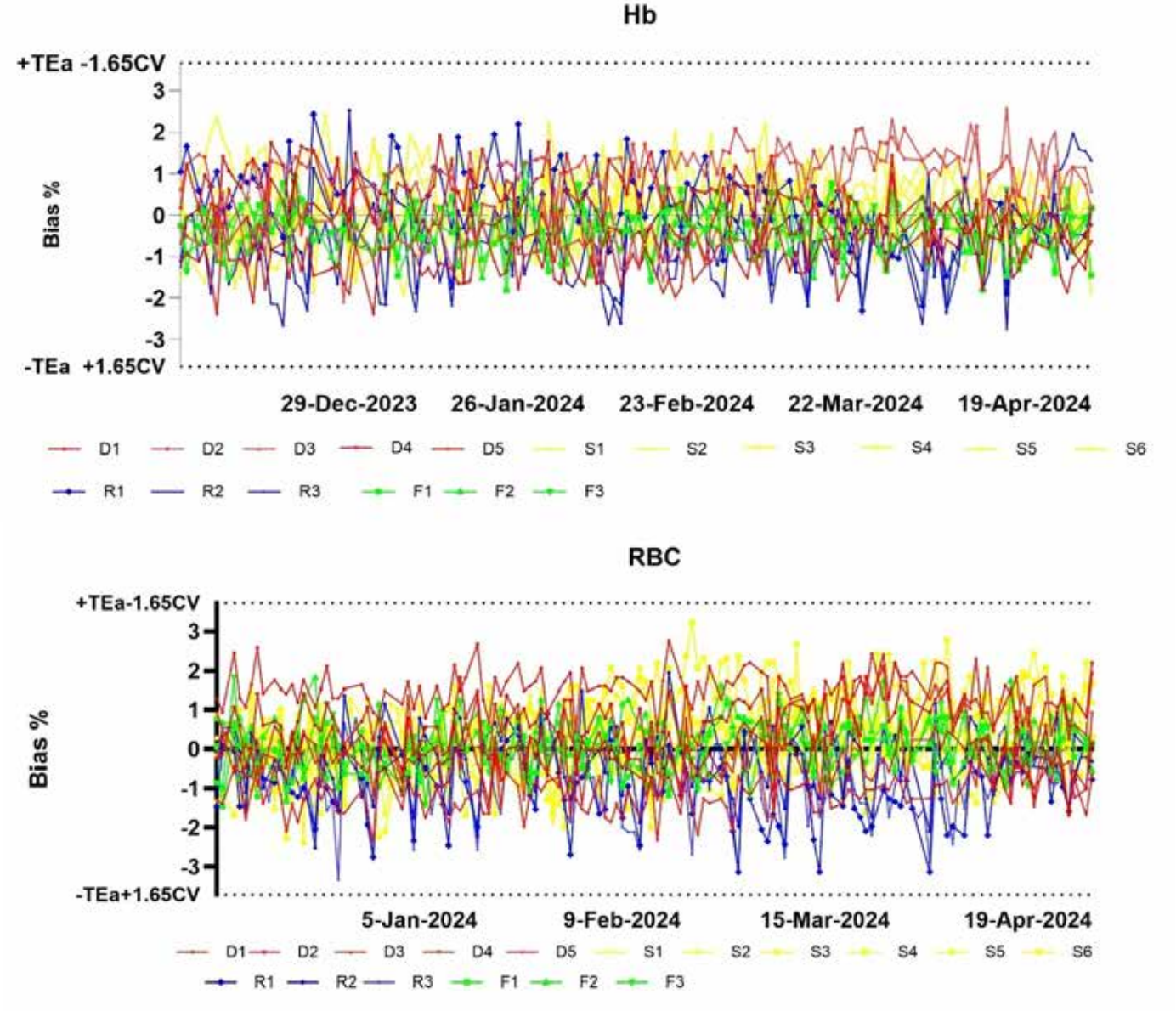


Figure 6 shows that there is no daily bias over the period studied.

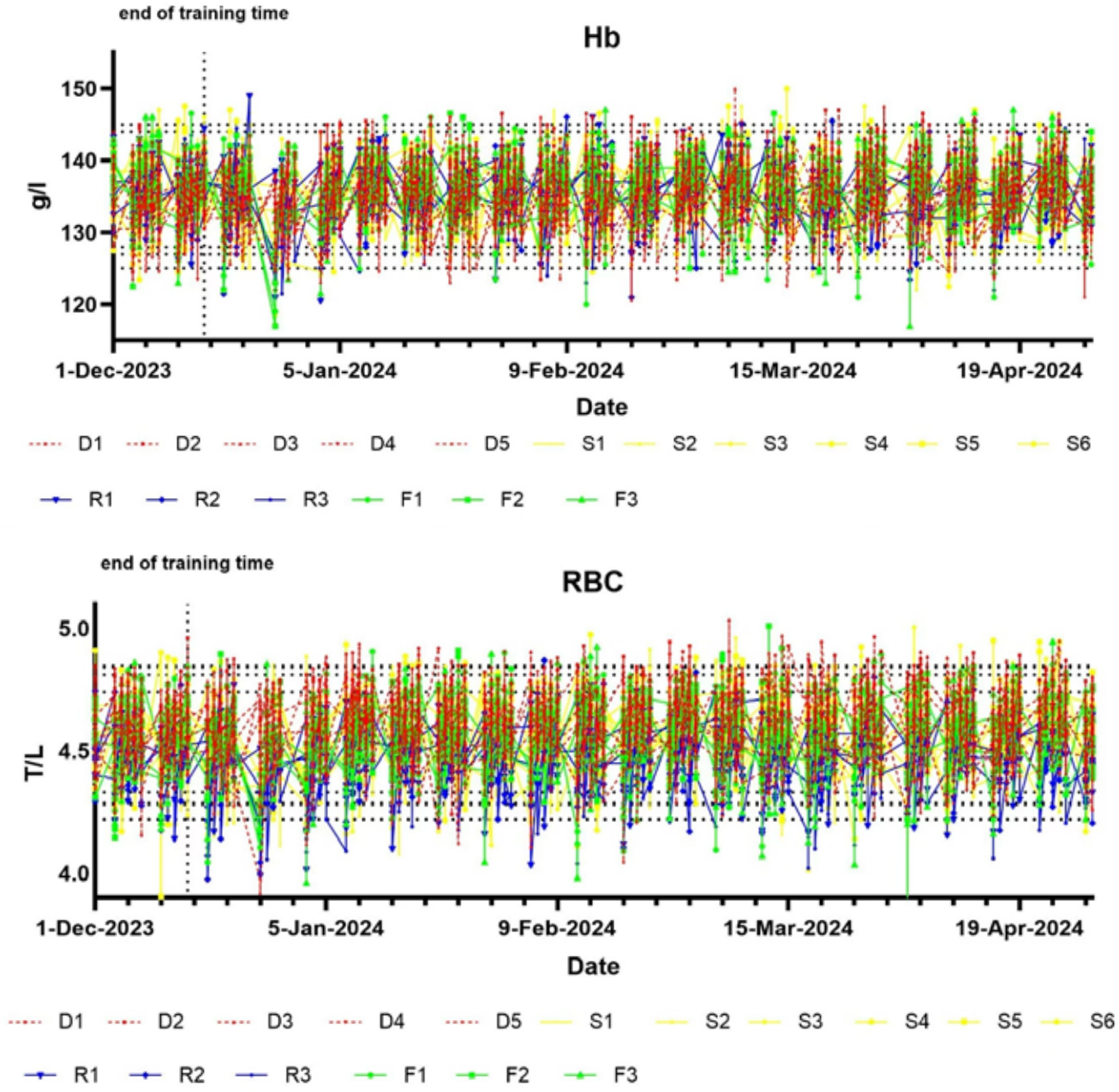
Imprecision

The percentile CV closest to the pooled CVs is consistently $CV_{intra}90$. These two CVs are not significantly different [19]. This is not the case, however, when considering $CV_{intra}50$ and $CV_{intra}95$ (see Supplementary Table 1). Therefore, it was set for the entire lifetime of the analyzer, unless the manufacturer informed us of a change in IQC manufacturing or packaging, or a global shift in the analyzer bias. In which case, these CVs should be revised.

Retrospective analysis of patient outcomes from moving median

We utilised the moving median of patient data to demonstrate that the model is effective in practice (Figure 7).

Figure 7: Evolution of patient medians for all analyzers with a block size of 50 and Winsorization from usual values (11.5 to 16.7 g/dl for Hb and 3.8 to 6 T/L for RBC).



The four analytical sites are identified by color. From December 1st to 15th, there was a training period. The black dotted lines represent the mean ± 2 SDs for each analysis site at the end of this test period.

There does not appear to be any seasonal effect over the analyzed period. All analyzers displayed a negative spike during the Christmas vacation, when most patients come from emergency departments or present serious pathologies that require frequent monitoring and explanation of lower hemoglobin levels. The revision of the IQC curves that day confirmed this hypothesis, making it possible to exclude a technical problem.
Supplementary Figure 1 demonstrates a simulation of the impact of + 1 SD bias on the seventeen analyzers and the error detection rate. For analyzers perfectly centered on the group mean before the introduced bias, the error will be detected at the first IQC point. The error will be immediately apparent for analyzers slightly above the group mean. However, not surprisingly, for analyzers running below the group mean (e.g., D4 and F2), the added bias cancels the negative bias, and there is no IQC alarm.
Supplementary data Figure 2 shows Violin plots for the patient medians of all analyzers with a block size of 50 and Winsorization outside reference intervals (11.5 to 16.7 g/dl for Hb and 3.8 to 6 T/L for RBC). The four analytical sites are identified by color. From December 1st to 15th, there was a training period. The colored dotted lines represent the median ± 2 SD.

Error detection
Once the $CV_{intra}90$ was determined, the alarm rates ±2 SD and rejection rates ±3 SD were calculated from the $SD_{intra}90$ for each analyzer. The rates of ± 2 $SD_{intra}90$ for hemoglobin are 4.2% (121/2894 QC points), and those of 3 $SD_{intra}90$ are 0.3% (8/2894 QC points). For RBC, the rates of ± 2$SD_{intra}90$ are 5.5% (178/2951 QC points), and those of 3 $SD_{intra}90$ are 0.5% (15/2951 QC points). These fall within the expected error detection range. Recall that the analyzers are centered on the mean of the peer group and not on their own mean. Thus, an analyzer exhibiting, for example, a slight downward shift, will trigger more -2s or -3s alarms than a perfectly centered analyzer with the same random error. It is this characteristic that makes our strategy effective for more quickly detecting the introduction of bias on one of the analyzers our instrument compared to the true value but also compared to other mirror analyzers (peer group average). See Supplementary Data Figure 3 for an example on an individual analyzer IQC.

Discussion
This paper aims to revisit IQC target selection in hematology, where a network of instruments is involved. An IQC strategy CLSI C24-Ed4 defines an SQC strategy as the "number of IQC materials to measure, the number of IQC results and the IQC rule to use at each IQC event, and the frequency of IQC events" [20]. Critical to the success of any IQC rule is the accuracy of the IQC target values of mean and acceptable range (IQC limits) [21].

Traditionally, many laboratories have determined these IQC targets using manufacturer-provided values on the IQC insert or by modifying these values [8]. This approach overlooks the fact that many laboratories operate multiple instruments to measure the same test. Each instrument has some inherent variation, and using different IQCs for every instrument is less optimal for managing patient risk. The optimal strategy in this situation is to use a single reference mean across all instruments, as shown in Figures 1 and 2.

As mentioned, setting accurate IQC targets is crucial for running an effective IQC strategy. The IQC targets (mean and CV) for the model were obtained from the manufacturer's website. These are dynamic and robust targets because of the contemporary instrument peer group sample size. The novel aspects of this investigation include using common IQC targets for all analyzers and utilising the $SD_{intra}90$ as the dispersion limit (Figure 5). This model has been applied to routine clinical chemistry networks [22].

The significant benefits of centering the analyzers on a consensus average from a robust peer group and common SD are:

- to detect and immediately correct a systematic error as soon as a new batch of IQC is set up (without waiting for a possible poor EQA result or a monthly retrospective outsourced IQC report with a poor SDI).
- to guarantee the comparability of intra- and inter-site analytical results via IQC data (without requiring exchanges of patient samples between technical sites.
- harmonize practices in terms of acceptance and rejection across the network.

In fact, given that the group average is the reference and the group CV is known, this is almost equivalent to performing a form of internal group quality assessment three times a day. This is why the daily bias was compared against the TEa, the dispersion due to random error, as a limit. However, some Westgard rules cannot be used in these circumstances (41S and 10x).

The $SD_{intra}90$ ($CV_{intra}90$) is a practical control limit. The total SD, through these two components, represents the overall dispersion of a method (mean dispersion and variance dispersion). For the network analyzers, it accurately reflects the overall performance of these instruments. The $SD_{intra}90$ is the maximum all-instrument imprecision achieved when all instruments have long-term stable performance, that is, no detected errors.

For any IQC strategy, it is essential not to neglect the critical importance of the run size, the number of IQC samples measured at each IQC event, and the placement of the IQC

samples [23–26]. The decrease observed in MaxE(NUF), or the Maximum number of patients affected in an undetected out-of-control condition, for a given control rule, becomes more significant as the number of control samples analyzed in each IQC event increases. This enables reaching the same target MaxE(NUF) (i.e., the same risk level for the patient) while analyzing a larger number of patient samples between IQC events, as the value of MaxE(NUF) obtained during an out-of-control condition is directly proportional to the run size [8].

The IQC rules used were standard. An assessment of intra-instrument bias was conducted before the model was implemented, and the $CV_{intra}90$ was used as the acceptable variation about the mean as this was closest to the pooled CV.

Validating the success of an IQC rule is difficult, but this was undertaken by assessing any significant statistical differences in daily IQC means and CVs for the different instruments. Patient moving medians were also evaluated for bias between instruments, and none was detected. The laboratories are enrolled in the national EQA scheme, and during the investigation period, no nonconforming results were found in the seven EQA cycles.

The model presented in this investigation provides a mechanism to assign the most statistically appropriate targets for the mean and allowable IQC value ranges to achieve the goal stated above. The model was validated prospectively.

Conclusion

Quality Control strategies must accommodate technological changes, even though the fundamental principle of reducing introduced variation remains unchanged. The increased number of samples processed in routine hematology laboratories has led to the use of multiple instruments to handle the workload, which needs a common IQC strategy to control for intra- and inter-instrument variation. It has added to the problem of ensuring comparability between measurement systems and managing bias between individual instruments. The goal of any IQC strategy needs to change to reduce patient risk, ensuring that patients obtain the same results regardless of the measuring instrument used in a laboratory or a network of laboratories [10]. The model presented focuses on identifying the bias between different instruments which can reduce variability between patient results.

Quality Control strategies continue to evolve in response to changing laboratory requirements. Most notably, consistent results are required when a patient sample is tested on different analyzers over time.

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Figure 4 was used with permission from Sysmex.

CRediT Author Statement

Conceptualization, Methodology: JMG, MB, TB.
Software: LT, MB
Validation, Analysis: LT, MB
Investigation: JMG, MB
Writing (Original draft, review and editing): TB, MB, JMG
Visualization: MB
Supervision, Project Admin: JMG, TB.

Declaration of Conflict of Interest

No conflicts of interest to declare.

Authors Disclosures

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Ethical Approval for Research

Not applicable for this research.

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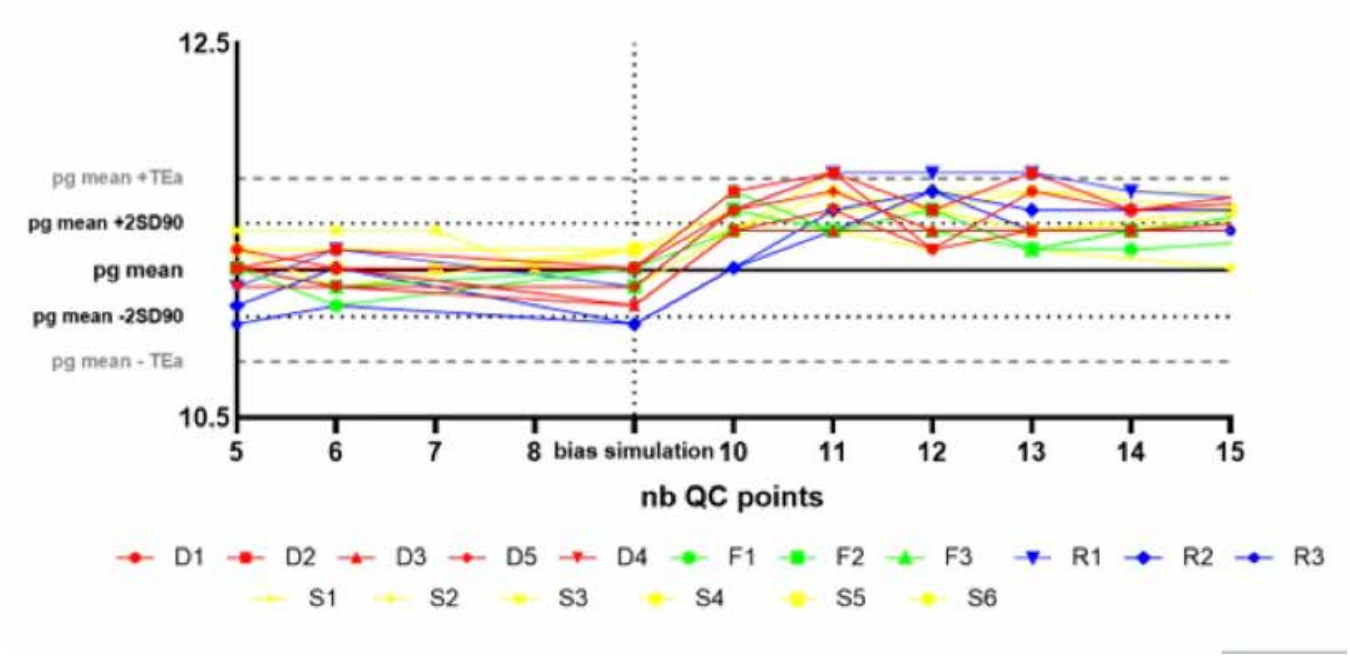
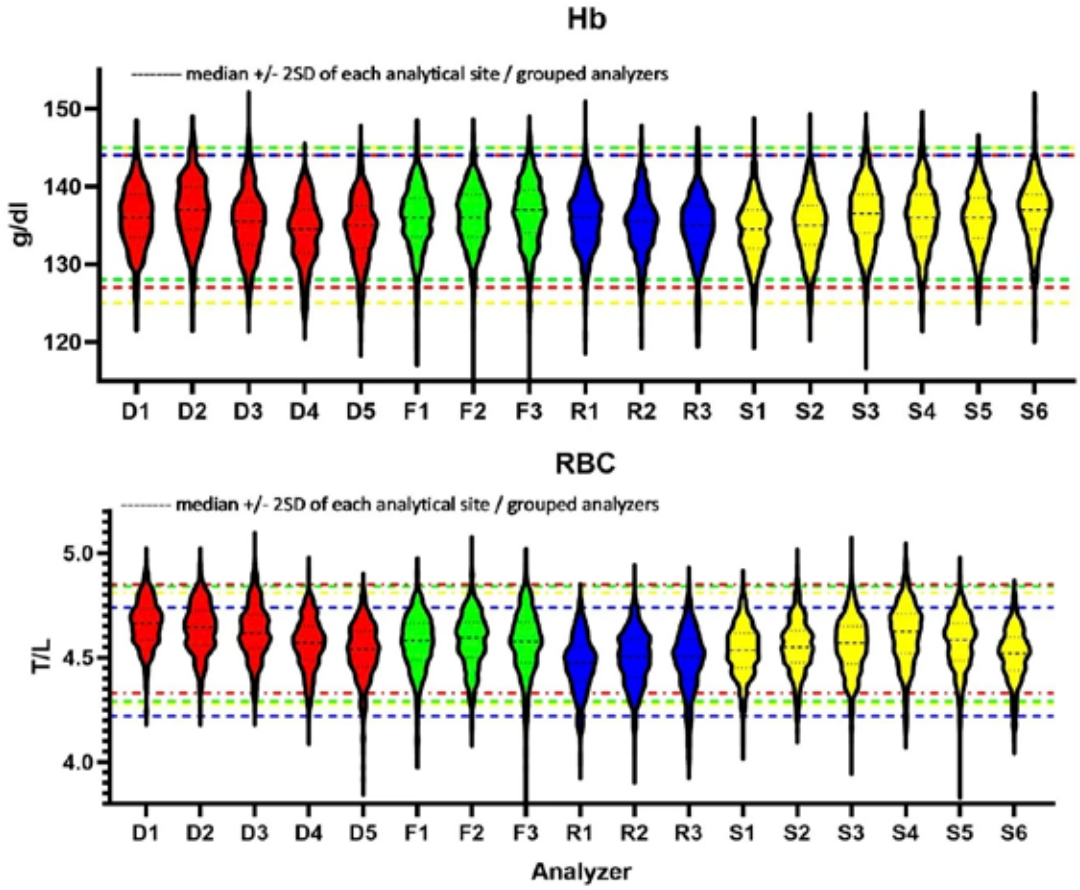
Supplementary Data

Supplementary Figure 1: Simulation of the impact of +1CVi bias on the seventeen analyzers and the error detection rate.

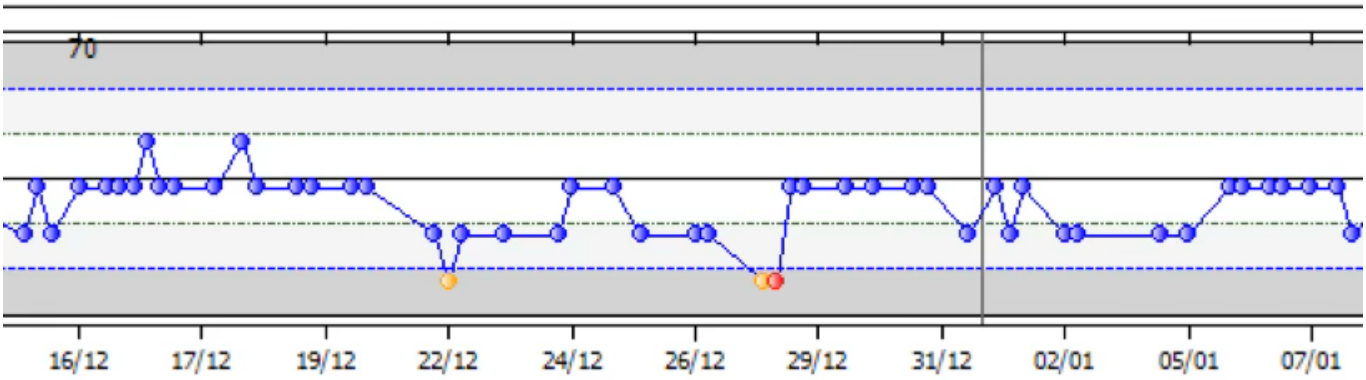


Supplementary Figure 2: Violin plots for the patient medians of all analyzers with a block size of 50 and Winsorization outside the reference interval (11.5 to 16.7 g/dl for Hb and 3.8 to 6 T/L for RBC).



The four analytical sites are identified by color. From December 1 to 15th, there was a training period. The colored dotted lines represent the median +/- 2 SD for each analysis site at the end of this test period.

Supplementary Figure 3. Example of IQC chart for one representative analyzer showing the impact of centering the IQC targets on the peer group data.



The analyzer exhibits a subtle systematic negative error that cannot be corrected with calibration, generating more -2S alarms.

Supplementary Table 1: Comparison of Pooled CV, CV_{50}, CV_{90} and CV_{95}.

Comparison of percentiled CV_{intra} with pooled CVs					
Parameter/level/Batch	**CV_{intra}90**	**pooled CV**	**CV_{intra}50 / pooled CV**	**CV_{intra}90/ pooled CV**	**CV_{intra}95/ pooled CV**
HB L2 3339	1,1	1,07	0,71	1,03	1,26
RBC L2 3339	1,2	1,13	0,78	1,06	1,22
HB L2 4023	1,1	1,03	0,74	1,07	1,31
RBC L2 4023	1,2	1,16	0,76	1,03	1,19

Research Article

Comparison of two analytical methods for HbA1c determination: HPLC ADAMS™ (ARKRAY A1c HA-8180T) versus CAPILLARYS 3 OCTA® (Sebia) capillary electrophoresis system

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Keywords

Capillary electrophoresis, glycated hemoglobin, HbA1c, HPLC, method comparison

Abstract

HbA1c is a valuable indicator for the diagnosis and therapeutic monitoring of diabetic patients. Our study aims to compare two methods of measuring HbA1c: capillary electrophoresis on the CAPILLARYS 3 OCTA ® (Sebia) with HPLC ADAMS™ (ARKRAY A1c HA-8180T) used routinely in our laboratory, to avoid any discrepancies in patient monitoring in case of changes in the HbA1c measurement method.

A total of 103 blood samples from adult patients received at the laboratory have been analyzed in parallel, singly, on both machines. The results show a good correlation between the two systems with a correlation coefficient of 0.991. The Bland and Altman difference diagram shows that the average bias between ADAMS A1c™ and CAPILLARYS 3 OCTA ® is 2.087 mmol/mol (95% CI: 1.7357 to 2.4390 mmol/mol) in IFCC units and 0.19% (95% CI: 0.1602 to 0.2262%) in the National Glycohemoglobin Standardization Program (NGSP) units, and that out of all the patients studied, only four had values outside the limits of the difference diagram. The latter shows a uniform dispersion of values across all the analyzed measurements, with over 95% of the differences between measurements falling within the range [-1.43; 5.61 mmol/mol] or [-0.14; 0.52%].

These results enable us to confirm the reliable transferability between the two techniques without compromising accuracy. Both machines can therefore be used interchangeably or as backup, ensuring homogeneous patient monitoring.

Introduction

Glycated hemoglobin corresponds to the main glycosylated portion of hemoglobin. This process results from the non-enzymatic and irreversible binding of glucose to one or two of the N-terminal valines of the β chain of hemoglobin. In the absence of interfering factors, the measurement of HbA1c reflects an individual's glycemic control over the 2 to 3 months preceding the sample collection [1] .

This places this parameter at the core of both the diagnosis and monitoring of diabetic patients, as well as the assessment of the risk of complications, as demonstrated by various large-scale studies such as the Diabetes Control and Complications Trial (DCCT) for type 1 diabetics and the United Kingdom Prospective Diabetes Study (UKPDS) for type 2 diabetics. These studies have established a correlation between controlled HbA1c levels and a reduced risk of long-term complications, particularly microvascular complications [2–4].

Several analytical methods are currently available for the measurement of HbA1c (chromatographic, electrophoretic, and immunochemical methods). The National Glycohemoglobin Standardization Program (NGSP) recognizes high-performance liquid chromatography (HPLC) using cation exchange as the reference method. Meanwhile, a working group of the International Federation of Clinical Chemistry (IFCC) has established a reference procedure based on reverse-phase HPLC coupled with mass spectrometry or capillary electrophoresis [5,6].

In the present study, we compared two analytical methods for the measurement of HbA1c :high-performance liquid chromatography (HPLC) using ion exchange and capillary electrophoresis.

The objective is to assess the transferability of results between the two automated systems, thereby ensuring consistency in patient monitoring when a change in the HbA1c measurement method occurs. This would allow both systems to be used in parallel or as backup instruments within the laboratory. Given the limited number of published evaluations of the recently introduced CAPILLARYS 3 OCTA system, this work provides timely and relevant data for clinical laboratories seeking to harmonize HbA1c measurements.

Materials and Methods

Patient Samples

The study included 103 venous whole-blood samples collected from adult patients referred to the biochemistry laboratory for HbA1c testing as part of diabetes diagnosis or follow-up. Given the pre-analytical stability of HbA1c, fasting was not required. Venous whole blood samples were collected in 4 ml EDTA Vacutainer tubes (Becton Dickinson) from both the hospital wards and the laboratory's reception and sampling center. The collected samples were stored at room temperature and analyzed in parallel on both automated analyzers on the same day of collection. The 103 patients included in our sample covered the measurement range defined by the manufacturer and exhibited normal hemoglobin chromatographic profiles.

Devices and measurement techniques

HbA1c measurements were compared using two automated systems: the HPLC-based ADAMS™ (ARKRAY A1c HA-8180T) and the recently implemented CAPILLARYS 3 OCTA® (Sebia).

The HPLC ADAMS™ (ARKRAY A1c HA-8180T) uses a high-performance liquid chromatography (HPLC) technique based on ion exchange, which relies on inorganic phosphate buffers (80 A, 80 B, and 80 CT) with increasing ionic strengths, which elute the different glycated and non-glycated fractions of the hemoglobin being analyzed. These fractions are then quantified by spectrophotometry using a dual wavelength (420/500 nm) [7] . This instrument provides a reportable HbA1c measurement range of 9–195 mmol/mol using IFCC units, corresponding to 3–20% on the NGSP scale, while the guaranteed analytical performance range extends from 22 to 117 mmol/mol , equivalent to 4.2 to 12.9 % [8] .

The CAPILLARYS 3 OCTA® system (Sebia), on the other hand, employs an automated capillary electrophoresis technique. This method is based on the separation of different hemoglobin fractions inside capillaries with very small diameters, subjected to a potential difference of several thousand volts at their ends. The fractions are detected directly on the cathode side at a wavelength of 415 nm [9] . Thanks to its high resolution, this method allows for the incidental detection of homozygous hemoglobin variants, which prevents erroneous HbA1c reporting in the absence of HbA. Analytical performance studies have reported that this analyzer provides linear HbA1c measurements across an approximate range of 25–199 mmol/mol , corresponding to 4.4–20.3% [10].

The results delivered by the two automated systems are expressed in NGSP (%) and IFCC (mmol/mol) units linked by the following equation:

HbA1c (NGSP/DCCT in %) = HbA1c (IFCC in mmol/mol) x 0.0915 + 2.152

Protocol and Statistics

A prospective analytical study was conducted on patient samples referred to the biochemistry laboratory for HbA1c testing, in accordance with the ethical principles outlined in the Declaration of Helsinki. Each sample was analyzed in series, with a single measurement per method, on the day of receipt. Data entry was performed using Excel 2016, and statistical analysis of the data was performed using MedCalc® software and jamovi (version 2.7.17). The study design followed the recommendations of the SH GTA 04 – Revision 02 protocol of the French Accreditation Committee (COFRAC) [11] .

The bias, concordance, and correlation between the two methods were assessed using the Bland-Altman plot and the Passing-Bablok and Deming regression tests.

Additional agreement analyses were conducted at clinically relevant HbA1c cutoffs of 39 mmol/mol (5.7%), 48 mmol/mol (6.5%), and 53 mmol/mol (7.0%). For each cutoff, samples

within ±3 mmol/mol (±0.3%) of the target value were selected to focus on the clinically critical range. Local bias between the two methods was determined, along with the corresponding standard deviation and 95% confidence interval. Classification agreement was assessed by categorizing results according to each cutoff and calculating the proportion of concordant classifications, with Cohen's kappa coefficient and McNemar's test.

Results

The study included 103 adult patients (60 females and 43 males; male-to-female ratio: 0.72). The mean age of the participants was 56.9 ± 16.1 years. The mean HbA1c values delivered by the two methods were 51.24 ± 18.26 mmol/mol (or 6.84 ± 1.66%) for the ADAMS A1c™ and 49.15 ± 17.88 mmol/mol (or 6.64 ± 1.63%) for the CAPILLARYS 3 OCTA®. Although CAPILLARYS 3 OCTA® produced slightly lower mean values, the difference was not clinically significant.

The equation of the Passing-Bablok regression (Figure 1) yielded the following equation:
Y (CAPILLARYS 3 OCTA ®) = -2.000000 + 1.000000 X (ADAMS A1c)
Similarly, the Deming regression produced the equation:
Y (CAPILLARYS 3 OCTA ®) = -1.0075 + 0.9789 X (ADAMS A1c)
The correlation coefficient was 0.991 ($P < 0.0001$).

Figure 1: Comparison between the two methods using the Passing and Bablok regression line.

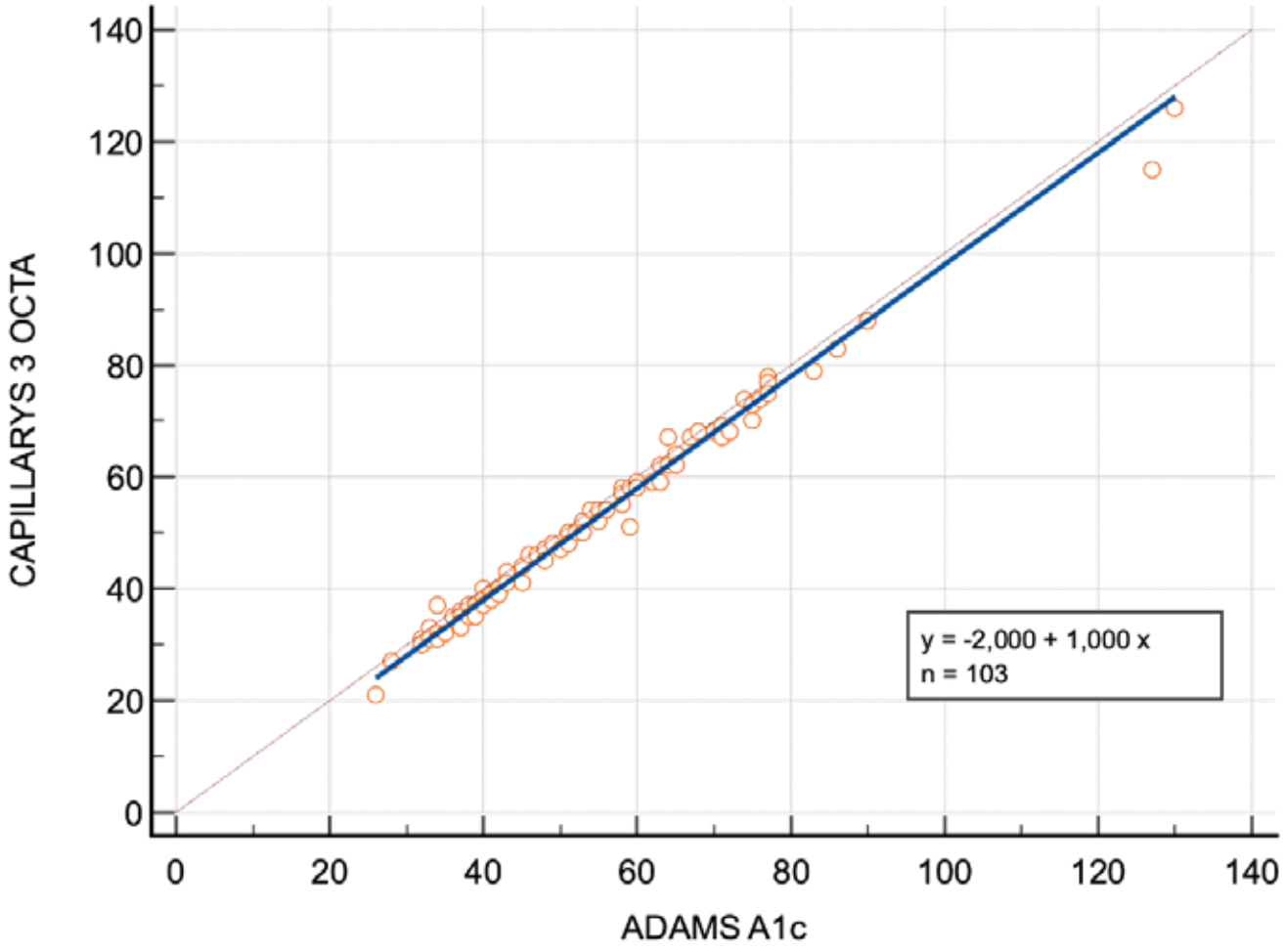
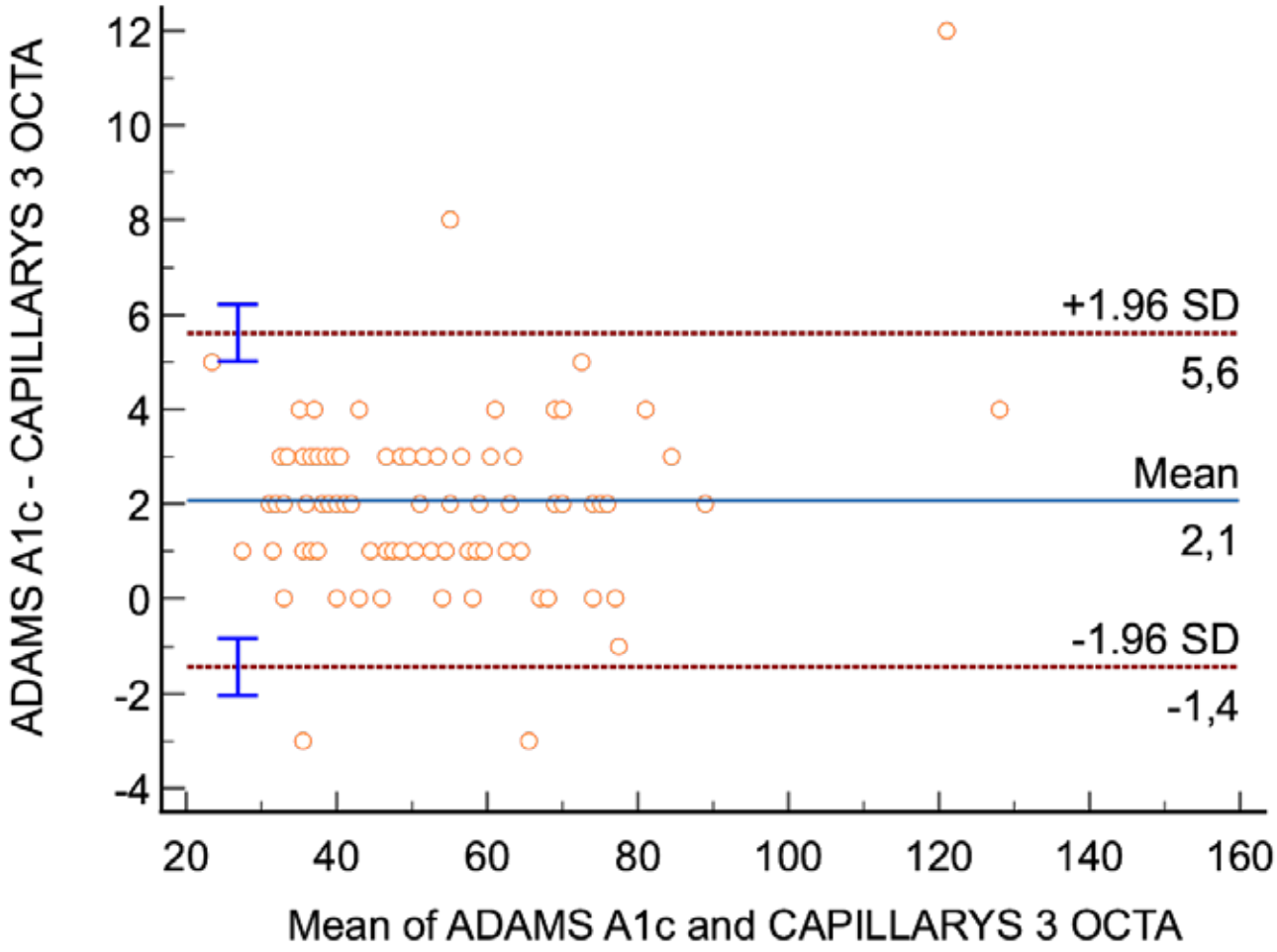


Figure 2: Comparison between the two methods using the Bland-Altman plot.



The Bland - Altman analysis revealed a mean bias of 2.09 mmol/mol (95 % CI: 1.74–2.44 mmol/mol) in IFCC units and 0.19 % (95 % CI: 0.1602–0.2262 %) in NGSP units between the ADAMS™ A1c HA-8180T and CAPILLARYS 3 OCTA® systems.

Among the 103 patients included in the study, only 3.8 % (4 patients) exhibited values falling outside the limits of the difference plot. The difference between HbA1c measurements using the two methods ranged from +1.96 SD to -1.96 SD, meaning that 95% of the differences between measurements fell within the range [-1.43; 5.61 mmol/mol] or [-0.14; 0.52%], respectively, in IFCC and NGSP units.

The agreement between the two methods was assessed at clinically relevant HbA1c cutoffs of 39 mmol/mol (5.7%), 48 mmol/mol (6.5%), and 53 mmol/mol (7.0%) (±3 mmol/mol). The mean biases were 2.21 mmol/mol (SD 0.91, 95% CI 1.89–2.52, N = 34), 1.83 mmol/mol (SD 1.27, 95% CI 1.03–2.64, N = 12), and 2.00 mmol/mol (SD 1.04, 95% CI 1.40–2.60, N = 14), respectively.

Classification concordance was high across all cutoffs, with 91.3% agreement at 39 mmol/mol (5.7%) (Cohen's $\kappa = 0.81$; McNemar $\chi^2 = 9.00$, $p = 0.003$), 96.1% at 48 mmol/mol (6.5%) ($\kappa = 0.92$; McNemar $p > 0.05$), and 95.1% at 53 mmol/mol (7.0%) ($\kappa = 0.90$; McNemar $\chi^2 = 5.00$, $p = 0.025$).

Discussion

HbA1c measurement is essential in the management of diabetic patients. However, the variety of analysis methods available on the market can lead to variations in the HbA1c values obtained. That is why it is essential to continue efforts to standardize and improve quality in order to guarantee reliable results and reduce inter-technical disparities.

Comparing methods is an essential part of the analytical evaluation of a measurement method. It allows the results produced by these methods to be compared and any potential bias between them to be identified. In the event of divergent results, it is crucial to investigate the causes and find appropriate solutions [5,12].

In our study, the mean HbA1c values for all samples measured by capillary electrophoresis were 49.15 ± 17.88 mmol/mol (6.64 ± 1.63%), slightly lower than those obtained by high-performance liquid chromatography (HPLC), which were 51.24 ± 18.26 mmol/mol (6.84 ± 1.66%). However, the difference between the average HbA1c results obtained by

HPLC on the ADAMS A1c™ and CAPILLARYS 3 OCTA® was only 0.2%, remaining well below the 1.4% acceptability threshold established by the French Health Products Safety Agency (AFSSAPS) guidelines. This result demonstrates the effectiveness of efforts to standardize HbA1c testing, even when the analytical methods rely on distinct measurement principles.

The Bland-Altman plot analysis of agreement between ADAMS A1c™ and CAPILLARYS 3 OCTA® was satisfactory. The mean bias was 2.087 mmol/mol (equivalent to 0.19%) between the two methods. The most significant differences were observed for normal and low HbA1c values as well as for very high values (>119 mmol/mol).

The difference diagram shows a uniform dispersion of values across all measurements analyzed, with more than 95% of differences falling within the limits of agreement. These limits range from [-1.43 to 5.61 mmol/mol] or [-0.14% to 0.52%] in IFCC and NGSP units, respectively.

Of the 103 samples analyzed, four showed a weak correlation between the results obtained on the two devices. These samples had HbA1c values ranging from 21 mmol/mol (4.1%) on the CAPILLARYS 3 OCTA® to 26 mmol/mol (4.6%) on the ADAMS A1c™ HPLC system, and between 115 mmol/mol (12.7%) on the CAPILLARYS 3 OCTA® and 127 mmol/mol (13.8%) on the ADAMS A1c™ automated system.

The discrepancies observed between the two methods, although limited in number, highlight the existence of occasional differences in HbA1c measurement between high-performance liquid chromatography (HPLC) and capillary electrophoresis. These differences may result from various methodological, biological, pre-analytical, or instrumental factors.

However, in our series, no major analytical interference was identified. Fetal hemoglobin (HbF) levels were within normal limits, no hemoglobin variants were detected, and no history of anemia or renal failure was reported in the patients concerned. These factors rule out the usual biological causes that could explain such a disparity between the two techniques.

Thus, the differences observed could be attributed to variations intrinsic to the analytical methods themselves, in particular differences in calibration, detection sensitivity, or signal processing between capillary electrophoresis systems.

Regarding correlation analysis, the two methods compared showed a strong correlation, with a correlation coefficient of 0.991. The Passing-Bablok equation establishes that: Y (CAPILLARYS 3 OCTA®) = -2.000000 + 1.000000 X (ADAMS A1c). Looking at the 95% confidence interval for the slope coefficient, we see that it encompasses the value of one. This indicates that there is no statistically significant difference between the slope value obtained and the value of one. Therefore, we can conclude that there is no significant proportional difference between the two methods [13].

In method comparison studies, global analytical agreement may mask clinically significant differences when measurements fall near decision thresholds that directly influence diagnosis and treatment. Therefore, particular attention was given to clinically relevant HbA1c cutoffs of 39 mmol/mol (5.7%), 48 mmol/mol (6.5%), and 53 mmol/mol (7.0%), which correspond to prediabetes identification, diabetes diagnosis, and commonly recommended therapeutic targets, respectively [14,15] . Assessing agreement specifically at these decision levels provides a more clinically meaningful evaluation of interchangeability between HbA1c measurement systems than overall performance metrics alone.

Even minor systematic bias or increased variability around these thresholds,particularly the diagnostic cutoff of 48 mmol/mol,may lead to patient misclassification and inappropriate clinical decisions. Similarly, discrepancies near the prediabetes or therapeutic targets may influence risk stratification and treatment adjustment.

Assessment of agreement at the clinically relevant HbA1c decision thresholds of 39, 48, and 53 mmol/mol demonstrated a small, consistent positive mean difference of approximately 2 mmol/mol. The stability of this deviation across all evaluated levels, together with its narrow confidence intervals, suggests a systematic yet limited analytical difference rather than random fluctuation. Notably, this magnitude remains well within the clinically acceptable limit of 5 mmol/mol (approximately 0.46% NGSP), in accordance with IFCC defined analytical performance specifications [16] .

Classification agreement was high at all three decision points, with concordance exceeding 90% and Cohen's kappa coefficients indicating strong to almost perfect agreement. Although the McNemar test reached statistical significance at 39 and 53 mmol/mol, this likely reflects a small but systematic directional shift rather than substantial clinical misclassification. At the 48 mmol/mol cutoff, which corresponds to the diagnostic threshold for diabetes, the absence of significant discordance further supports the clinical interchangeability of the two methods in routine practice.

Numerous previous comparisons of automated systems using similar techniques have been conducted. Sriwimol et al. [13] compared HbA1c values obtained by capillary electrophoresis on the Capillarys 3 Tera system (Sebia) with HPLC on the ADAM TM A1c HA-8180V (Arkray) on 270 samples from subjects with normal hemoglobin profiles and no detectable hemoglobin variants on the electropherograms or chromatograms. The results of this study showed good agreement, highlighting a significant consistency and correlation between the two methods, indicating that they are comparable and interchangeable.

The work carried out by Khashoggi et al. [17] also compared high-performance liquid chromatography (HPLC) on Tosoh HLC-723 G11 in VAR mode with a capillary electrophoresis instrument (Sebia Capillarys 2 Flex Piercing). The analysis covered 250 randomly selected patient samples, including some hemoglobin variants (HbS, HbE, and HbD). The results revealed a significant correlation (r = 0.99). However, the Tosoh HLC-723 G11 yielded higher values in the low HbA1c

range and lower values in the high range, making it difficult to use these two automated systems interchangeably in the monitoring of diabetic patients.

Nevertheless, Dupuy et al. [18] remains one of the few studies to our knowledge that has compared the CAPILLARYS 3 OCTA® system recently launched by Sebia with an HPLC system: the Tosoh Bioscience HLC®-723G8. They successfully demonstrated a good correlation between the two methods (r= 0.995), which is consistent with the results obtained in our comparison.

A limitation of our work lies in the absence of hemoglobin variants among the analyzed samples, which prevented assessment of potential interferences caused by abnormal hemoglobins. This aspect warrants further investigation in future studies to evaluate the performance of both systems in the presence of hemoglobinopathies.

Conclusion

Our study demonstrates good agreement between HbA1c results delivered by ADAMS™ (ARKRAY A1c HA-8180T) and CAPILLARYS 3 OCTA®. These results allow us to confirm reliable transferability between the two techniques without loss of accuracy. The consistency observed at major diagnostic and therapeutic decision thresholds further supports their comparability in routine clinical practice. The two automated systems can be used in parallel or as backups while maintaining consistent patient monitoring. This study is part of a quality policy required by the ISO EN 15189 standard, to which medical laboratories must be fully committed.

Conflict of interest disclosure

The authors declare no conflicts of interest.

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Nisma Douzi: Conceptualization, Methodology, Writing - Original Draft, Visualization.Oussama Grari : Writing - Review & Editing, Visualization . Imad-Eddine El Khamlichi: Formal analysis. Soufiane Beyyoudh: Formal analysis. Dounia El Moujtahide: Supervision. El-houcine Sebbar: Resources, Supervision. Mohammed Choukri : Resources, Supervision.

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Research Article

Laboratory Professionals' Perspectives on Artificial Intelligence in Laboratory Medicine: Insights from a National Survey in Albania

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Keywords

Artificial Intelligence, Laboratory Medicine, Laboratory Professionals, Perspectives, Survey

Abstract

Introduction: While Artificial Intelligence (AI) is transforming Laboratory Medicine, successful AI integration depends on the readiness of healthcare professionals. This study aimed to assess the perspectives of Albanian laboratory professionals toward AI integration in medical laboratories.

Methods: We conducted a cross-sectional, voluntary, and anonymous survey using Google Forms. The survey link was distributed to all members of the Albanian Society of Clinical Biochemistry and Laboratory Medicine and their affiliated staff. The survey explored General Information and Demographics, Digital Properties and Health Data Access, and Perspectives on Artificial Intelligence in Medical Laboratories. Responses were automatically collected over four weeks and were analyzed to investigate laboratory professionals' perspectives.

Results: A total of 220 laboratory professionals completed the survey. 30% of participants were laboratory doctors and 70% were laboratory technicians. Participants expressed a generally optimistic outlook on AI integration in medical laboratories, believing it could streamline routine workflows and save time (74%), simplify repetitive tasks (70%), reduce work-related stress (61%), improve analytical accuracy and precision (57%), and reduce costs and enhance efficiency (49%). The main barriers to AI integration were considered high cost of implementation, the lack of appropriate IT infrastructure, the lack of specialized staff, and ethical considerations. Significant differences were observed among various subgroups, but interest in AI training prevailed among the majority of respondents.

Conclusion: This survey highlights a generally positive perspective on AI among laboratory professionals in Albania, alongside a strong interest in AI education. According to the survey respondents, strengthening digital infrastructure and promoting training programs will be essential for AI integration in laboratory medicine.

Introduction

Artificial Intelligence (AI) is increasingly transforming Laboratory Medicine, offering tools that enhance diagnostic accuracy, automate repetitive tasks, and support clinical decision-making [1, 2]. Applications such as machine learning, image recognition, and natural language processing have shown promise in areas like quality control, test selection, results prediction, generation and interpretation, and workflow optimization [3, 4, 5]. However, the adoption of AI in laboratory settings is uneven across countries and healthcare systems. Progress is often hindered by fragmented digital infrastructure, limited interoperability, and a lack of national strategies for digital health integration [6, 7].

In addition to technical capacity, successful AI integration relies on the readiness, acceptance, and engagement of healthcare professionals. The active contribution of laboratory professionals is key to providing accurate data analysis and interpretation [1]. Human trust toward AI's potential plays a central role in determining how digital tools are used in practice and real-world implementation, yet studies examining professionals' awareness, attitudes, and training needs related to AI remain limited [8, 9].

Understanding the current level of knowledge, digital preparedness, and perceived barriers is essential to developing national policies and regulatory frameworks that support the responsible modernization of the healthcare system [6]. This study aimed to assess the perspectives of Albanian laboratory professionals toward AI integration in medical laboratories, using a national cross-sectional survey to explore attitudes, digital infrastructure, and training interest.

Materials and methods

The voluntary and anonymous survey was conducted using Google Forms. The survey link was distributed via email and WhatsApp to all members of the Albanian Society of Clinical Biochemistry and Laboratory Medicine (ASoLaM). In Albania, medical laboratories are legally required to have a Technical Director who is a licensed Medical Doctor specialized in Laboratory Medicine, all of whom are members of ASoLaM. These Technical Directors were asked to distribute the survey to their affiliated staff and confirmed the dissemination of the survey link. The target population included all laboratory doctors (Medical Doctors, MD, specialized in Laboratory Medicine) and technicians currently practicing in Albania.

A non-probability, voluntary response sampling method was employed. The required sample size was calculated using an open-source calculator (OpenEpi, Version 3.01) based on the following assumptions: an estimated laboratory professional population size of 2000 individuals, a 5% margin of error, and a 99% confidence level. This calculation yielded a required minimum sample size of 154 participants. This sample size calculation was performed for the overall study population; analyses conducted at the subgroup level were exploratory and not specifically powered. Although a maximum number of respondents was encouraged, participation remained entirely voluntary throughout the survey period.

The questionnaire was reviewed by two laboratory medicine experts for face and content validity and pre-tested by five laboratory professionals to ensure clarity and comprehensibility.

This study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki [10]. As the survey was anonymous, voluntary, involved no medical intervention, posed no risks to participants, and collected no identifiable personal data, ethics committee approval was not required. Data collection and handling complied with relevant data protection regulations.

The final questionnaire consisted of 25 questions, organized into three sections:

1. General Information and Demographics (six questions)
2. Digital Properties and Health Data Access (five questions)
3. Perspectives on Artificial Intelligence in Medical Laboratories (fourteen questions).

A working definition of Artificial Intelligence was introduced at the beginning of the survey. Responses were automatically collected over a four-week period (July 16 to August 13, 2024). Data analysis was conducted using Microsoft Excel 2016 (Microsoft Corp., Redmond, WA, USA) and IBM SPSS Statistics v.27 (IBM Corp., Armonk, NY, USA). Survey results were tallied for each question and expressed as percentages. For multiple response questions, percentages were calculated for each response option and may exceed 100%. Analyses were stratified by age, gender, professional role (doctors vs. technicians), laboratory type (public vs. private), laboratory size (<500 vs. >/=500 samples/day), and accreditation status (yes vs. no/in process). Differences in response distribution across these subgroups were analyzed using the Pearson Chi-square test. Two-sided p-values <0.05 were considered statistically significant, with results interpreted cautiously in the context of multiple comparisons.

Results

General Information and Demographics

A total of 220 laboratory professionals completed the survey. Participants' ages ranged from 21 to 66 years, with a mean age of 37.9 years (standard deviation [SD]:10.7). The highest number of responses was recorded in the 21-30 and 31-40 age groups, comprising 69 and 68 individuals, respectively.

Regarding workplace settings, 71% were employed in public laboratories, including:

- 43.5% in University Hospital Center laboratories,
- 46% in Regional Hospital laboratories,
- 9% in Municipality Hospital laboratories and
- 1.5% in Ambulatory Polyclinic laboratories.

The remaining 29% worked in private laboratory settings. Concerning quality standards, most of the respondents worked in laboratories accredited according to ISO 15189. Participant characteristics are summarized in Table 1.

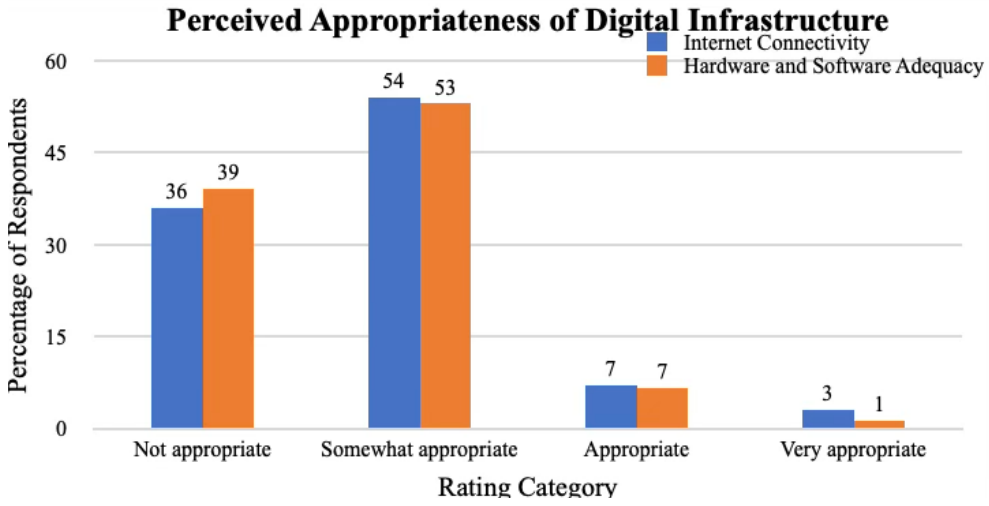
Table 1: Characteristics of survey respondents (n=220).

Characteristic		No.	%
Sex			
	Male	21	9,5
	Female	199	91,5
Age (years)			
	21-30	69	31,4
	31-40	68	30,9
	41-50	49	22,3
	51-60	31	14,1
	61-70	3	1,3
Role			
	Laboratory Doctor	66	30
	Laboratory Technician	154	70
Workplace type			
	Public	156	71
	Private	64	29
ISO 15189 Accreditation Status			
	Yes	121	55
	No	25	11,4
	In process	74	33,6
Sample Volume (/day)			
	>/=500	54	24,5
	<500	166	75,5

Digital Properties and Health Data Access

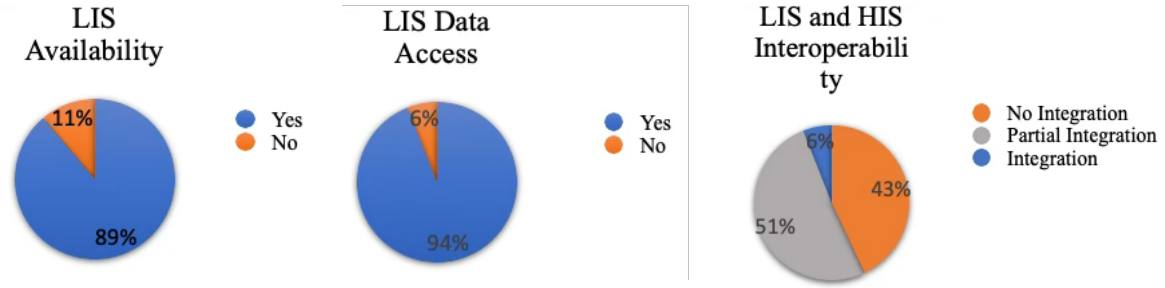
Participants were asked to evaluate the quality of digital infrastructure in their laboratories. Their perceptions of the appropriateness of internet connectivity (speed and stability), and the adequacy of available hardware and software are presented in Figure 1.

Figure 1: Perceived Appropriateness of Digital Infrastructure for AI Integration.



A Laboratory Information System (LIS) was reported to be implemented in the laboratories of 89% of respondents. Results on professionals' ability to extract patients' laboratory data from the LIS, as well as on system interoperability, both partial integration with data from other diagnostic departments (e.g., microbiology or pathology) and integration with other institutional health databases, are presented in Figure 2.

Figure 2: LIS availability, data accessibility, and systems interoperability.



When asked about their access to supplementary clinical data, 33% of respondents stated that they lacked access to data beyond laboratory results. Alternatively, 12% of the participants declared they could access patients' electronic health records, 48% received relevant information from the referring clinicians, and 18% obtained data directly through outpatients' anamneses.

AI-related applications were:

- Auto-verification of laboratory test results (18%),
- Digital image analysis (15%),
- Pre-analytics (13%), and
- Reviewing patients' risk profiles for certain conditions (9.5%).

Perspectives on Artificial Intelligence in Medical Laboratories

Participants' perspectives on the use of artificial intelligence in medical laboratories are reported in this section. In addition to overall descriptive results, subgroup analyses were performed to assess differences according to gender, age, professional role, laboratory type, laboratory size, and laboratory accreditation status.

Knowledge Level of AI Technologies

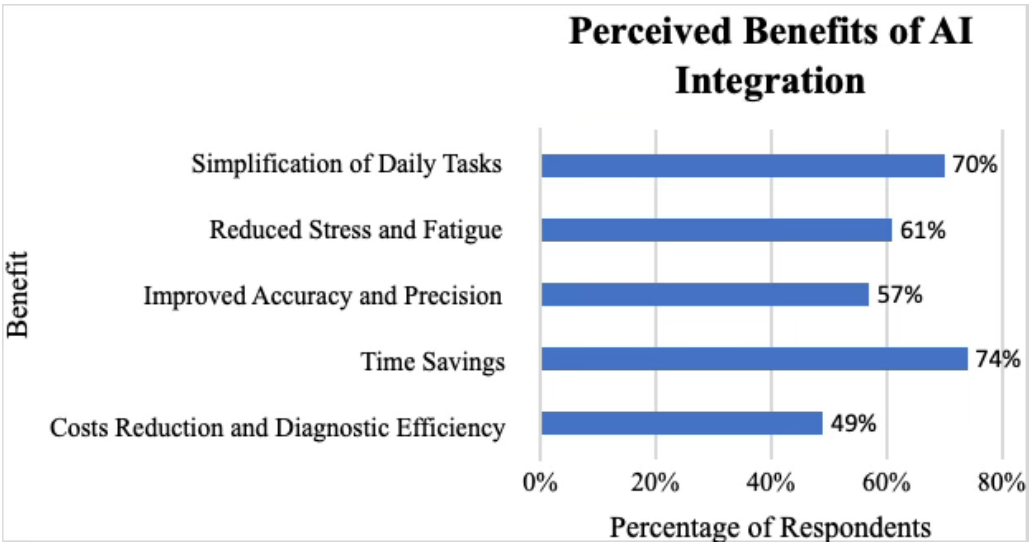
Laboratory professionals were asked to self-assess their knowledge level of AI technologies and evaluate the appropriateness of their knowledge. 56% declared they possessed an appropriate or very appropriate level of knowledge. No statistically significant differences in self-reported AI knowledge level were observed across demographic subgroups. A total of 46% stated there were AI elements already in use in their laboratories. The most frequently cited current

Participants identified laboratory sections that could benefit the most from AI implementation, including Hematology (52%), Clinical Chemistry (43%), Immunology (38%), and Molecular Biology (38%).

Perceived Benefits of AI Integration

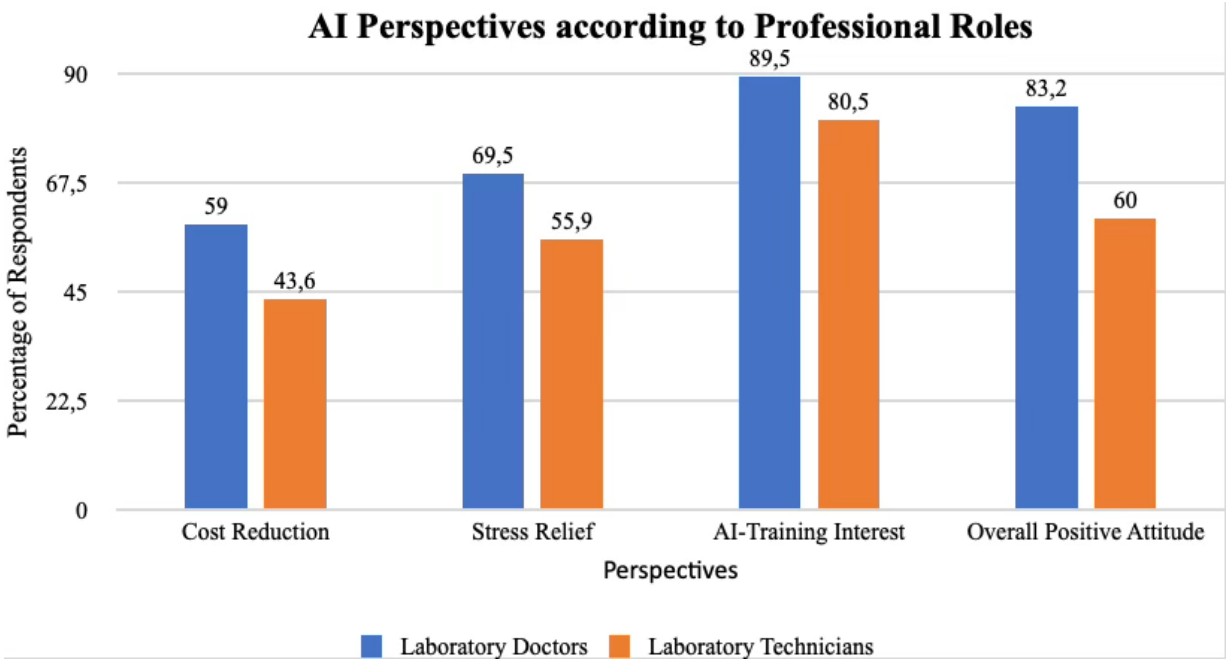
Participants expressed a generally optimistic outlook on AI integration in medical laboratories. Nearly half of the survey respondents (49%) believed AI implementation could reduce operational costs and enhance diagnostic efficiency. 74% stated that AI would streamline routine workflows and save time. Additionally, 57% expected that AI would improve analytical accuracy and precision, while 61% believed AI implementation could reduce work-related stress and fatigue. Furthermore, 70% of participants anticipated that AI would simplify daily and repetitive tasks, thereby allowing personnel to devote more time to creative or interpretive activities.

Figure 3: Perceived Benefits of AI Integration among Laboratory Professionals.



Comparisons across subgroups showed that statistically significant differences in perceived AI benefits were observed only for professional role and laboratory size.
Laboratory Doctors more strongly associated AI implementation in medical laboratories with cost reduction (p=0.03) and stress relief (p=0.04). A more prominent AI training interest (p=0.04) and an overall more positive attitude toward AI (p<0.01) were observed among laboratory doctors compared with laboratory technicians.

Figure 4: Differences in AI Perspectives according to Professional Roles of the Respondents.



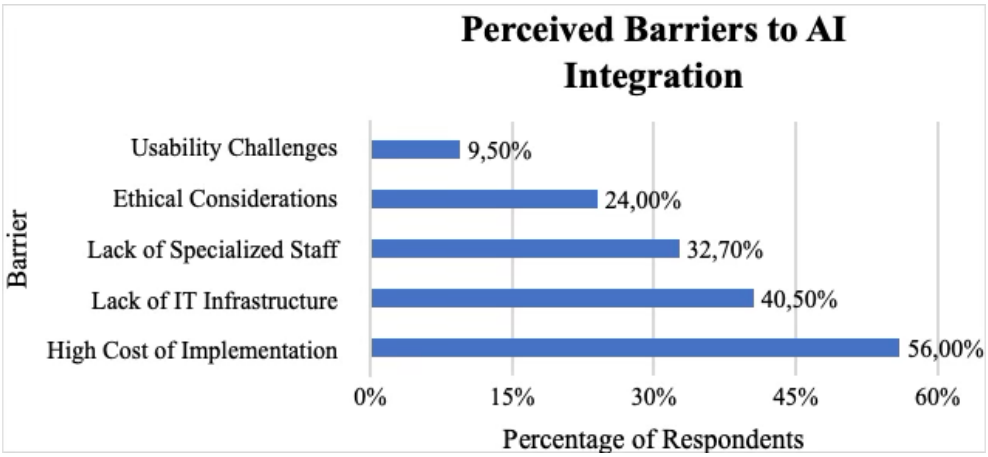
Significant differences also emerged based on laboratory size. Professionals working in laboratories processing more than 500 samples/day more frequently believed that AI would:

- Reduce operational costs (p=0.007),
- Improve analytical accuracy and precision (p=0.01),
- Save time (p=0.04), and
- Reduce work-related stress and fatigue (p=0.02), compared to those working in smaller laboratories.

Perceived Barriers to AI Integration

The survey investigated the perspectives of the participants on AI integration barriers. 39% of the respondents rated the barriers as "Important" or "Maximal". The most frequently cited barrier was the high cost of initial implementation (56%). Other notable barriers included a lack of appropriate IT infrastructure, a lack of specialized staff, and ethical considerations. No statistically significant differences were observed in perceived barriers to AI integration across demographic subgroups of the study population.

Figure 5: Perceived Barriers to AI Integration among laboratory professionals.



Concerns about Job Displacement

Regarding potential job displacement due to AI:

- 31% of professionals expressed concern about losing their jobs due to AI replacement,
- 43% were not concerned,
- 26% were uncertain and selected "I don't know".

Laboratory technicians reported greater concern about AI-related job loss than laboratory doctors (p=0.02). Respondents under the age of 40 expressed significantly greater concern about potential job loss due to AI-driven replacement compared to those aged 40 and above (p=0.04). This apprehension was particularly pronounced among laboratory professionals aged 21-30 years, of whom 43.5% reported concern about losing their job as a result of AI implementation. No statistically significant differences were observed among the other subgroups regarding concerns about job displacement.

AI Training Interest and General Attitude toward AI

When queried about their interest in AI-focused training, 84% of respondents indicated willingness to participate. Overall, 67% of all survey participants exhibited a positive general attitude toward AI in Laboratory Medicine. Subgroups' analysis revealed a more positive attitude toward AI among female professionals (p=0.003). Doctors expressed greater interest in AI training (p=0.004) and a more positive general attitude toward AI (p=0.004) compared with technicians. Participants working in private laboratories demonstrated a more positive attitude toward AI than those employed in public laboratories (p=0.04). Similarly, professionals working in larger laboratories showed a more positive attitude toward AI (p=0.03). Participants who rated their AI knowledge as appropriate or very appropriate also exhibited a more positive attitude toward AI in Laboratory Medicine (p=0.001).

Discussion

This national survey provides valuable insights into Albanian laboratory professionals' perspectives on AI implementation in Laboratory Medicine. The results reveal a broadly positive outlook toward AI, with a majority of respondents recognizing its potential to enhance laboratory efficiency, improve analytical accuracy, and reduce workload-related stress. Consistent with our findings, Ardon et al. reported that 64% of surveyed laboratory professionals supported the development of AI augmented diagnostic tools, and most frequently believed AI can reduce errors and enhance efficiency [11]. Similarly, Paranjape et al. found that 90% of respondents expressed positive attitudes toward the value of AI in the diagnostic laboratory medicine [9].

Despite prevailing positive attitudes toward AI, our findings highlight significant gaps between awareness and actual implementation, as well as concerns about costs, infrastructure, and workforce readiness. The current use of AI in Albanian laboratories appears limited, with most respondents working in environments lacking full digital integration and interoperability between LIS and Hospital Information Systems (HIS); 11% of them even work in laboratories without a LIS. Comparable issues were identified in previous European surveys. Nearly 50% of respondents declared having no access to data outside their LIS, according to a comprehensive survey of AI adoption in European laboratory medicine by Cadamuro et al. on behalf of the European Federation of Clinical Chemistry and Laboratory Medicine Working Group on Artificial Intelligence [12]. Similar lack of interoperability was also reported by 65% of participants of the Bellini et al. survey on Italian clinical laboratories [13]. Since it is widely acknowledged the symbiosis between digital transformation and interoperability, this digital fragmentation likely represents a key bottleneck for AI adoption [14, 15].

Barriers to AI implementation, especially concerns about high costs of computational infrastructure, lack of IT support, fear of job displacement, and ethical issues, were generally consistent with previous observations [3, 16]. In line with our findings, the major obstacles identified by Bellini et al. were inadequate infrastructure and a lack of specialized software, followed by poor data integration. However, respondents generally agreed that there is not one single obstacle but many challenges to focus on [13]. While only 31% of our participants clearly expressed concern about job displacement due to AI, fear of job loss was the main concern according to Ardon et al [11]. Furthermore, 75% of respondents in Shami et al.'s [17] survey believed AI would affect future hiring opportunities, with 44.2% thinking lab staff would be replaced by AI. Addressing these foundational barriers will be critical for any effort to scale AI integration in medical laboratories through coordinated national strategies that include investments in digital infrastructure and regulatory frameworks.

An important outcome of this study was the variation in AI perspectives across different subgroups. Laboratory technicians were more worried than laboratory doctors about job loss due to AI replacement. Younger professionals exhibited significantly greater concern about potential job displacement than their older counterparts. These results may reflect differences in professional experience, job security, and long-term career outlook. Younger respondents, particularly those in the early stages of their careers, may perceive themselves as more vulnerable to technological disruptions and less established in roles requiring advanced decision-making, critical thinking, and creative problem-solving, which are less likely to be automated and replaced by AI [4]. In contrast, more experienced professionals might anticipate that the widespread AI integration will occur gradually and not significantly impact their remaining years in the workforce. Additionally, it is believed there will be no replacement for physicians' intuition and expertise gained after many years of professional experience [4]. Respondents from larger laboratories expressed significantly higher expectations for AI benefits, compared with laboratory professionals employed in laboratories processing fewer samples per day. This difference may partly reflect the

greater availability of IT infrastructure and digital systems in larger laboratories, making it difficult to disentangle the effects of laboratory size from those of technological readiness on expectations toward AI. Notably, interest in AI training was significantly more pronounced among laboratory doctors than technicians, reflecting differing roles and expectations within diagnostic workflows. These disparities emphasize the need for tailored communication and training strategies that address specific professional roles and institutional contexts.

The strong interest in AI-focused training shown by the majority of participants reflects a clear willingness to engage with emerging technologies and underscores the need to incorporate AI into medical and laboratory science education. In this study, 84% of respondents indicated readiness to participate in training programs related to AI, reflecting a high level of awareness regarding its growing relevance in Laboratory Medicine. Similar attitudes have been previously reported in other studies, with most respondents showing interest in AI training courses [12, 13]. The medical education system still lacks adequate AI training and understanding of AI methodology, representing a significant barrier in the medium and long-term [16]. As it is evident from our results and previous research, there is a need to introduce and incorporate AI into medical curricula [9, 13]. Integration of foundational AI concepts into undergraduate curricula and postgraduate training, along with offering continuing professional development opportunities, can help reduce knowledge gaps, promote appropriate adoption, and support the ethical implementation of AI in clinical settings [9,13, 14].

Strength of the study

To the best of our knowledge, this study is the first survey of AI perspectives and attitudes within the medical community in Albania. This survey offers a timely insight into the perspectives of laboratory professionals regarding the integration of AI in Laboratory Medicine. A key strength lies in its national scope and design, encompassing professionals from a variety of healthcare settings, including public and private laboratories, as well as regional and university centers. This diversity enhances the generalizability of the findings within the Albanian laboratory context. The inclusion of a broad range of participants by age, role, and institution type contributes to the robustness of the dataset and enables relevant subgroup comparisons. Furthermore, the questionnaire was structured to capture both perceived benefits and concerns related to AI, enabling a balanced investigation of attitudes across different professional demographics.

Limitations of the study

While this survey provides a valuable baseline for assessing AI readiness in Albanian medical laboratories, several limitations must be acknowledged. First, the data are self-reported and may be influenced by social desirability bias or varying levels of understanding of artificial intelligence, potentially leading to misinterpretation of AI-related questions or the misclassification of conventional automation tools as AI. A further limitation is the possibility that professionals employed in the same laboratory discussed the survey among themselves, probably leading to shared interpretations of the questions or influenced responses, potentially biasing the study's results. The observed gender imbalance, with 90.5% of respondents being female, may reflect the actual workforce composition in Albanian laboratories. However, the voluntary nature of participation may limit the generalizability of the findings. Additionally, the survey did not assess the technical specifications or implementation level of AI tools currently in use, restricting insights into practical AI integration. These limitations highlight the need for future research to further evaluate the effectiveness of AI in clinical workflows, assess the outcomes of structured AI training programs, and monitor changes in perceptions as digital maturity evolves.

Conclusion

This national survey highlights a generally positive perspective on AI among laboratory professionals in Albania, alongside a strong interest in AI-focused training and education. These findings offer a foundation for future regulatory policies and educational initiatives aimed at advancing AI-enabled diagnostic services in Albania and similar healthcare systems.

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Artificial Intelligence Usage Statement

The authors declare the use of AI tools (ChatGPT, OpenAI, San Francisco, CA, USA, and Grammarly Inc., San Francisco, CA, USA) to assist in grammar checking, editing the language, and improving clarity in the writing of this manuscript. All scientific content and interpretations remain the sole responsibility of the authors.

Declaration of Conflict of Interest

The authors declare no conflict of interest.

Ethical Approval

This study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. As the survey was anonymous, voluntary, involved no medical intervention, posed no risks to participants, and collected no identifiable personal data, ethics committee approval was not required.

Author's Disclosures

The authors have nothing to disclose.

Ethical Principles Compliance

This study complies with the ethical principles for medical research involving human subjects, in accordance with the Declaration of Helsinki.

Credit Author Statements

Helena Lame: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing-Original Draft, Writing - Review & Editing, Visualization, Project administration
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Data Availability Statement

The dataset generated and analyzed during this study is available from the corresponding author upon reasonable request.

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Survey: Artificial Intelligence in Laboratory Medicine
'Artificial Intelligence refers to computer systems that mimic human cognition and are capable of performing tasks that typically require human intelligence.'

I. General Information and Demographics (six questions)

1. Respondent's professional profile
a. Laboratory Doctor
b. Laboratory Technician

2. Gender
a. Male
b. Female
c. Other

3. Age

4. Laboratory type
a. University Hospital Center laboratory
b. Regional Hospital laboratory
c. Municipality Hospital laboratory
d. Ambulatory Polyclinic laboratory
e. Private Laboratory

5. ISO 15139 accreditation status of your laboratory
a. Accredited
b. Not accredited
c. In the accreditation process

6. Sample volume per day processed by your laboratory
a. >/= 500
b. < 500

II. Digital Properties and Health Data Access (five questions)

7. How do you evaluate the speed and stability of the internet connection in your laboratory?
a. Very appropriate
b. Appropriate
c. Somewhat appropriate
d. Not appropriate

8. How do you evaluate the adequacy of hardware and software available in your laboratory?
a. Very appropriate
b. Appropriate
c. Somewhat appropriate
d. Not appropriate

9. Which patients' clinical data can you access:
a. I have no access to patients' clinical data
b. Patients' electronic health records
c. Clinical data from the referral clinicians
d. Clinical data obtained directly through the outpatients' anamneses

10. Can you access and extract patients' laboratory data from the LIS?
a. Yes
b. No

11. Is there integration between the LIS and other health databases within your institution?
a. No integration
b. Partial integration (with data from other diagnostic departments, e.g., microbiology or pathology)
c. Integration (with data from other health databases within your institution)

III. Perspectives on Artificial Intelligence in Medical Laboratories (fourteen questions)

12. How do you evaluate your knowledge level of Artificial Intelligence:
a. Very appropriate
b. Appropriate
c. Somewhat appropriate
d. Not appropriate

13. Is Artificial Intelligence actually used in your laboratory?
a. Yes
b. No

14. Which AI-related applications are used in your laboratory?
(open text)

15. Which laboratory sections could benefit the most from AI applications? (multiple choice)
a. Clinical Chemistry
b. Immunochemistry
c. Coagulation
d. Hematology
e. Immunology
f. Urinalysis
g. Microbiology
h. Pathology
i. Molecular Biology
j. Point of Care Testing
k. Other (specify)

16. Do you think AI implementation could reduce costs and enhance diagnostic efficiency?
a. Yes
b. No
c. I don't know

17. Do you think AI implementation would streamline routine workflows and save time?
a. Yes
b. No
c. I don't know

18. Do you think that AI implementation would improve analytical accuracy and precision?
a. Yes
b. No
c. I don't know

19. Do you think that AI implementation could reduce work-related stress and fatigue?
a. Yes
b. No
c. I don't know

20. Do you think that AI would simplify daily tasks, allowing personnel to devote more time to creative activities?
a. Yes
b. No
c. I don't know

21. How do you evaluate the barriers of AI implementation in your laboratory?
a. Minimal
b. Moderate
c. Important
d. Maximal

22. What are the barriers to AI implementation in your laboratory? (multiple choice)
a. High cost of implementation
b. Lack of IT infrastructure
c. Lack of specialized staff
d. Ethical considerations
e. Usability challenges
f. Other (specify)

23. Are you concerned about job loss due to AI replacement?
a. Yes
b. No
c. I don't know

24. Are you interested in an AI training course?
a. Yes
b. No
c. I don't know

25. How is your general attitude toward AI in Laboratory Medicine?
a. Positive
b. Negative
c. I don't know

Research Article

Triple Point Pooled Sera (TriPPS) QC for Laboratory Analyte Error Detection: A Machine Learning based Quality Control in Laboratory

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Keywords

Serum, Machine Learning, Quality Control, Medical Errors/ prevention & control, Clinical Laboratory Techniques

Abstract

Background: Reliable internal quality control (IQC) is vital for ensuring analytical accuracy in clinical laboratories. Conventional rule-based QC systems, such as Westgard and Levey–Jennings, often exhibit retrospective detection and limited sensitivity to small but clinically meaningful shifts. This study introduces the Triple-Point Pooled Sera (TriPPS) Quality Control system, a novel, machine-learning-based framework integrating in-house pooled sera with adaptive algorithms for enhanced error detection.

Methods: Residual patient sera were pooled to create stable, matrix-relevant IQC material for 60 consecutive analytical days. Sodium and potassium were used as representative analytes. Three complementary machine learning models were applied: k-Nearest Neighbour (k-NN) for trend detection, Isolation Forest (IF) for random error identification, and Gaussian Process Regression (GPR) for systematic bias modeling. Controlled ±1% daily biases and stochastic random errors were introduced to simulate analytical drift. Detection lag, sensitivity, and anomaly classification were evaluated.

Results: The k-NN algorithm effectively identified trend errors within 0–2 days of bias onset, while IF accurately detected random fluctuations with minimal false positives. GPR modeled nonlinear systematic drift with high fidelity, capturing bias progression that is overlooked by linear methods. The integration of pooled sera enhanced the system's stability, reproducibility, and cost efficiency across all error types.

Conclusion: The TriPPS system demonstrates a scalable, data-driven approach to laboratory quality control by combining pooled sera with machine learning algorithms. This framework enhances analytical vigilance, facilitates proactive error identification, and provides a practical, resource-efficient solution for real-time QC monitoring in clinical chemistry laboratories.

Introduction

Precise and accurate biochemical testing is crucial for patient management; as clinical decisions often rely on laboratory-generated data. Therefore, robust internal quality control (IQC) systems are crucial for ensuring analytical accuracy, minimizing reporting errors, and safeguarding patient safety [1, 2]. IQC consistently assesses intra-laboratory variability to ascertain whether analytical variations remain below acceptable thresholds and detect possible deviations before they affect outcomes. Despite the prevalent use of commercial control materials, their elevated cost, lot-to-lot variability, and sporadic unavailability continue to pose significant obstacles, especially for laboratories in low- and middle-income regions [3]. Conversely, in-house pooled sera have emerged as a practical and dependable alternative, providing a matrix equivalent to native patient samples while significantly reducing costs and logistical reliance [1, 4, 5]. Pooled sera have demonstrated significant efficacy in detecting both random and systematic analytical mistakes, therefore maintaining analytical consistency over prolonged monitoring intervals [4].

Considering their evident potential, pooled-sera-based IQC programs continue to focus significantly on conventional statistical process control methodologies, like Levey-Jennings plots and Westgard multi-rule criteria [6, 7]. Although these approaches have been fundamental to clinical laboratory quality control for decades, they are fundamentally retroactive, identifying out-of-control occurrences only after a substantial deviation has transpired. Analytical alterations may remain unnoticed for several hours or even days prior to intervention [8, 9]. Furthermore, these traditional rule-based systems have restricted sensitivity to minor but clinically significant variations, resulting in misleading confidence in analytical stability [10–13]. The growing intricacy of automated laboratory systems, combined with increased sample throughput, has exacerbated manual validation procedures, thereby heightening the likelihood that minor or transient errors go undetected [10]. These constraints underscore the urgent necessity for more flexible, immediate, and data-informed methodologies in laboratory quality assurance.

Recent advances in machine learning (ML) present a significant opportunity to address these limitations. Machine learning algorithms possess the capability to independently learn from historical data patterns, identify intricate, non-linear relationships, and detect anomalies with significantly greater sensitivity compared to conventional quality control rules [10, 12, 14, 15]. In the context of laboratory analytics, machine learning can enhance the early detection of random noise and systematic drift, thereby connecting statistical process control with predictive maintenance [10]. Incorporating machine learning into standard quality control procedures has the potential to enhance the reliability of analytical outcomes and establish ongoing, intelligent monitoring systems in clinical laboratories.

This study presents and assesses the Triple-Point Pooled Sera (TriPPS) Quality Control system, an innovative machine-learning-based QC model designed for the detection of laboratory analyte errors. The TriPPS approach integrates pooled sera as a cost-effective, matrix-relevant control material alongside a suite of machine learning algorithms, such as k-Nearest Neighbour (k-NN), Isolation Forest (IF), and Gaussian Process Regression (GPR), to identify and categorize analytical deviations. The term “Triple-Point” in TriPPS reflects the integration of three complementary analytical layers within a unified QC framework: (1) matrix-relevant pooled sera as the material foundation, (2) multi-domain error detection encompassing trend, random, and systematic deviations, and (3) three distinct machine learning algorithms optimized for each error category. This tripartite architecture enables simultaneous surveillance across multiple dimensions of analytical instability. This study seeks to create a comprehensive, scalable QC framework that simultaneously tackles trend, random, and systematic errors, thereby enhancing analytical vigilance and resilience in clinical chemistry laboratories.

Methodology

The study was conducted in the Department of Biochemistry at All India Institute of Medical Sciences. As only discarded samples were used, the Institutional Ethical Committee waived the need for ethical clearance.

Preparation of Pooled Sera

The estimated requirement of serum for each analyte across the study duration was approximately 100 µL per run; thus, for a 60-day evaluation period, a total of 6 mL of pooled sera was required. The pooled sera were prepared on a single day (Day 0) using residual and discarded patient serum samples collected from the clinical biochemistry laboratory. Based on patient reports, only samples falling within or close to the reference intervals were selected to ensure representative analytical performance. Samples exhibiting visible haemolysis, lipemia, icterus, or insufficient volume were excluded to preserve analytical integrity.

Although individual samples were not screened for infectious pathogens such as HIV, Hepatitis B, or Hepatitis C, owing to the retrospective and pooled nature of the work, strict adherence to universal biosafety precautions was maintained. Personnel used gloves, laboratory coats, and face shields, and followed institutional protocols for hand hygiene and surface disinfection. The collected sera were thoroughly homogenized to ensure compositional uniformity, then aliquoted into 60 sterile 250 µL microcentrifuge tubes using calibrated micropipettes. All aliquots were immediately frozen at –5 °C in a dedicated container to maintain analyte stability throughout the 60-day quality-control (QC) period. For each analytical day, one aliquot was thawed and analysed in parallel with commercial QC material, serving as an internal stability comparator.

Analytical Workflow and Data Acquisition

Each pooled-sera aliquot was assayed using the routine biochemistry analyzer under identical operational conditions to minimize instrument-related variation. Daily analyses were performed twice, once in the morning and once in the evening , to simulate two independent QC runs and to assess possible temporal drift. Electrolytes, specifically sodium and potassium, were chosen as model parameters due to their analytical robustness and relevance in monitoring instrument performance. Raw measurement data were recorded for each run and compiled into a longitudinal dataset spanning 60 consecutive days.

To evaluate the models' detection capability, systematic and random errors were artificially induced. A controlled linear bias of ±1% per day was introduced into the dataset to simulate analytical drift (trend error). In contrast, stochastic random errors were injected at predefined intervals to represent transient measurement noise. Each day's run was labeled as Normal, Amber (with a deviation of more than 75% from the baseline mean), or Red (with a deviation of more than 90% from the baseline mean). These percentage thresholds were selected to represent graded deviation severity relative to the induced bias magnitude, allowing discrimination between moderate and high analytical instability. The cut-offs were intentionally set conservatively to enhance sensitivity during proof-of-concept validation rather than to replicate specific regulatory decision limits.

Machine-Learning Framework

Three complementary machine-learning algorithms were employed to identify analytical anomalies: k-Nearest Neighbour (k-NN) for trend detection, Isolation Forest (IF) for random error detection, and Gaussian Process Regression (GPR) for systematic bias modelling.

k-Nearest Neighbour (k-NN) for Trend Detection

The k-NN model was used to capture gradual changes in the pooled-sera measurements over time. A sliding-window length of 3 days and a neighbourhood size of 1 were adopted after sensitivity testing for optimal performance. The algorithm computed local similarity among consecutive runs based on Euclidean distance, flagging deviations that exceeded predefined drift thresholds. Detection output was expressed as the percentage of Red and Amber days, as well as the lag between the onset of bias and the first detection event.

Isolation Forest (IF) for Random-Error Detection

The IF algorithm, an ensemble-based anomaly detection approach, isolates data points through recursive random partitioning of the feature space. Each measurement's anomaly score was determined by its average path length across a forest of randomly constructed trees (n = 100). Points with shorter path lengths corresponded to higher likelihoods of anomalies. The model was trained on bias-free baseline data and subsequently applied to datasets containing injected random noise to quantify sensitivity and false-flag rate.

Gaussian Process Regression (GPR) for Systematic-Bias Analysis

GPR was employed to model structured, non-linear deviations resulting from gradual bias induction. Using a radial-basis (Gaussian) kernel, the regression function estimated posterior mean predictions and associated uncertainty intervals for each daily observation. Divergence between observed and predicted values was interpreted as evidence of systematic drift. The flexibility of GPR enabled comparison of linear versus non-linear bias progression across both electrolytes.

Evaluation Metrics and Visualization

For each algorithm, performance was assessed using the proportion of flagged Red and Amber days, the lag (in days) to first detection, and visual inspection of anomaly or residual plots. Model outputs were summarized numerically for sodium and potassium across morning and evening sessions. All analyses were performed using Python (version 3.12) with standard scientific libraries , NumPy, pandas, scikit-learn, and matplotlib. The reproducible codebase was validated using repeated subsampling to ensure stability of detection patterns.

Results

This study consisted of a continuous 60-day assessment of pooled serum samples for electrolytes, with a focus on sodium and potassium as indicative analytes of analytical stability. We examined the morning and evening batches separately to determine if there was any variation in measurement drift throughout the day. The k-Nearest Neighbor (k-NN) model was initially utilized to identify linear trends induced by a controlled ±1% bias per day. Categorizing variations into Amber (>75%) and Red (>90%) zones allowed it the potential to measure the model's sensitivity and the period it takes to calculate flags.

The k-NN-based trend detection framework showed the same level of accuracy for both electrolytes. A +1% per-day trend for sodium led to 80% Red-day identification in morning runs and 93.3% in evening runs. A -1% trend, on the other hand, led to 73.3% and 100% detection, respectively. Potassium exhibited a comparable trend, achieving full (100%) detection of negative drift during evening sessions, with an average delay of 0–2 days prior to the initial alert. These results demonstrate that the model rapidly adapts to new analytical biases. This has been summarized in Table 1.

Table 1: Analysis of Trend Detection by k-NN method.

Electrolyte	Run	Error Type	No of Red Days	Red Days (%)	No of Amber Days	Amber Days (%)	First Day of Error Detected	Lagged Days
Sodium	Morning	Trend +1%/ day	12	80	2	13.3	47	1
	Morning	Trend -1%/ day	11	73.3	2	13.3	48	2
	Evening	Trend +1%/ day	14	93.3	1	6.7	46	0
	Evening	Trend -1%/ day	15	100	0	0	46	0
Potassium	Morning	Trend +1%/ day	13	86.7	1	6.7	47	1
	Morning	Trend -1%/ day	13	86.7	2	13.3	46	0
	Evening	Trend +1%/ day	12	80	2	13.3	47	1
	Evening	Trend -1%/ day	15	100	0	0	46	0

The IF algorithm was subsequently utilized to detect random error occurrences within aggregated sera datasets. IF effectively separated sporadic anomalies from background analytical variation by successively segmenting the feature set and defining points with short mean path lengths. In potassium runs, random error induction caused sharp, isolated spikes in anomaly scores that matched the injected noise exactly. In sodium runs, similar short-term changes were found without over-flagging. These observations validate the adaptability of IF in identifying stochastic instability. Figure 1 and 2 illustrate this.

Figure 1: Detection of Random Error in Potassium Pooled Sera Measurement by IF.

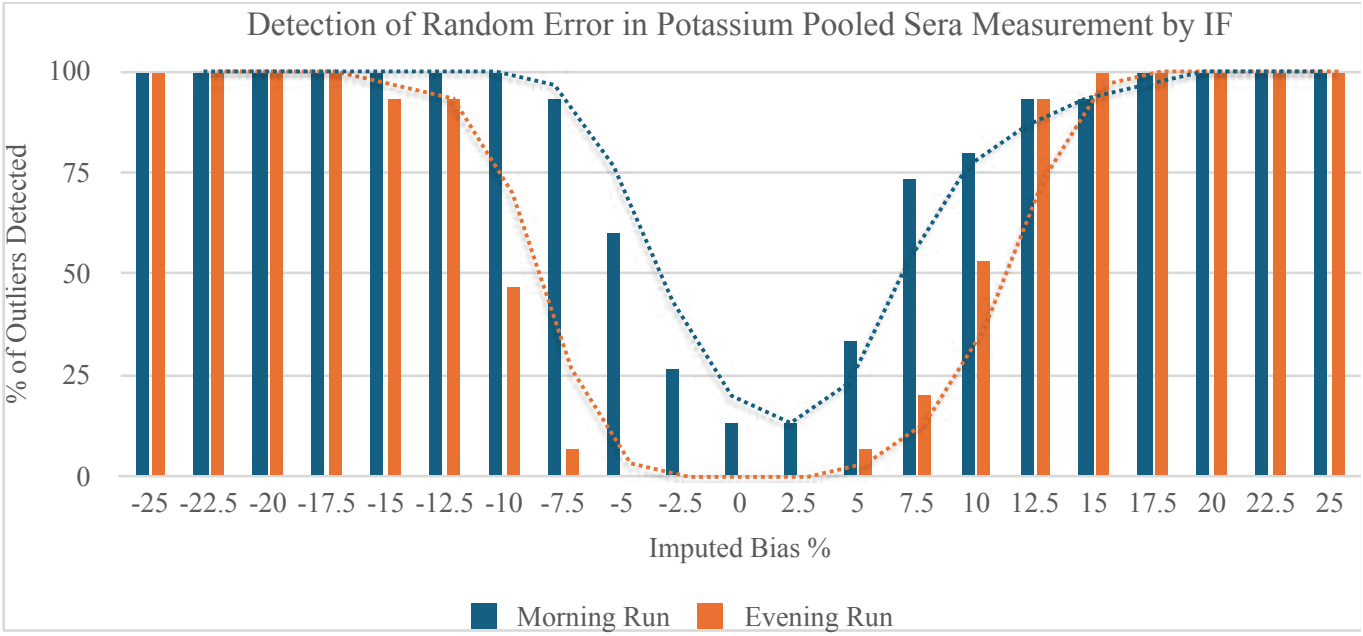
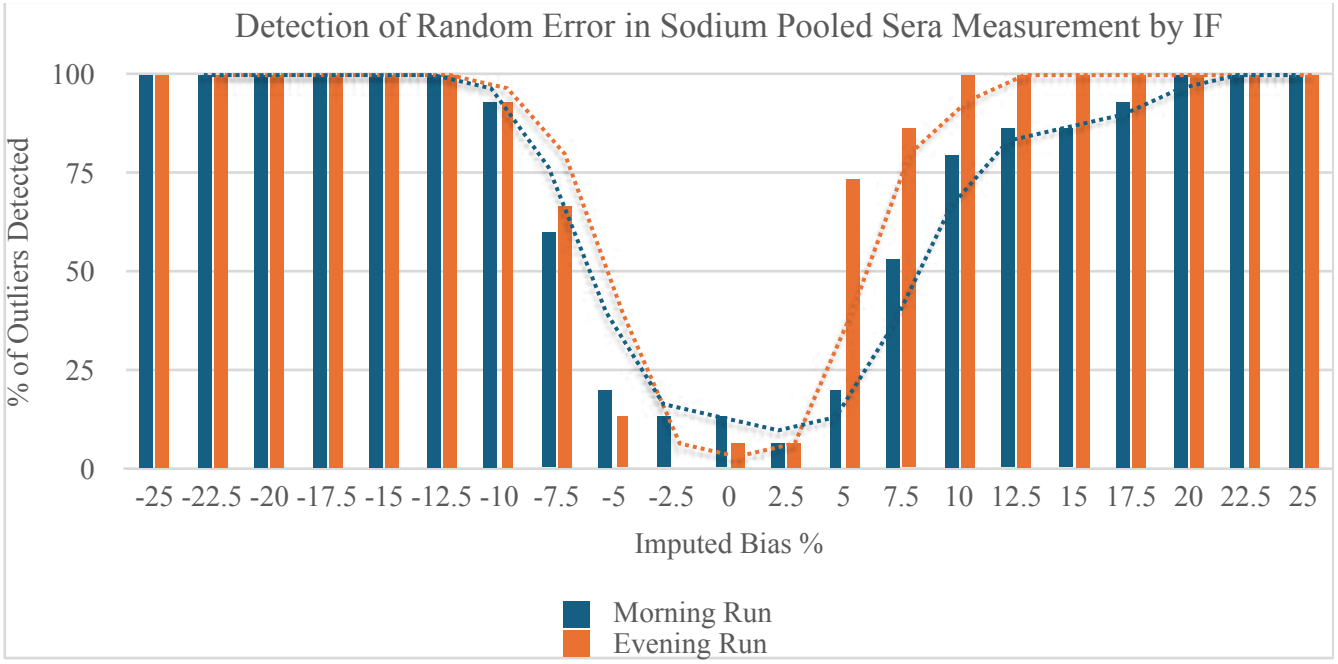


Figure 2: Detection of Random Error in Sodium Pooled Sera Measurement by IF.



Next, Gaussian Process Regression (GPR) was used to examine systematic bias in datasets that had been purposely imputed. In potassium, GPR recorded the progressive deviation of estimated versus measured values as the bias magnitude increased, showing uniform, non-linear shift dynamics. Sodium acted similarly, and Gaussian kernels accurately mapped the residual expansion over time. These results highlight GPR's capacity to model structured deviations that evade detection by linear approaches alone. Figure 3 and 4 depict this.

Figure 3: Systemic Error Detection in Biased Imputation of Potassium by GPR.

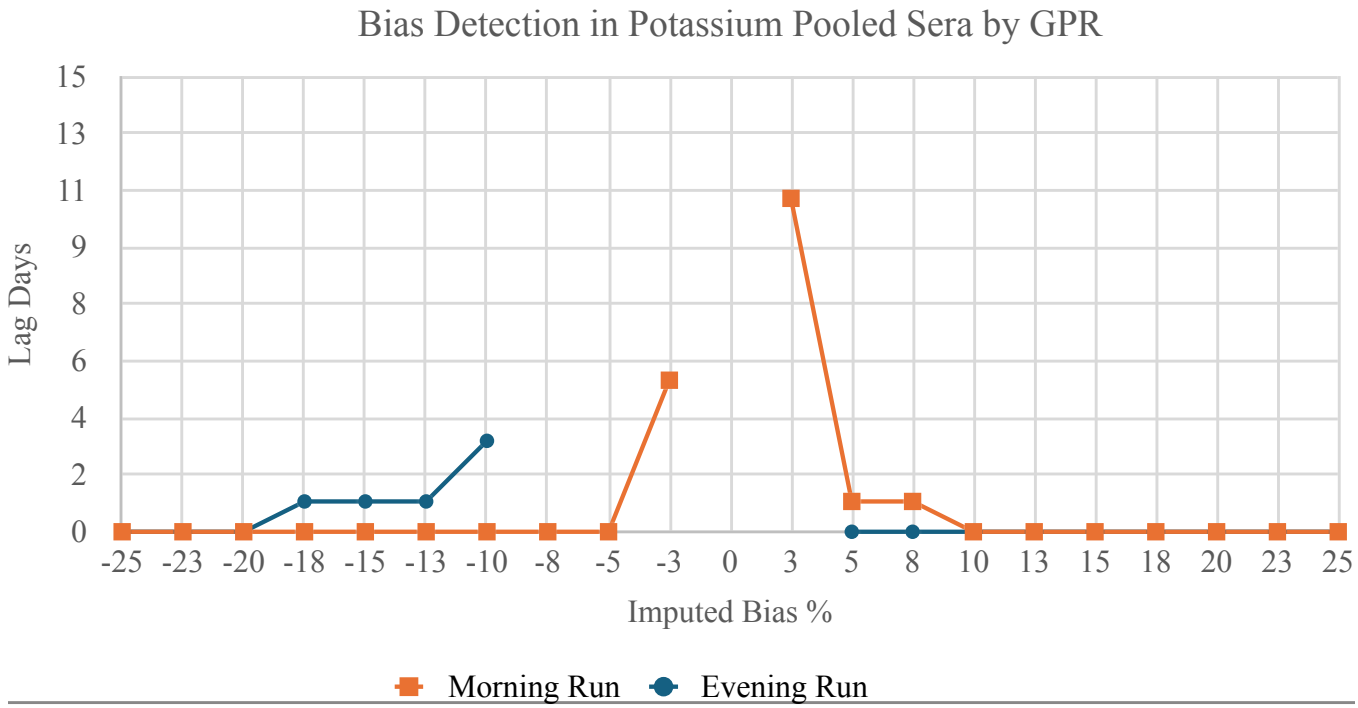
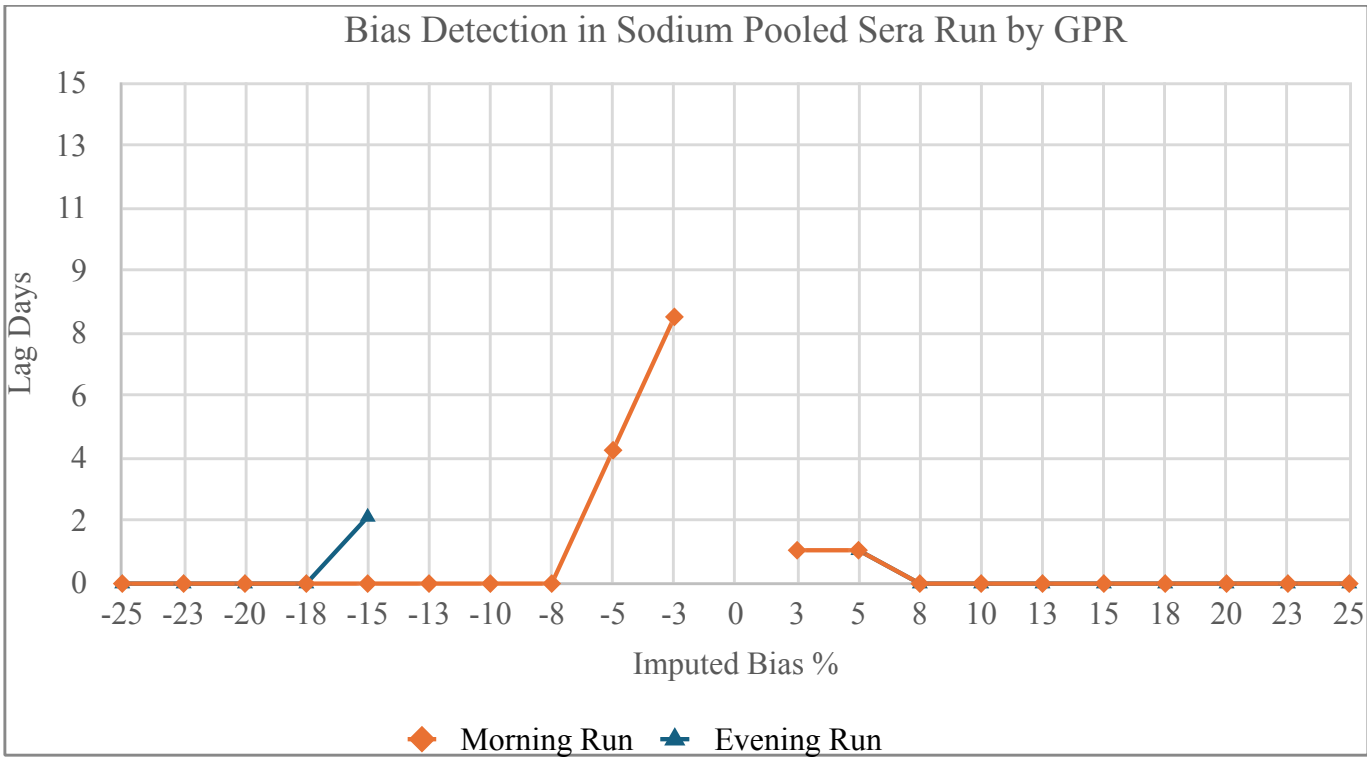
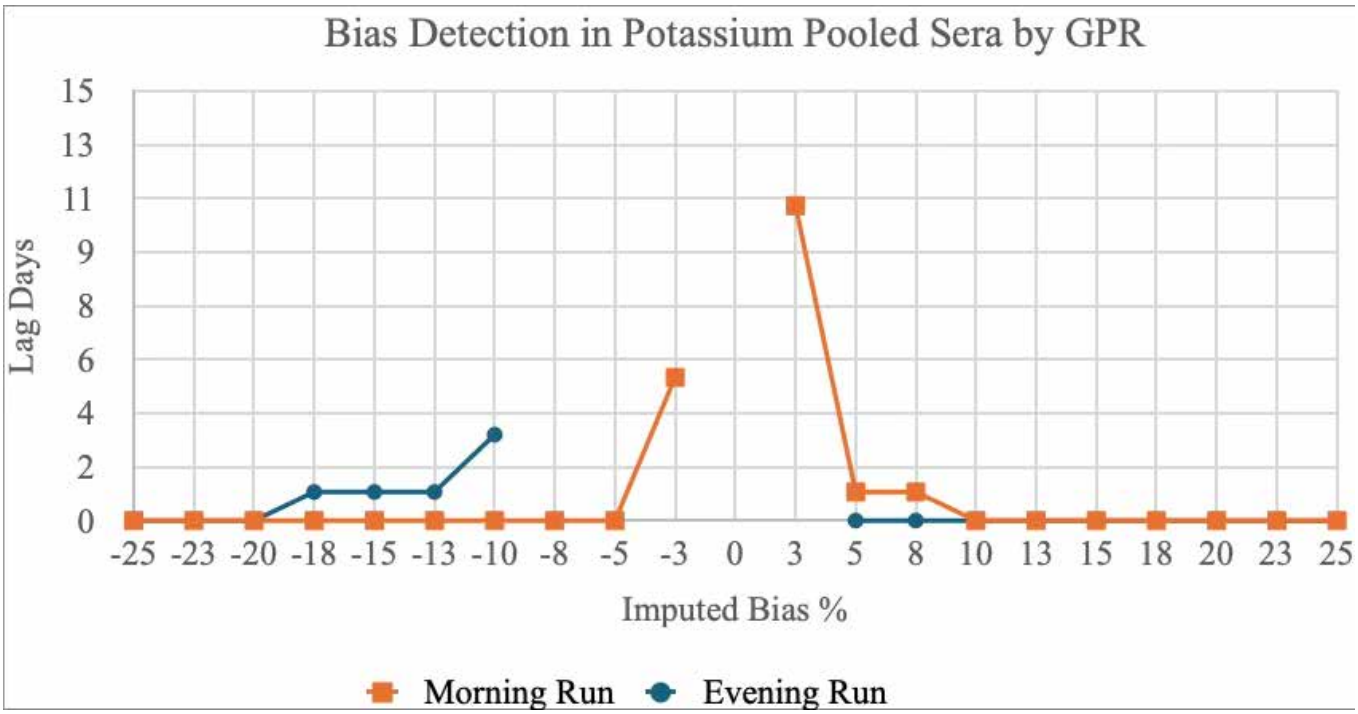


Figure 4: Systemic Error Detection in Biased Imputation of Sodium by GPR.





Discussion

This study presents and assesses the Triple-Point Pooled Sera (TriPPS) Quality Control system, a contemporary machine learning framework designed to enhance the detection of laboratory analyte errors. Through the integration of pooled sera, a cost-effective, matrix-relevant internal control, alongside a suite of complementary algorithms, the TriPPS system creates a multi-layered framework for thorough analytical monitoring. In this study, the k-Nearest Neighbour (k-NN) method was utilized for trend detection, the Isolation Forest was applied for identifying random errors, and the Gaussian Process Regression (GPR) was employed for modeling systematic bias. The system exhibited strong sensitivity in detecting anomalies across various error types by intentionally introducing linear trends and stochastic random errors. The collective findings indicate that the TriPPS model has the potential to enhance analytical vigilance and resilience in clinical chemistry laboratories, especially in environments where resource constraints limit the regular use of commercial control materials.

The enhanced features of machine-learning-driven quality control systems effectively tackle numerous persistent challenges associated with traditional IQC approaches. Traditional rule-based systems, such as Westgard rules or Levey–Jennings charts, depend on retrospective detection and exhibit limited sensitivity to minor but clinically significant deviations, resulting in a delay in identifying analytical instability [10]. In high-throughput automated laboratories, the reliance on manual validation exacerbates this issue, heightening the likelihood that minor drifts or temporary errors remain undetected [10]. Machine learning facilitates data-driven, real-time surveillance by employing adaptive modeling techniques [16, 17]. Through the analysis of historical data and the recognition of complex, non-linear relationships, machine learning-driven quality control frameworks are capable of identifying analytical drift or bias with enhanced precision and efficiency [18]. This capability facilitates the prompt identification of errors associated with calibration drift as well as pre-analytical and analytical disturbances, including sample contamination from intravenous fluids or anticoagulants, delays in analysis, or incorrect blood sample handling. Similar results have been observed in investigations assessing Machine Learning Internal Quality Control (MLiQC) systems, which have shown enhanced bias detection and early warning sensitivity compared to patient-based real-time QC approaches [12]. The convergence of these findings highlights the significant potential of machine learning in transforming quality control approaches from a reactive stance to a proactive monitoring system.

The integration of triple-point pooled sera into the TriPPS framework significantly enhances its practical applicability and methodological strength. Pooled sera, obtained from leftover patient samples, provide a cost-effective and compositionally genuine substitute for commercial controls, addressing challenges associated with cost, availability, and variability between lots [4, 5]. Their biological resemblance to patient matrices guarantees that the analytical performance metrics derived from pooled sera more precisely represent actual clinical scenarios. Previous investigations have demonstrated that pooled sera are effective in detecting both random and systematic analytical errors over extended periods [12, 19]. The TriPPS model enhances this foundation by integrating the stability of pooled sera with algorithmic intelligence, resulting in a cohesive system that can concurrently identify both stochastic and directional errors. This dual-layered integration connects material-based stability assurance with computational vigilance.

The application of ML in this scenario is designed not to replace traditional IQC methods, but to enhance and update them. Integrating statistical process control with predictive maintenance allows for the early identification of emerging noise or drift, thereby evolving quality control into a system of continuous learning [20, 21]. These systems can independently identify unusual runs, anticipate calibration issues, and prioritize samples for manual examination, thus enhancing analytical precision and operational effectiveness [18]. The enhancement of conventional quality control methods leads to fewer undetected errors, quicker corrective measures, and heightened patient safety [10, 12]. Additionally, intelligent automation enables laboratory staff to focus on complex interpretive and troubleshooting activities, thereby enhancing productivity and diagnostic accuracy. Essentially, ML enhances traditional QC methods by integrating decision-support intelligence into the analytical workflow [19]. Nonetheless, various factors need to be examined prior to the widespread adoption of ML-driven QC frameworks. Establishing uniformity in the processes of model development, validation, and reporting is crucial for achieving reproducibility and comparability of algorithms across different laboratories [10, 18]. The integrity of data is essential, as any inconsistencies in data entry, lack of complete metadata, or variations before analysis can undermine the learning process of models and their interpretability. Moreover, the potential dependence on algorithmic results without human supervision, along with ongoing concerns regarding regulatory compliance and data ethics, presents both practical and ethical challenges to clinical application [20, 21]. The primary advantage of this study is its ability to combine pooled sera with machine learning algorithms, yielding a cost-effective, matrix-relevant, and adaptive quality control system. The TriPPS framework effectively identifies random, systematic, and trend-related errors, showcasing robust analytical sensitivity and practical applicability.

However, the results stem from simulated bias and controlled error conditions that utilize only two electrolytes, potentially failing to encompass the full spectrum of real-world analytical variability. Comprehensive validation across a variety of analytes, instruments, and laboratory settings is necessary to

establish the general applicability of this method. It is important to emphasize that the present validation was conducted under controlled, simulated bias and noise conditions to enable systematic performance comparison across algorithms. While this approach allows precise quantification of detection lag and sensitivity, real-world analytical disturbances, such as reagent lot shifts, calibration instability, or instrument maintenance effects, may exhibit more complex patterns and require prospective validation. Moreover, the aspects of long-term stability, operator variability, and reagent-lot effects have not been thoroughly evaluated and require further exploration before routine application.

A logical next phase of investigation would include prospective validation across multi-analyte panels, multiple instrument platforms, and extended monitoring periods incorporating natural laboratory perturbations. Such expansion would determine robustness across different analytical principles and operational environments. Practical implementation would require integration within laboratory middleware or LIS environments capable of automated data streaming and algorithm execution in parallel with conventional QC modules. Transparent audit trails, version control of models, and predefined override mechanisms would be necessary to align with regulatory expectations for AI-assisted diagnostic systems. By focusing on these elements, we can ensure the secure and efficient integration of AI-powered quality control, allowing laboratories to transition from a reactive to a predictive operational framework.

Conclusion

The Triple-Point Pooled Sera (TriPPS) Quality Control system demonstrates how the integration of in-house pooled sera with machine learning algorithms can significantly enhance the detection of analytical errors in clinical laboratories. The integration of k-Nearest Neighbour, Isolation Forest, and Gaussian Process Regression allows the framework to effectively identify random, systematic, and trend-related deviations with minimal lag. This method provides a budget-friendly, relevant, and flexible solution that enhances conventional internal quality control practices, facilitating a shift from identifying errors after the fact to implementing proactive, data-informed laboratory monitoring. Extensive validation involving a variety of analytes and real-time clinical datasets will be crucial for positioning TriPPS as a fundamental element of smart quality assurance in laboratory medicine.

Ethical Approval

This study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. As only anonymized, discarded patient serum samples were used for pooled preparation, without any direct patient involvement or identifiable data, the Institutional Ethics Committee of the Department of Biochemistry, All India Institute of Medical Sciences, Bhubaneswar, waived the requirement for formal ethical approval.

Conflict of Interest

The authors declare that they have no conflicts of interest related to this study. No financial, personal, or professional relationships influenced the conduct or reporting of this research.

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Data Availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request. All data have been anonymized to protect patient confidentiality and comply with institutional data governance policies.

Author Contributions (CRediT Statement)

PD: Conceptualization, Writing – Review & Editing, Supervision. SN: Methodology, Software, Formal Analysis, Writing – Original Draft, Project Administration. SR: Investigation, Data Curation, Resources. BKK: Investigation, Data Curation, Resources. CRP: Investigation, Data Curation, Resources. DP: Investigation, Data Curation, Resources. TR: Investigation, Data Curation, Resources.

All authors reviewed and approved the final version of the manuscript.

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Review article

Circulating netrin-1 levels in type 2 diabetes mellitus: A systematic review and meta-analysis

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Keywords

Netrin-1, Type 2 diabetes mellitus, Biomarker, Meta-analysis

Abstract

Background: Netrin-1, a laminin-related guidance cue protein with emerging immunomodulatory roles, has shown conflicting associations with type 2 diabetes mellitus (T2DM) in human studies. We conducted a systematic review and meta-analysis to evaluate circulating netrin-1 levels in individuals with T2DM compared to healthy controls.

Methods: A comprehensive search of MEDLINE/PubMed, Scopus, and Europe PMC up to March 17, 2025, was performed for studies reporting netrin-1 levels in T2DM patients. Eligible studies were observational and provided extractable quantitative data. Study quality was assessed using the Newcastle-Ottawa Scale and JBI checklist. Standardized mean differences (SMDs) with 95% confidence intervals (CIs) were calculated using a random-effects model. Heterogeneity, subgroup, sensitivity, and publication bias analyses were performed using R software.

Results: Twenty studies involving 1,798 articles were included. Pooled analysis revealed significantly elevated netrin-1 levels in T2DM (SMD = 0.57; 95% CI: 0.03–1.11; $p = 0.0393$), with substantial heterogeneity ($I^2 = 96.4\%$). Subgroup analyses indicated geographic and diagnostic criteria as key moderators. Egyptian studies and those using ADA guidelines reported increased netrin-1, while Indian and WHO-based studies reported reductions. The 95% prediction interval (–2.03 to 3.18) reflected wide variability. No significant publication bias was detected (LFK index = –0.49).

Conclusion: This meta-analysis supports a potential link between netrin-1 and T2DM, suggesting its role as a biomarker of metabolic inflammation. However, substantial heterogeneity and study-level differences necessitate further standardized, longitudinal research to clarify its clinical relevance.

Introduction

Type 2 diabetes mellitus (T2DM) is a rapidly growing global health concern, characterized by chronic hyperglycemia resulting from insulin resistance and progressive β-cell dysfunction. According to recent estimates, approximately 537 million adults worldwide (aged 20–79 years) are currently living with diabetes, a number projected to rise to 643 million by 2030 and 783 million by 2045 [1]. The pathophysiology of T2DM is multifactorial, involving metabolic dysregulation, lipotoxicity, and chronic low-grade inflammation. Sustained elevations in glucose and free fatty acid levels contribute to β-cell apoptosis and heightened production of pro-inflammatory mediators, which further exacerbate insulin resistance and disease progression [2].

Netrin-1 (NTN-1), originally identified as a member of the neuronal guidance cue family, has garnered increasing attention for its immunomodulatory and tissue-protective properties. This secreted laminin-related protein exerts both chemoattractive and chemorepulsive effects, mediated through interactions with a diverse set of receptors, including UNC5 homologs (UNC5A-D), deleted in colorectal carcinoma (DCC), neogenin-1 (NEO-1), A2B adenosine receptor (A2BAR), CD146, and integrins [3,4]. Although first discovered in the central nervous system, netrin-1 is widely expressed in peripheral tissues such as the vascular endothelium, pancreas, liver, spleen, kidney, and lungs [5]. Emerging evidence suggests its involvement in various inflammation-associated diseases, including cardiovascular and hepatic disorders, cancer, obesity, and ischemia-reperfusion injury, highlighting its systemic role in immune regulation and tissue homeostasis [6-9].

In the context of metabolic disease, netrin-1 has been implicated in attenuating vascular inflammation by reducing endothelial adhesion and monocyte infiltration- processes central to atherogenesis. Experimental models also demonstrate its antiangiogenic and vasodilatory effects via enhanced nitric oxide production, suggesting a protective role in ischemic and inflammatory settings [10]. Given these properties, netrin-1 may serve as a key link between metabolic inflammation and the development or progression of T2DM.

Despite these mechanistic insights, clinical studies evaluating circulating netrin-1 levels in patients with T2DM have yielded inconsistent results. Some investigations report elevated levels in diabetic individuals, potentially as a compensatory anti-inflammatory response [11, 12], while others demonstrate reduced levels, implying a possible deficit in endogenous protection [13, 14]. A prior meta-analysis by Behnoush et al. (2023) synthesized data from 19 studies investigating netrin-1 levels across diverse glycemic states and diabetes-related complications. However, only 8 of these studies specifically focused on individuals with T2DM, thereby limiting the strength of conclusions that could be drawn for this population [15].

To address this gap, the present systematic review and meta-analysis synthesizes evidence from 20 eligible studies investigating circulating netrin-1 levels in individuals with T2DM. By integrating these data, we aim to provide a comprehensive evaluation of the association between netrin-1 and T2DM, with the goal of elucidating its potential utility as a biomarker or therapeutic target in diabetes-associated inflammation and metabolic dysregulation.

Materials and Methods

The study adhered to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [16] and was prospectively registered with the International Prospective Register of Systematic Reviews (PROSPERO) under reference number CRD42023449622.

Search strategy and study selection

MEDLINE/PubMed, Scopus, and Europe PMC databases were systematically searched for studies reporting netrin-1 levels in individuals with type 2 diabetes mellitus, covering the period from the inception of these databases until 17 March 2025. The search was restricted to human studies and publications in the English language. The following search terms were utilized: “Type 2 Diabetes Mellitus”, “Diabetes Mellitus”, “Type 2 Diabetes”, “Diabetes”, “Diabetics”, “Type 2 DM”, “T2DM”, “T2D”, “Netrin-1”, “NTN1” and “NT-1”. A detailed search strategy for each database is presented in Supplementary Table 1, additionally, the bibliographies of relevant articles were manually searched. Titles and abstracts of all retrieved articles were independently screened by all reviewers to identify articles for full-text review and subsequent inclusion. Discrepancies were resolved through consensus-based discussion. Articles that satisfied the inclusion criteria were selected for the meta-analysis.

Eligibility criteria

Inclusion Criteria: The PECOS format was employed to delineate the inclusion criteria: P (Population): individuals diagnosed with type 2 diabetes mellitus and healthy control participants; E (Exposure): patients with type 2 diabetes mellitus; C (Comparator): non-diabetic healthy control subjects; O (Outcome): levels of netrin-1; and S (Study Design): observational studies that provide clear and extractable data regarding netrin-1.

The exclusion criteria were as follows:

1. Publications categorized as case reports or reviews (including systematic reviews or meta-analyses), editorials, commentaries, and conference abstracts;
2. Studies involving animal models or cell lines;
3. Papers that did not provide raw data;
4. Research articles lacking full text; and
5. Unpublished or ongoing trials. The inclusion and exclusion criteria were established following extensive discussions among the authors of this study.

Data extraction

Two independent researchers (RKM and VR) carried out the initial data extraction process. The information obtained encompassed the first author's name, year of publication, country of study, study design, diagnostic criteria for T2DM, the number of T2DM cases and healthy controls, netrin-1 levels in both groups, participant age, and other pertinent details. In instances of missing or incomplete data, the corresponding author of the respective study was contacted for clarification. A third investigator (MKM) subsequently verified the accuracy of the extracted data. Any discrepancies identified were resolved through discussion among all investigators.

Quality assessment

The methodological quality of the included case-control studies was assessed using the Newcastle-Ottawa Scale (NOS), which evaluates studies across three domains: selection, comparability, and outcome assessment. A maximum score of 9 points could be awarded. Based on the NOS score, studies were categorized as low quality (≤4 points), moderate quality (5–6 points), or high quality (≥7 points) [17]. For cross-sectional studies, the Joanna Briggs Institute (JBI) Critical Appraisal Checklist for Analytical Cross-Sectional Studies was utilized. Studies that received ≤49% "yes" responses were deemed to possess a high risk of bias (low quality), those with 50–69% were classified as having a moderate risk of bias (moderate quality), and those with ≥70% were considered to have a low risk of bias (high quality) [18]. Two reviewers (RKM and VR) conducted an independent assessment of the study quality. A third reviewer (MKM) verified these evaluations, and any discrepancies were resolved through consensus discussions.

Statistical analysis

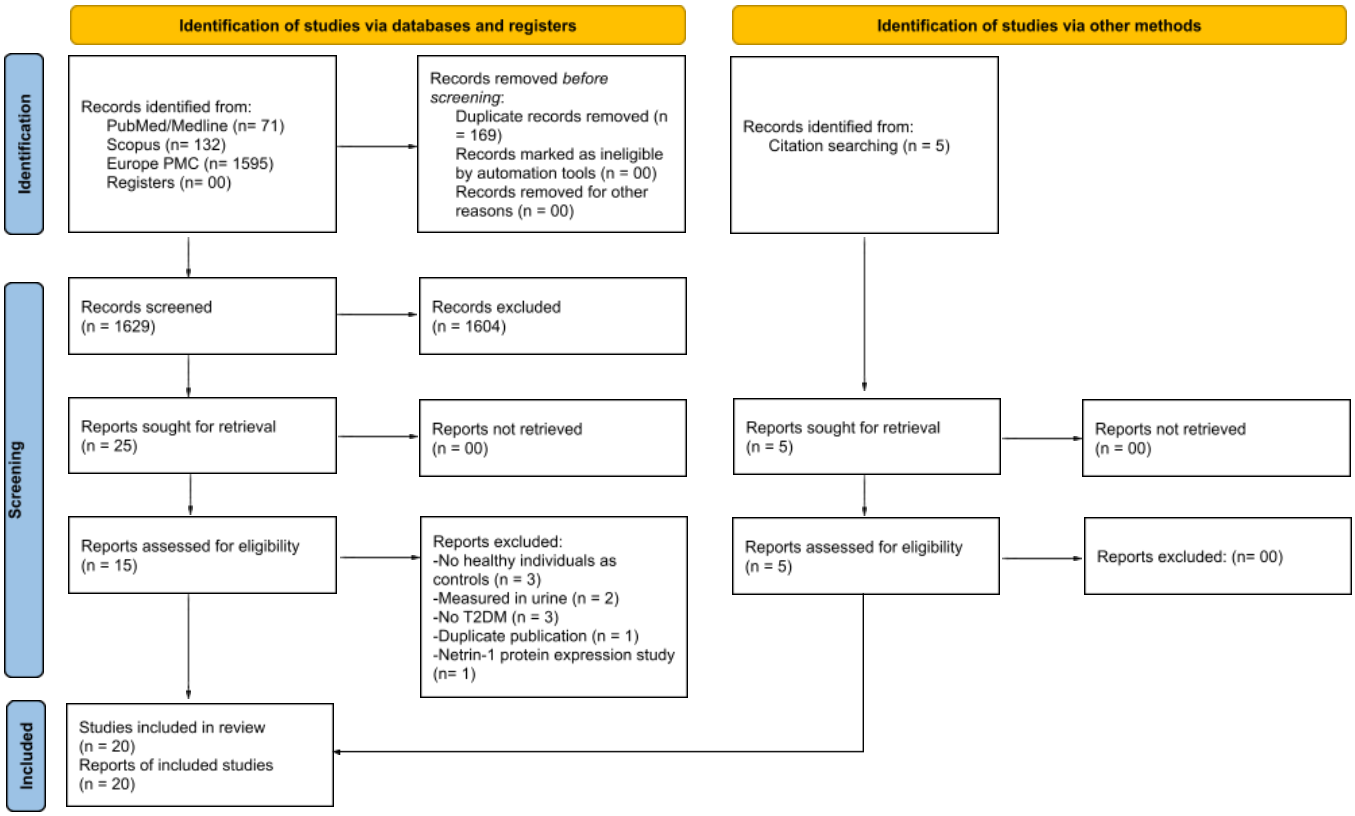
All statistical analyses were conducted using R software (version 4.4.1), specifically employing the "meta" and "metasens" packages. The comparison of circulating netrin-1 concentrations between individuals diagnosed with T2DM and healthy controls was performed by calculating standardized mean differences (SMD) along with 95% confidence intervals (CIs). Furthermore, a 95% prediction interval (PI) was included to provide an estimate of the potential range of netrin-1 values in future studies. A random-effects model was selected for the meta-analysis to accommodate the anticipated clinical heterogeneity among the included studies. For datasets presented as medians with interquartile ranges (IQRs), these values were converted into means and standard deviations (SDs) using established conversion techniques available through the meta-analysis accelerator platform [19]. To evaluate the consistency and reliability of the findings, sensitivity analyses were conducted. Study heterogeneity was assessed utilizing the Cochrane Q test and I^2 statistic, with thresholds of $p < 0.10$ and $I^2 > 50\%$ denoting substantial heterogeneity. To explore sources of heterogeneity, subgroup analyses were performed based on geographical region, diagnostic criteria, sample size, biological sample type, and study design. To assess potential publication bias, Doi plots were visually inspected, and asymmetry was quantitatively measured employing the Luis Furuya-Kanamori (LFK) index [20]. The interpretation of the LFK index adhered to established guidelines: values ranging from −1 to +1 were deemed to reflect symmetry, values between ±1 and ±2 indicated slight asymmetry, and values exceeding ±2 were indicative of substantial asymmetry. A p-value of less than 0.05 was considered statistically significant unless otherwise specified.

Results

Characteristics of included studies

A total of 1,798 articles were initially identified through the implemented search strategy. Following the removal of duplicates, 1,629 records remained for screening. Of these, 25 articles were subjected to full-text review, leading to the exclusion of 10 studies based on the criteria outlined in Figure 1. Furthermore, an additional five studies were identified through citation tracking. Ultimately, 20 studies met all inclusion criteria and were incorporated into the systematic review and meta-analysis [11-14, 21-36]. The comprehensive selection process is illustrated in the PRISMA flow diagram (Figure 1).

Figure 1: Shows the PRISMA flow diagram for the study selection process.



Of the studies included in the analysis, seven were from Egypt [12, 21, 22, 24, 28, 32, 34], five from Iraq [11, 13, 14, 31, 33], two from India [27, 35], and one each from Mexico [23], China [25], Spain [26], Bulgaria [29], Turkey [30], and Korea [36]. Thirteen studies employed a cross-sectional design [14, 21-23, 25, 27-29, 32-36], while seven followed a case-control format [11-13, 24, 26, 30, 31]. Ten studies diagnosed T2DM using the American Diabetes Association (ADA) criteria [11, 12, 21-24, 26, 34-36], one used World Health Organization (WHO) guidelines [25], and the remaining nine did not specify their diagnostic approach [13, 14, 27-33]. Netrin-1 levels were assessed in serum samples in 18 studies [11-14, 21-24, 27-36] and in plasma samples in 2 studies [25, 26]. The publication years of the included studies ranged from 2016 to 2024. Detailed characteristics of each study are summarized in Table 1. The quality results of included studies are also presented in Table 1.

Table 1: Baseline characteristics of studies included in meta-analysis.

Author	Year	Country	Study design	Diagnostic criteria of diabetes	Type 2 DM		Controls		Sample	Quality	Main findings
					Age (Years) Mean±SD or Range	N	Age (Years) Mean±SD or Range	N			
Al-Shakour et al. [11]	2024	Iraq	C-C	ADA	53.60 ± 9.10	81	52.60 ± 9.67	79	Serum	High	The average serum Netrin-1 level was significantly elevated in patients with type 2 diabetes mellitus compared to the control group.
Assi et al. [13]	2021	Iraq	C-C	NR	54.44±11.190	45	49.21±14.91	45	Serum	Moderate	Netrin-1 levels were significantly decreased in newly diagnosed patients with type 2 diabetes compared to the control group.

Badran et al. [21]	2024	Egypt	C-S	ADA	54.90±10.18	60	54.29 ±10.64	30	Serum	High	Patients with type 2 diabetes and diabetic nephropathy (DN) had significantly lower levels of Netrin-1 compared to those with type 2 diabetes without nephropathy; however, there was no statistically significant difference between the DN group and the control group.
Elkholy et al. [22]	2021	Egypt	C-S	ADA	52.10±9.54	135	50.3±8.1	45	Serum	High	The mean serum netrin-1 levels were significantly elevated in diabetic nephropathy (DN) patients with microalbuminuria and macroalbuminuria compared to the control group. Among these, the highest levels were found in patients with macroalbuminuria, and the differences between these groups were statistically significant. However, no statistically significant difference in netrin-1 levels was found between normoalbuminuric type 2 diabetes mellitus (T2DM) patients and the control group.
Fadel et al. [12]	2021	Egypt	C-C	ADA	50.36 ±7.57	100	52.22 ± 8.72	80	Serum	High	Patients with uncomplicated type 2 diabetes mellitus (T2DM) and those with complications both showed significantly increased netrin-1 levels compared to the control group. Additionally, patients with complicated T2DM had significantly higher netrin-1 levels compared to those without complications.
Garcia Galindo et al. [23]	2023	Mexico	C-S	ADA	51.3±5.5	30	22.8 ± 4.3	30	Serum	High	Ntn1 levels were significantly elevated in newly diagnosed patients with type 2 diabetes.

Ibrahem et al. [24]	2024	Egypt	C-C	ADA	53.24 ± 11.76	34	45.60 ± 10.60	20	Serum	High	Serum netrin-1 levels were significantly higher in patients with type 2 diabetes mellitus compared to the control group.
Khazeil et al. [14]	2020	Iraq	C-S	NR	54.11±2.44	58	42.7 ± 2.1	30	Serum	Moderate	Netrin-1 levels were found to be lower on average in individuals with type 2 diabetes mellitus (T2DM) compared to the control group.
Liu et al. [25]	2016	China	C-S	WHO	52.7 (11.08)	30	52.96 (11.65)	26	Plasma	High	Netrin-1 levels were significantly decreased in patients with type 2 diabetes mellitus compared to the control group.
Mentxaka et al. [26]	2022	Spain	C-C	ADA	47±2	41	42±5	18	Plasma	High	Circulating levels of NTN-1 were significantly elevated in individuals with obese type 2 diabetes compared to lean control individuals.
Mondal et al. [27]	2024	India	C-S	NR	50.96±9.57	72	43.07±10.53	45	Serum	Moderate	Serum netrin-1 concentrations were markedly lower in patients with type 2 diabetes without small nerve fiber damage (DN-) and with small nerve fiber damage (DN+) compared to the control group.
Naiem et al. [28]	2024	Egypt	C-S	NR	53.53±8.60	90	53.07±8.4	30	Serum	High	Plasma netrin-1 levels are significantly increased in patients with diabetic nephropathy, including those with microalbuminuria or macroalbuminuria, highlighting its potential as an early biomarker for the diagnosis of diabetic nephropathy.
Nedeva et al. [29]	2020	Bulgaria	C-S	NR	54.18±10.86	39	49.38±12.12	42	Serum	High	Lower levels of Netrin-1 were reported in patients with newly diagnosed type 2 diabetes compared to the healthy control group.

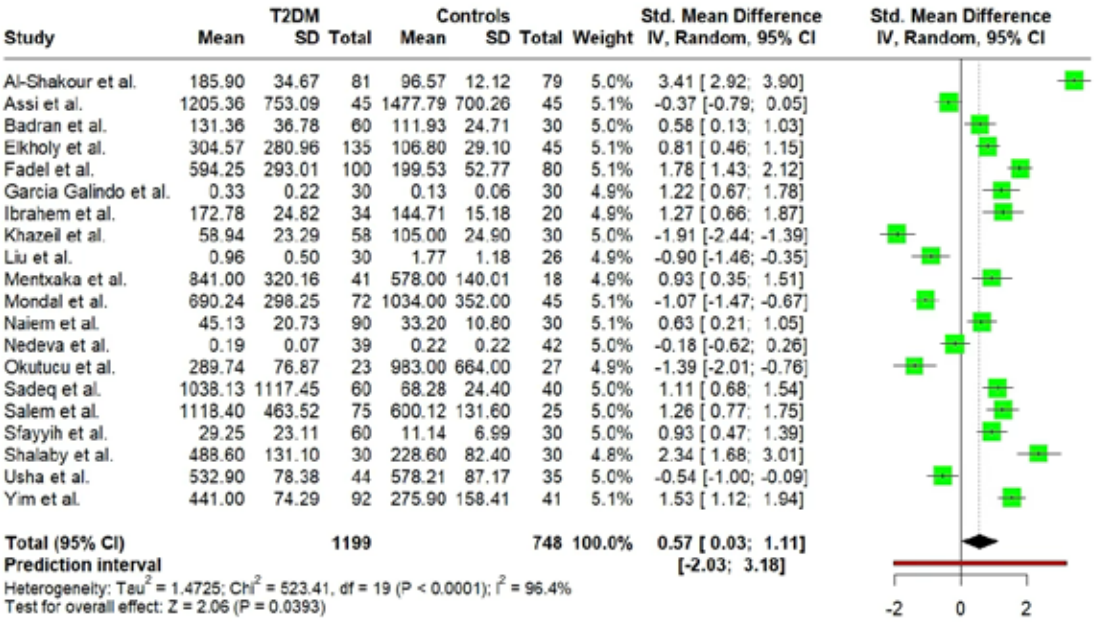
Okutucu et al. [30]	2021	Turkey	C-C	NR	67.35±10.32	23	65.74 ± 7.17	27	Serum	High	Patients with proliferative and non-proliferative diabetic retinopathy exhibited significantly lower serum netrin-1 levels compared to the control group.
Sadeq et al. [31]	2021	Iraq	C-C	NR	54.18±8.11	60	53.42±9.93	40	Serum	High	Netrin-1 levels were significantly elevated in patients with type 2 diabetes mellitus (with and without diabetic retinopathy) compared to healthy controls.
Salem et al. [32]	2022	Egypt	C-S	NR	49.96±8.37	75	48.32 ± 8.21	25	Serum	Moderate	Netrin-1 levels were significantly higher in diabetic patients with macroalbuminuria and microalbuminuria compared to normoalbuminuric patients and the control group.
Sfayyih et al. [33]	2024	Iraq	C-S	NR	R= 34-56	60	R= 34-56	30	Serum	Moderate	Netrin-1 levels were significantly elevated in patients with type 2 diabetes compared to the healthy control group.
Shalaby et al. [34]	2021	Egypt	C-S	ADA	44.7±6.2	30	44.3 ±7.3	30	Serum	High	Serum netrin-1 concentrations were elevated in individuals with newly diagnosed type 2 diabetes.
Usha et al. [35]	2023	India	C-S	ADA	49.38±2.29	44	47.78±2.69	35	Serum	High	There was no significant difference in serum netrin-1 levels between patients with type 2 diabetes mellitus and healthy controls.
Yim et al. [36]	2018	Korea	C-S	ADA	52.6±13.4	92	40.9± 14.5	41	Serum	High	Serum netrin-1 levels were significantly elevated in subjects with type 2 diabetes compared to healthy controls.

C-C: Case-Control; C-S: Cross-Sectional; ADA: American Diabetes Association; WHO: World Health Organization; NR: Not Reported; N: Number of T2DM/Control Subjects; SD: Standard Deviation.

Meta-analysis

Using a random-effects model, the pooled SMD in netrin-1 levels between T2DM and control groups was 0.57 (95% CI: 0.03 to 1.11, p = 0.0393), indicating a statistically significant elevation of netrin-1 in patients with T2DM. Substantial heterogeneity was observed among the included studies (I^2 = 96.4%, τ^2 = 1.4725, p < 0.0001), suggesting considerable variability in study results. The 95% prediction interval ranged from –2.03 to 3.18, indicating that the effect size in future studies may vary and, in some cases, may not demonstrate a significant difference or may even favor controls. Figure 2 displays a forest plot comparing netrin-1 values between patients with T2DM and those without.

Figure 2: Forest plot showing comparison of circulating netrin-1 levels between T2DM and controls.

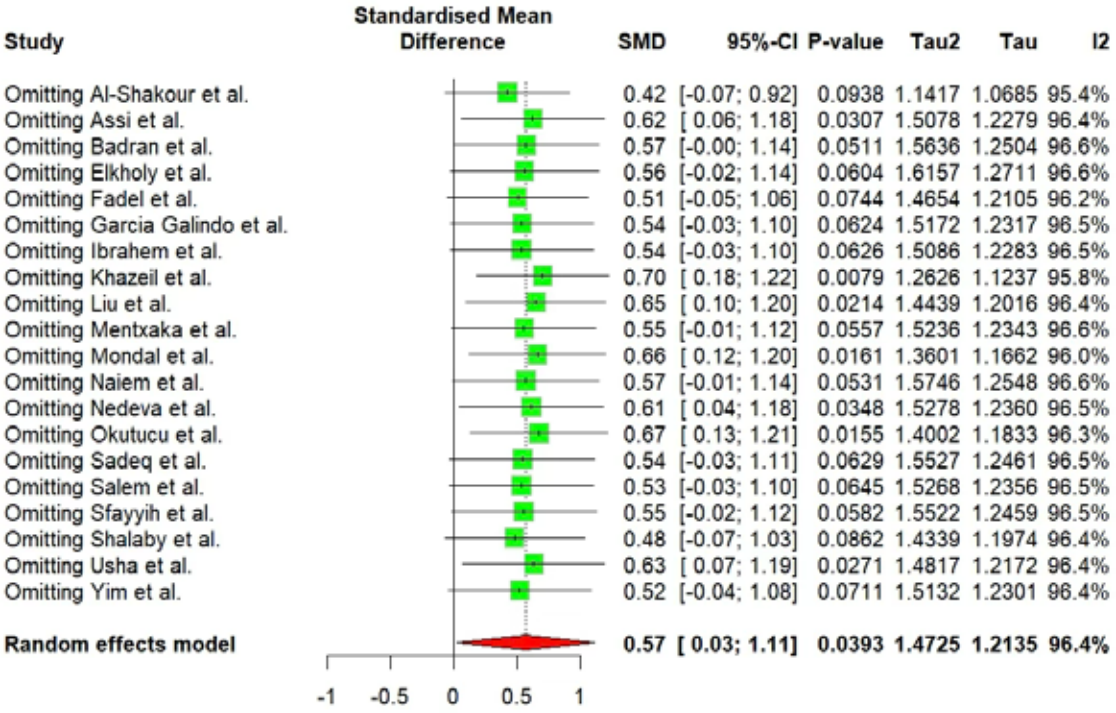


A drapery plot was also constructed to visually represent the meta-analysis findings by displaying each study's p-value in relation to its corresponding effect size (Supplementary Figure 1).

Sensitivity analysis

Across all iterations, the pooled SMD remained positive and relatively stable, ranging from 0.42 (95% CI: –0.07 to 0.92) upon exclusion of Al-Shakour et al. [11] to 0.70 (95% CI: 0.18 to 1.22) when Khazeil et al. [14] was excluded. Although none of the exclusions meaningfully changed the direction of the overall effect, the statistical significance of the association approached non-significance when studies such as Al-Shakour et al. [11], Badran et al. [21], Elkholy et al. [22], Fadel et al. [12], Garcia Galindo et al. [23], Ibrahem et al. [24], Mentxaka et al. [26], Naiem et al. [28], Sadeq et al. [31], Salem et al. [32], Sfayyih et al. [33], Shalaby et al. [34], and Yim et al. [36] were excluded individually. Heterogeneity remained consistently high across all iterations (I^2 = 95.4% to 96.6%, Tau^2 = 1.3601 to 1.6157), indicating persistent between-study variability regardless of individual study omission (Figure 3).

Figure 3: Results of leave-one-out method in sensitivity analysis.



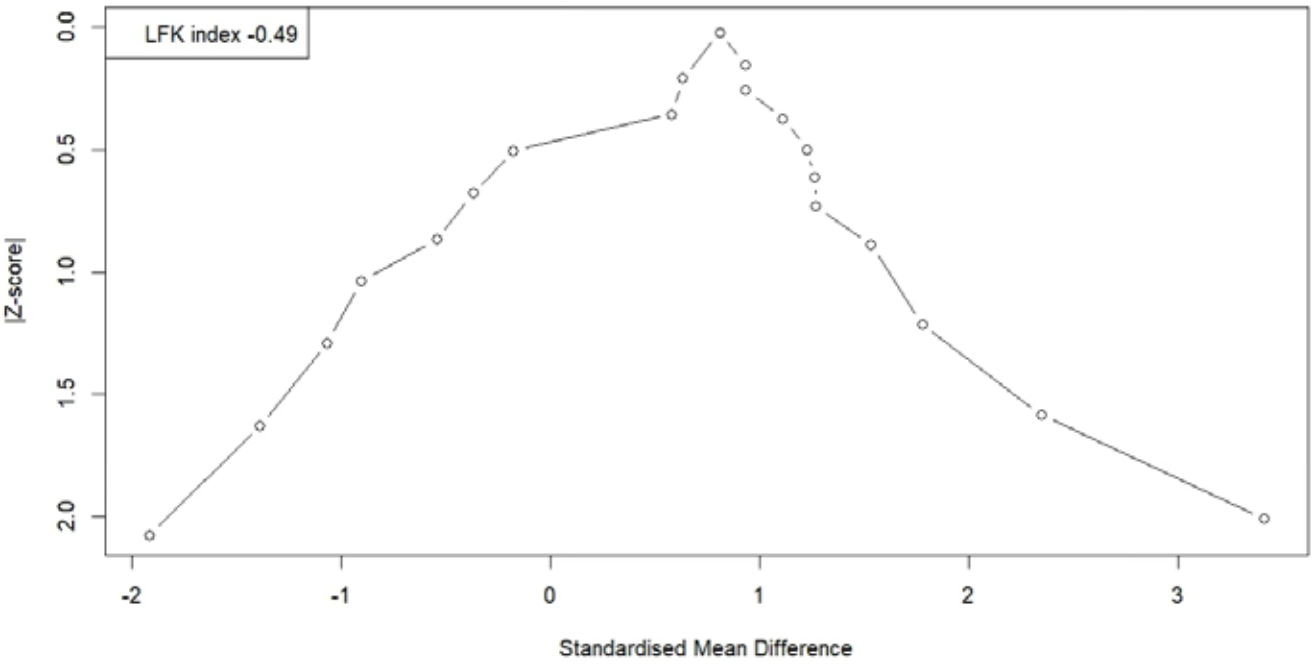
Subgroup analysis

When stratified by country, studies from Egypt showed a significantly elevated pooled SMD in netrin-1 levels among individuals with T2DM compared to controls (SMD: 1.21; 95% CI: 0.77 to 1.66), whereas studies from India demonstrated a significantly decreased pooled effect (SMD: –0.82; 95% CI: –1.33 to –0.31). Studies from Iraq and other countries yielded non-significant pooled effects. The difference among country subgroups was statistically significant ($\chi^2 = 34.60$, $p < 0.0001$), suggesting that geographic location may be a key moderator of effect size (Supplementary Figure 2). Subgroup analysis by diagnostic criteria revealed that studies adhering to the ADA criteria reported a significant increase in netrin-1 levels (SMD: 1.33; 95% CI: 0.68 to 1.97), while studies with unreported criteria showed no significant difference (SMD: –0.10; 95% CI: –0.82 to 0.61), and studies using WHO criteria reported significantly lower netrin-1 levels in T2DM (SMD: –0.90; 95% CI: –1.46 to –0.35). The difference among diagnostic subgroups was also statistically significant ($\chi^2 = 26.72$, $p < 0.0001$) (Supplementary Figure 3). When categorized by sample size, studies with ≥100 participants showed a significant association (SMD: 1.18; 95% CI: 0.35 to 2.00), whereas studies with <100 participants reported a non-significant effect (SMD: 0.16; 95% CI: –0.48 to 0.79). However, the difference between these subgroups was marginally non-significant ($\chi^2 = 3.69$, $p = 0.0546$) (Supplementary Figure 4). Subgroup analysis by biological sample type revealed a significant association in serum-based studies (SMD: 0.63; 95% CI: 0.06 to 1.21), whereas plasma-based studies did not show a significant association (SMD: 0.01; 95% CI: –1.79 to 1.81); nonetheless, this difference was not statistically significant ($\chi^2 = 0.42$, $p = 0.5188$) (Supplementary Figure 5). Finally, analysis by study design showed non-significant associations in both case-control studies (SMD: 0.97; 95% CI: –0.10 to 2.03) and cross-sectional studies (SMD: 0.36; 95% CI: –0.24 to 0.95), with no significant subgroup difference observed ($\chi^2 = 0.96$, $p = 0.3265$) (Supplementary Figure 6). These findings suggest that geographic origin and diagnostic criteria may be major contributors to the observed heterogeneity, while sample size, sample type, and study design appear to have a more modest impact.

Publication bias

Publication bias was assessed utilizing the Doi plot in conjunction with the LFK index. The Doi plot illustrates a symmetrical distribution of studies around the central axis, with a calculated LFK index of –0.49. This value falls within the acceptable range of ±1, suggesting the absence of significant asymmetry in the Doi plot (Figure 4). Consequently, there is no evidence of publication bias influencing the pooled results of netrin-1 levels in individuals diagnosed with T2DM.

Figure 4: Doi plot showing no evidence of publication bias.



Discussion

This systematic review and meta-analysis synthesize contemporary evidence concerning circulating netrin-1 levels in individuals diagnosed with T2DM. The pooled analysis demonstrated a statistically significant elevation of netrin-1 levels in T2DM patients when compared to healthy controls, indicating a potential role for netrin-1 as a biomarker for diabetes-related inflammation and metabolic dysfunction. Nonetheless, considerable heterogeneity across studies and inconsistent findings in subgroup analyses underscore the complexity of netrin-1's biological role and the impact of various study characteristics.

Our findings diverge from the meta-analysis conducted by Behnoush et al. [15], which found no overall difference in netrin-1 levels between patients with diabetes and healthy controls. However, they did report significantly elevated netrin-1 levels in diabetic individuals with microalbuminuria and macroalbuminuria compared to those with normoalbuminuric. Furthermore, they observed reduced netrin-1 levels in individuals with prediabetes compared to healthy controls. In contrast, our results align with several individual studies that document increased serum netrin-1 concentrations in patients with T2DM and its associated complications. For instance, Elkholy et al. [22] demonstrated significantly elevated netrin-1 levels in patients suffering from diabetic nephropathy, with both serum and urinary measurements suggesting its potential utility for early detection. Mentxaka et al. [26] similarly found increased circulating netrin-1 levels in patients with obesity and T2DM, which exhibited a positive correlation with insulin and HOMA-IR, and a negative correlation with QUICKI. Fadel et al. [12] reported higher netrin-1 levels in T2DM patients experiencing vascular complications, thereby underscoring its diagnostic utility in conjunction with inflammatory markers such as VCAM-1. Additionally, Garcia Galindo et al. [23] established a positive correlation between netrin-1 levels and hs-CRP, further emphasizing its role in T2DM-related low-grade inflammation. Despite these findings, discrepancies persist in the literature. Usha et al. [35] reported no significant difference in netrin-1 levels between T2DM patients and controls; however, they identified a significant negative correlation between netrin-1 and QUICKI. Yim et al. [36] observed elevated serum netrin-1 levels in individuals with impaired fasting glucose or T2DM, interpreting this as a potential compensatory response. Their analysis also revealed significant positive correlations with fasting glucose, HbA1c, and HOMA-IR. Conversely, studies conducted by Liu et al. [25] indicated lower plasma netrin-1 levels in newly diagnosed T2DM patients, suggesting that netrin-1 expression may be diminished in the early stages of the disease or as a result of impaired compensatory mechanisms. In their investigation, netrin-1 was negatively associated with fasting and postprandial glucose, HbA1c, triglycerides, and HOMA-IR. Similarly, Nedeva et al. [29] reported significantly lower serum netrin-1 levels in individuals with obesity, prediabetes, and T2DM compared to healthy controls. These inconsistencies may arise from variations in population characteristics, duration of diabetes, sample sizes, disease phenotypes, and methodological differences, particularly regarding the use of various ELISA kits for netrin-1 measurement. Furthermore, the diagnostic criteria employed appeared to influence the results; studies utilizing ADA guidelines generally reported elevated netrin-1 levels, whereas

those employing WHO criteria or unspecified diagnostic standards exhibited inconsistent or inverse trends.

Biologically, netrin-1 exhibits dual roles, functioning as an anti-inflammatory mediator by inhibiting leukocyte adhesion and migration, while also being implicated in insulin resistance and metabolic dysfunction [15]. Garcia Galindo et al. [23] proposed a mechanistic model in which a loss of receptor affinity (e.g., Unc5b) leads to unchecked secretion of netrin-1, thereby amplifying inflammatory cascades via high-sensitivity C-reactive protein (hs-CRP) and nuclear factor kappa B (NF-κB), which contribute to insulin resistance and the progression of T2DM. Furthermore, preclinical studies highlight tissue-specific effects; for instance, Ramkhelawon et al. [37] demonstrated that in obese mice, the expression of netrin-1 and UNC5B was elevated in visceral adipose tissue, promoting macrophage retention and inflammation. Deletion of hematopoietic netrin-1 improved insulin sensitivity by reducing adipose inflammation. Wu et al. [38] further suggested that the impact of netrin-1 varies depending on receptor subtype and concentration, adding an additional layer of complexity. Notably, netrin-1 levels also appear to vary with body composition. Nedeva et al. [8] observed that netrin-1 is elevated in lean individuals with diabetes but decreased in their obese counterparts, which may partly account for the inconsistent findings across clinical studies. Despite these mechanistic insights, a critical gap remains in the literature: no longitudinal studies have yet evaluated changes in netrin-1 levels throughout the course of diabetes development or insulin resistance. Consequently, the precise role of netrin-1 in the pathophysiology of T2DM remains incompletely elucidated.

The findings from the subgroup analyses further highlight the variability in circulating netrin-1 levels across diverse studies. Notably, research conducted in Egypt consistently reported elevated levels of netrin-1 among individuals with T2DM, whereas studies from India-including those by Mondal et al. [27] -tended to indicate reduced levels. This geographical variation may reflect underlying differences in population genetics, environmental exposures, or disease phenotypes. Furthermore, the choice of diagnostic criteria appeared to influence the observed associations: studies utilizing the ADA guidelines more frequently demonstrated elevated netrin-1 levels, whereas those employing WHO criteria or lacking specific diagnostic standards often exhibited null or inverse associations.

Despite the overall positive association observed in our findings, the results were characterized by considerable heterogeneity. The sensitivity analysis indicated that no single study exerted a disproportionate influence on the overall results; however, the statistical significance of the findings diminished upon the exclusion of studies such as Al-Shakour et al. [11], Badran et al. [21], Elkholy et al. [22], Fadel et al. [12], Garcia Galindo et al. [23], Ibrahem et al. [24], Mentxaka et al. [26], Naiem et al. [28], Sadeq et al. [31], Salem et al. [32], Sfayyih et al. [33], Shalaby et al. [34], and Yim et al. [36]. The extensive prediction interval (–2.03 to 3.18) and the accompanying drapery plot visualization further elucidate the variability and dispersion of effect estimates, thereby reinforcing the existence of genuine differences between studies.

Interestingly, our analysis did not reveal any evidence of publication bias, as supported by the symmetrical Doi plot and an LFK index of –0.49, which falls within the threshold for no asymmetry. This enhances confidence in the overall findings despite heterogeneity.

This meta-analysis is the most comprehensive to date focusing exclusively on circulating netrin-1 in T2DM, incorporating diverse geographic populations, sample types, and study designs. The use of rigorous subgroup and sensitivity analyses enhances the reliability and transparency of findings. Additionally, visualization tools such as forest plots, drapery plots, and Doi plots provide multiple perspectives on data distribution and bias. However, several limitations must be acknowledged. First, the majority of the studies included in this review were cross-sectional in nature, which restricts the ability to draw causal inferences. Second, substantial heterogeneity persists despite subgroup analyses. Third, discrepancies among the studies were noted, which may be attributable to a variety of factors, including differences in sample size, patient characteristics, and measurement techniques. Additionally, there may be variations in the duration of diabetes and its progression among the patient groups. Lastly, the results may have been influenced by the conversion of non-normally distributed data to a normal distribution, potentially leading to variations in the findings.

Conclusion

In conclusion, while elevated netrin-1 levels are associated with T2DM in pooled analyses, variability across studies warrants cautious interpretation. Future research should aim for standardized methodologies, larger multicentre cohorts, and longitudinal designs to clarify netrin-1's role in the pathophysiology and progression of type 2 diabetes.

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Declaration of Conflict of interests

The authors declare that they have no conflict of interest regarding the publication of this article.

Ethical approval

This study is a systematic review and meta-analysis. No ethical approval is required.

Credit Author Statements

Roshan Kumar Mahat: Conceptualization, Methodology, Software, Formal analysis, Writing - Original Draft, Writing - Review & Editing, Supervision, Project administration.
Vedika Rathore: Methodology, Investigation, Data Curation, Validation, Writing - Review & Editing.
Mritunjay Kumar Mishra: Methodology, Investigation, Data Curation, Writing - Review & Editing.
All authors have read and approved the final manuscript.

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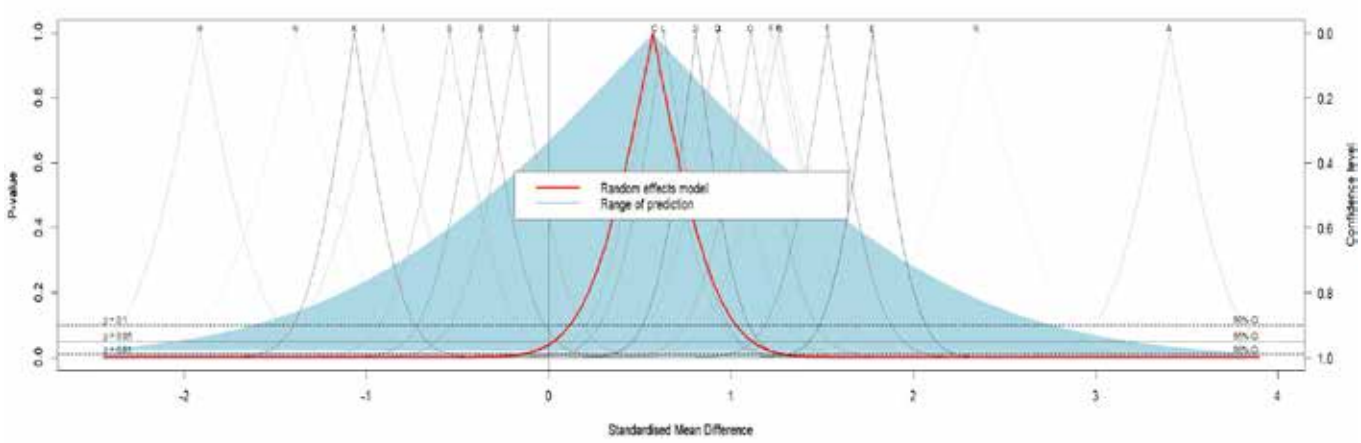
Supplementary files

Supplementary Table 1: Search strategies in databases.

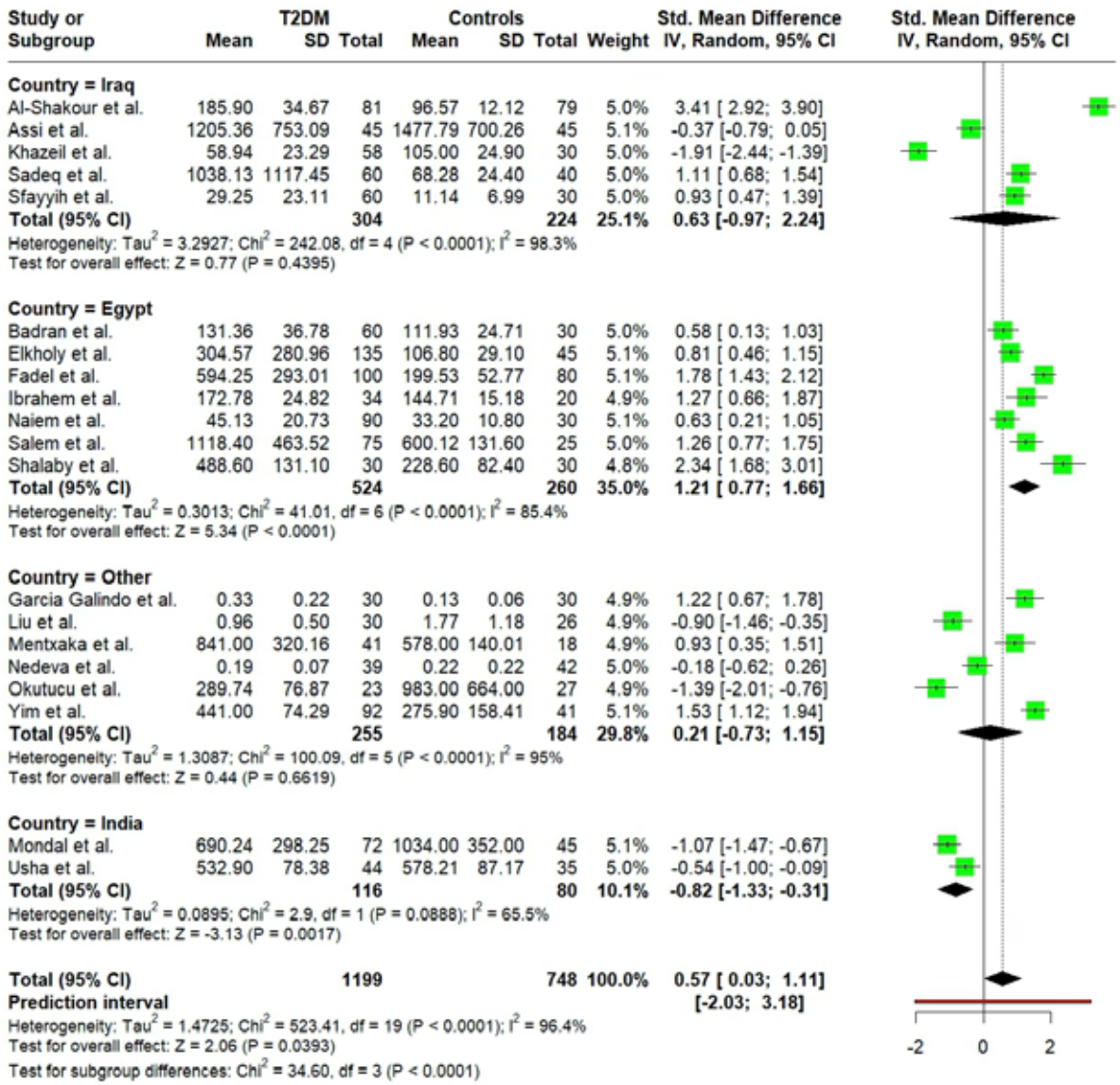
Databases	Search terms	No of articles identified
PubMed/Medline	("Type 2 Diabetes Mellitus"[All Fields] OR "Diabetes Mellitus"[All Fields] OR "Type 2 Diabetes"[All Fields] OR "Diabetes"[All Fields] OR "Diabetics"[All Fields] OR "Type 2 DM"[All Fields] OR "T2DM"[All Fields] OR "T2D"[All Fields]) AND ("Netrin-1"[All Fields] OR "NTN1"[All Fields] OR "NT-1"[All Fields])	71
Scopus	TITLE-ABS-KEY (("Type 2 Diabetes Mellitus" OR "Diabetes Mellitus" OR "Type 2 Diabetes" OR "Diabetes" OR "Diabetics" OR "Type 2 DM" OR "T2DM" OR t2d) AND ("Netrin-1" OR "NTN1" OR "NT-1"))	132
Europe PMC	(“Type 2 Diabetes Mellitus” OR “Diabetes Mellitus” OR “Type 2 Diabetes” OR “Diabetes” OR “Diabetics” OR “Type 2 DM” OR “T2DM” OR T2D) AND (“Netrin-1” OR “NTN1” OR “NT-1”)	1595

Supplementary Figure 1: Drapery plot of meta-analysis.

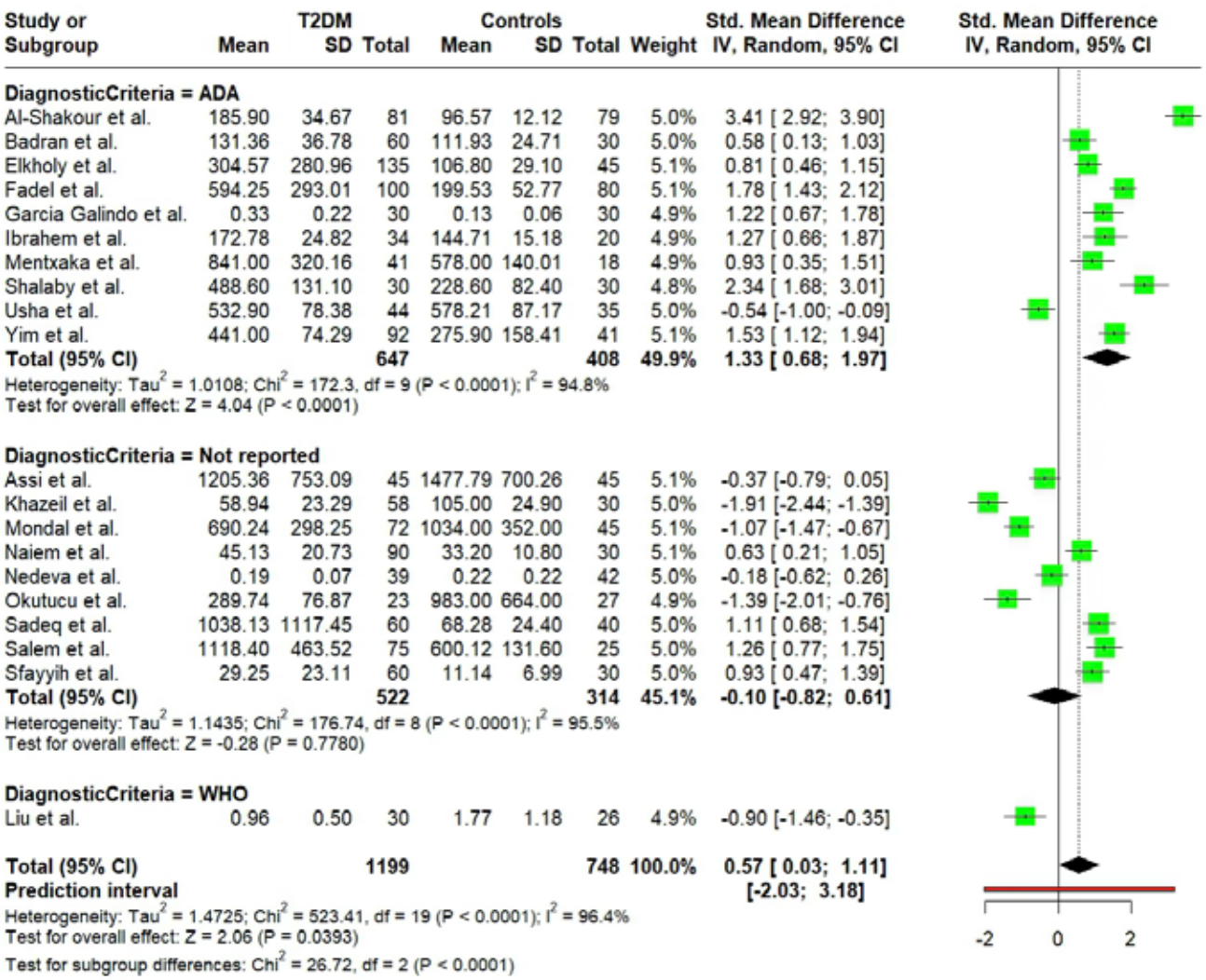


A: Al-Shakour et al. [11]; B: Assi et al. [13]; C: Badran et al. [21]; D: Elkholy et al. [22]; E: Fadel et al. [12]; F: Garcia Galindo et al. [23]; G: Ibrahem et al. [24]; H: Khazeil et al. [14]; I: Liu et al. [25]; J: Mentxaka et al. [25]; K: Mondal et al. [27]; L: Naiem et al. [28]; M: Nedeva et al. [29]; N: Okutucu et al. [30]; O: Sadeq et al. [31]; P: Salem et al. [32]; Q: Sfayyih et al. [33]; R: Shalaby et al. [34]; S: Usha et al. [35]; Yim et al. [36].

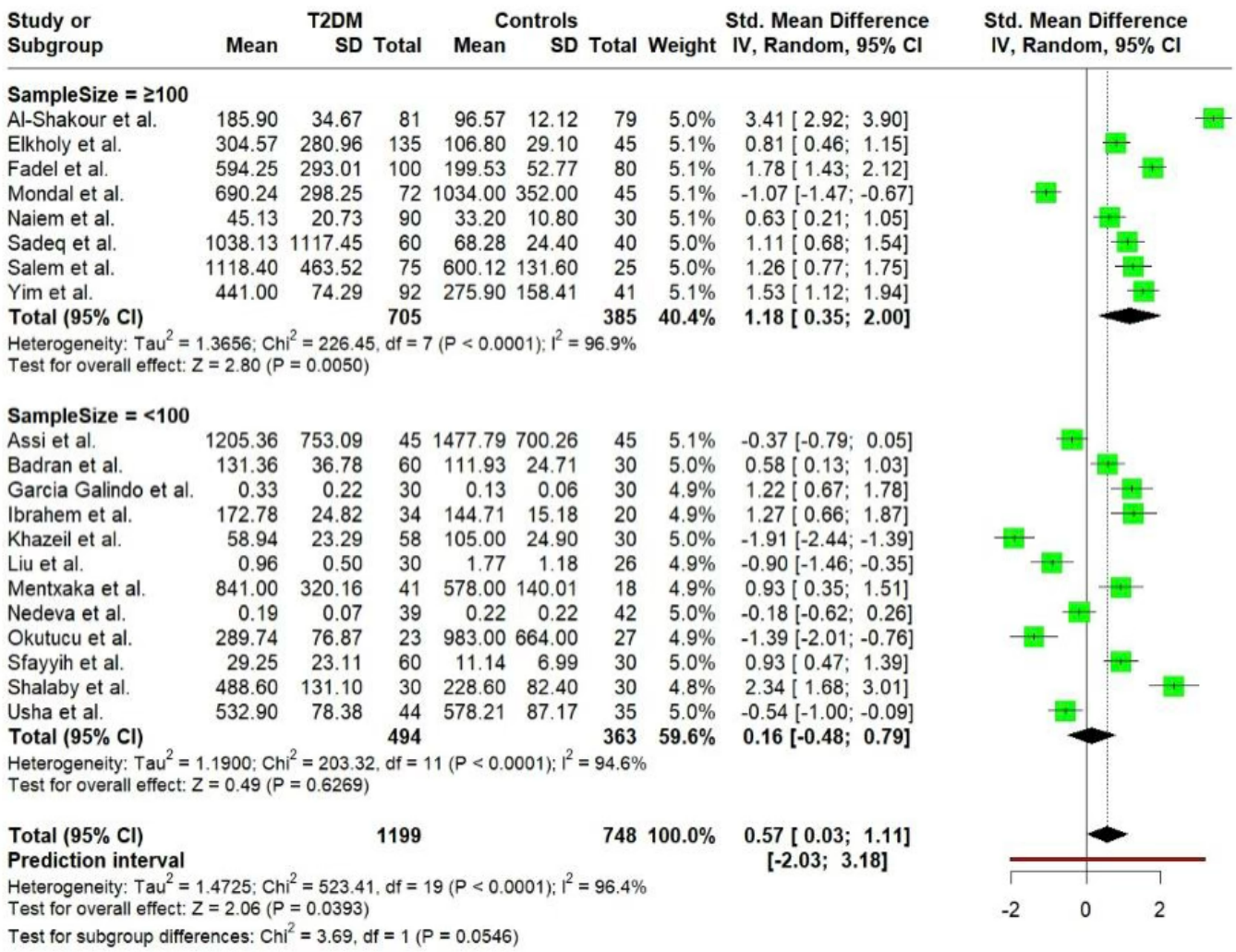
Supplementary Figure 2: Subgroup analysis by geographical region (country).



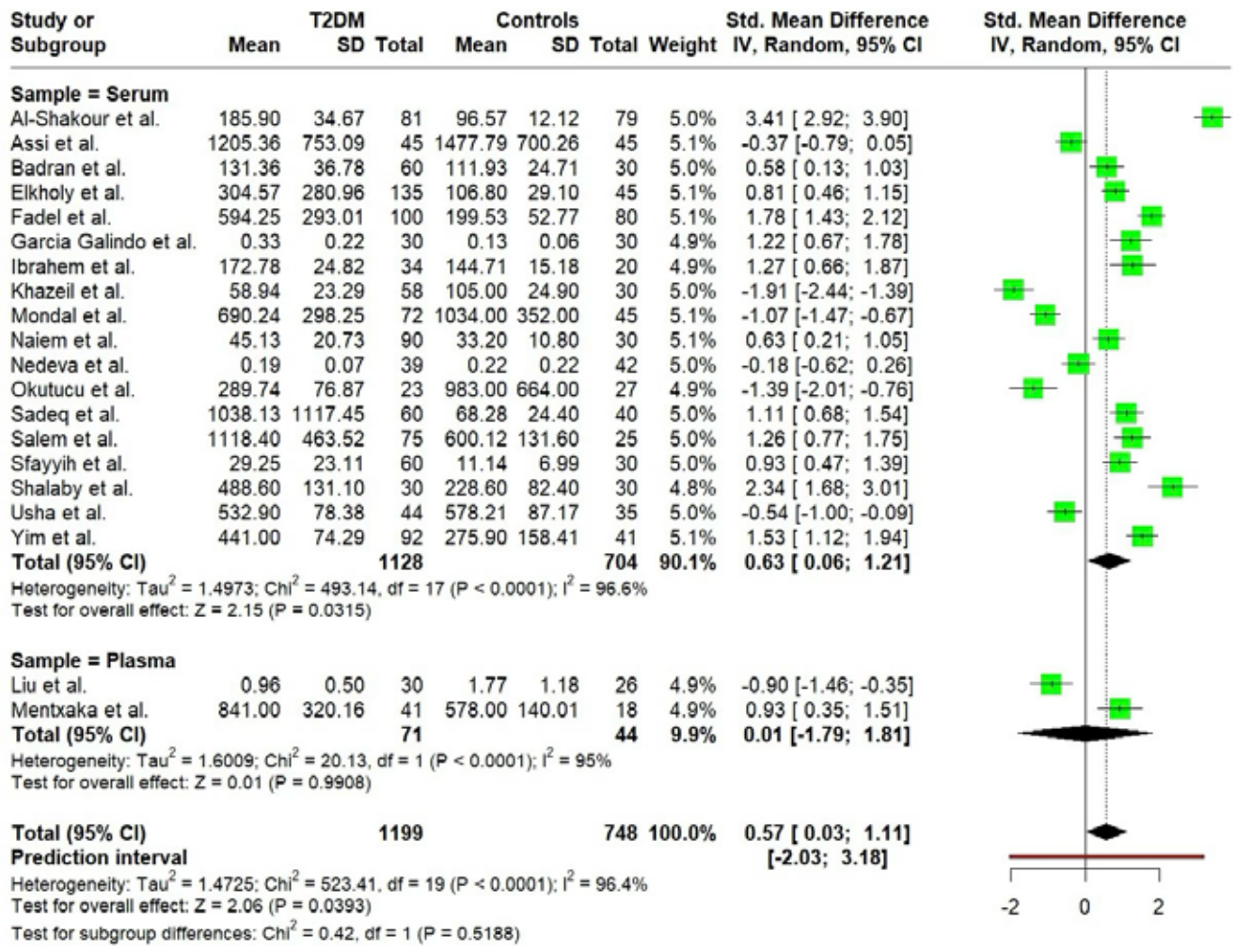
Supplementary Figure 3: Subgroup analysis by diagnostic criteria.



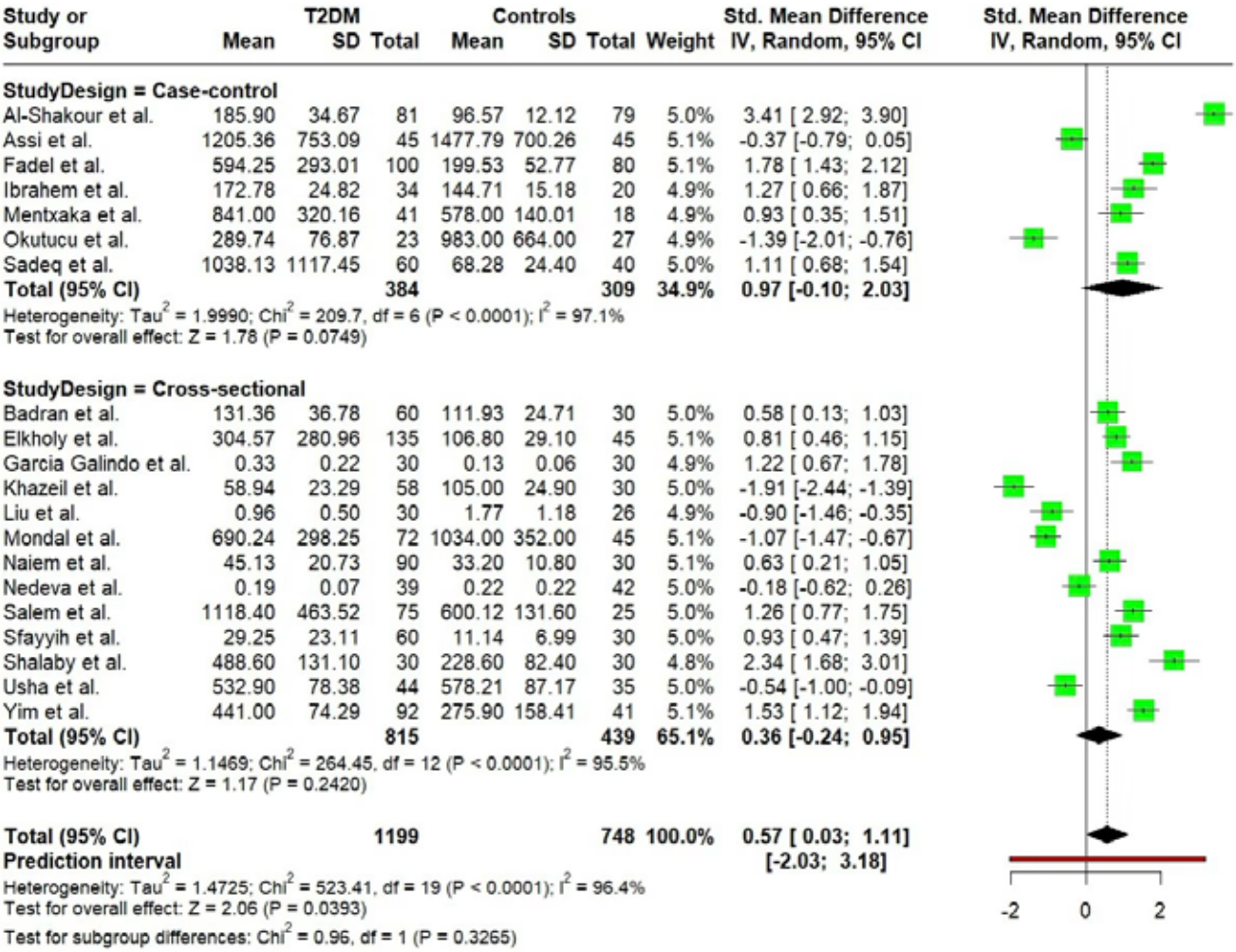
Supplementary Figure 4: Subgroup analysis by sample size.



Supplementary Figure 5: Subgroup analysis by biological sample type.



Supplementary Figure 6: Subgroup analysis by study design.



Rapid Communication

Beneficial Intelligence in Laboratory Medicine: Aligning Human and Artificial Intelligence for Value-Based Outcomes

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Keywords

Beneficial Intelligence, Artificial Intelligence, Value-Based Laboratory Medicine, Outcomes, Ethics

Abstract

The integration of artificial intelligence (AI) into healthcare and laboratory medicine is reshaping diagnostics, workflows, and patient management. Yet, technological progress alone cannot ensure meaningful outcomes. The concept of Beneficial Intelligence (BI), defined as the synergy of human and artificial intelligence (H + A = B), emphasizes that technology must be guided by human purpose, ethics, and empathy. BI reframes AI not as a replacement for human expertise but as an augmentation that enables laboratory professionals to deliver care that is accurate, sustainable, and patient-centered. In alignment with value-based healthcare, BI prioritizes outcomes that matter most-clinical, operational, economic, and societal. Laboratory medicine provides a fertile ground for this framework, where digitalization, automation, and machine learning models already enhance diagnostics, risk stratification, and decision support. However, responsible adoption requires validation against patient outcomes, adherence to structured evaluation frameworks and continuous human oversight. Ultimately, Beneficial Intelligence is not only a technical model but a mindset: a commitment to ensure that the alliance of human wisdom and AI fosters equitable, efficient, and sustainable healthcare for the future.

Introduction

The rapid integration of artificial intelligence (AI) into healthcare and laboratory medicine offers unprecedented opportunities to transform patient care, streamline workflows, and optimize outcomes [1,2]. Yet, there is a growing awareness that AI alone cannot guarantee meaningful progress. Human judgment, empathy, and ethical responsibility remain indispensable. The concept of Beneficial Intelligence (BI) embodies the synergy of human intelligence (H) and artificial intelligence (A), expressed as H + A = B. BI should not be understood merely as a technical framework, but as a way of thinking. It is a concept that redefines how human and artificial intelligence are integrated, emphasizing that technology must be guided by human purpose, ethics, and empathy. BI emphasizes that optimal healthcare outcomes are achieved when human and artificial intelligence work together to serve the well-being of patients and society. In laboratory medicine, BI aligns closely with the principles of value-based healthcare, prioritizing outcomes that matter to patients while ensuring sustainability and efficiency.

The Case for Beneficial Intelligence

Laboratory medicine is experiencing a paradigm shift shaped by digitalization, automation, and AI. Machine learning models, particularly in diagnostics, have demonstrated improved accuracy in areas such as the detection of myocardial infarction through algorithms like CoDE-ACS [2]. These tools enhance risk stratification, accelerate decision-making, and reduce unnecessary admissions. Yet, their full potential is realized only when combined with human oversight and contextual judgment. As highlighted in the European AI Act, human oversight is a prerequisite for high-risk AI deployment in healthcare [2]. BI thus reframes the discussion: rather than replacing human expertise, AI should augment it, enabling clinicians and laboratory specialists to deliver care that is both technologically advanced and ethically grounded.

Value-Based Laboratory Medicine as the North Star

The goal of laboratory medicine is not the performance of individual tests, but their impact on patient outcomes [3]. Outcome-based studies demonstrate how laboratory information supports effective management, improves treatment pathways, and reduces adverse events. However, important gaps remain in linking test results directly to clinical outcomes, underscoring the need for multidisciplinary approaches and robust study designs [3]. Value-based laboratory medicine seeks to optimize the clinical utility of tests while ensuring sustainability and patient-centeredness [4].

In this context, BI provides a framework to integrate AI-driven analytics with human expertise in order to define, measure, and achieve outcomes that truly matter-clinical, operational, and societal. Yet, for AI to be trusted and deployed responsibly, its performance must be validated against outcomes and assessed with existing frameworks such as the EFLM checklist [1]. This checklist emphasizes laboratory-specific challenges, including analytical variability, harmonization, metadata, and reproducibility. BI aligns with this structured approach, embedding ethical, technical, and methodological rigor into AI adoption. Crucially, the human role remains central: only professionals can set meaningful outcome measures, interpret results within context, and ensure that technology ultimately serves patients. This partnership between human judgment and artificial intelligence is essential to foster trust, ensure compliance, and advance equitable implementation

Trends Shaping Beneficial Intelligence

Emerging technologies such as hyperautomation, point-of-care testing, and wearable devices are expanding access to diagnostics and prevention [5]. Coupled with exposome research and omics integration, laboratory medicine is moving toward personalized and proactive healthcare. AI facilitates the identification of patterns and predictive biomarkers, while human expertise ensures that interventions are patient-centered and ethically sound. This duality exemplifies the promise of BI: technology with a human purpose, addressing both efficiency and equity.

Principles of Beneficial Intelligence

BI is rooted in a few core principles:

- **Technology with Purpose**: AI informs, but conscience decides.
- **Humans augmented, not replaced**: machines provide the map, humans choose the destination.
- **Well-being as the true metric**: success is measured in human benefit, not just technical performance.
- **Continuous co-learning**: each interaction between human and artificial intelligence strengthens the other.

The integration of BI must be evaluated through the lens of value-based outcomes.

These include:

- **Clinical outcomes**: improved diagnosis, timely interventions, reduced morbidity and mortality.
- **Operational outcomes**: streamlined workflows, optimized test utilization, and reduced errors.
- **Economic outcomes**: cost-effectiveness, reduced hospital admissions, and better allocation of resources.
- **Societal outcomes**: equitable access, patient empowerment, and sustainable healthcare systems. By prioritizing these outcomes, Beneficial Intelligence ensures that technology serves the common good.

Conclusion

Beyond algorithms and workflows, Beneficial Intelligence is first and foremost a mindset. It is a concept that reminds us that technology alone cannot deliver value; it is the alliance of human judgment and artificial intelligence that generates outcomes aligned with the well-being of patients and society. Beneficial Intelligence reframes the role of AI in healthcare and laboratory medicine. The equation H + A = B illustrates

the alliance between human wisdom and artificial intelligence, a partnership that fosters outcomes aligned with value-based healthcare. Laboratory professionals are uniquely positioned to lead this transformation, ensuring that technological advances are harnessed responsibly, ethically, and sustainably. By embracing Beneficial Intelligence, laboratory medicine can become a cornerstone of patient-centered, efficient, and equitable healthcare in the decades to come. Its success, however, will depend on continuous evaluation & audit, transparent governance & collaborative governance, innovation & ethical compliance, finally the commitment of professionals to balance progress with accountability and patient trust.

Declaration of Conflict of Interest

The authors declare that they have no conflicts of interest related to the content of this manuscript.

Ethical Approval and Compliance with Ethical Standards

This article does not involve any new studies with human participants or animals performed by any of the authors.
All considerations discussed are based on previously published literature and conceptual analysis.
Therefore, ethical approval and informed consent were not required.
The manuscript is fully compliant with the ethical principles of the Declaration of Helsinki.

Authors' Disclosures

The authors declare that they have no financial or personal relationships that could inappropriately influence or bias the content of this work.

CRediT Author Contribution Statement

Damien Gruson: Conceptualization; Writing – original draft; Writing – review & editing; Visualization; Supervision.
Pradeep Dabla: Writing – review & editing; Critical revision of the manuscript for important intellectual content.
All authors have read and approved the final version of the manuscript and agree to be accountable for all aspects of the work.

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Data Availability Statement

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Case Report

Plasma Cell Neoplasm Mimicking Metastatic Bone Disease in a Breast Cancer Survivor: A Case Report Highlighting the Role of Serum Protein Electrophoresis

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Keywords

Albumin-to-globulin ratio, breast cancer, bone lesions, plasma cell neoplasm, serum protein electrophoresis

Abstract

Breast cancer, a prevalent solid tumor, is the most common cancer among women worldwide. Hematological malignancies, such as acute myeloid leukemia, myelodysplastic syndrome, and acute lymphoblastic leukemia, are more frequent in breast cancer survivors, while plasma cell neoplasms are less common. We report the case of a 44-year-old woman with breast cancer who underwent neoadjuvant chemotherapy and surgery, presenting one year later with bone lesions and anemia. Initially suspected to be metastatic bone disease, the findings of elevated serum total protein and globulins prompted serum protein electrophoresis, which revealed a distinct M band in the gamma region, suggesting a plasma cell neoplasm. Further evaluation confirmed this diagnosis, and treatment was initiated. This case underscores the importance of considering elevated total protein, globulins, and an altered albumin-to-globulin ratio as primary bone marrow disorders, such as plasma cell neoplasms, in breast cancer patients with bone lesions.

Introduction

Plasma cell neoplasm is a systemic malignancy characterized by the uncontrolled proliferation of monoclonal plasma cells in the bone marrow, resulting in the production of nonfunctional immunoglobulins or immunoglobulin light chains, known as M-protein. Diagnosing this condition is challenging due to nonspecific symptoms, such as bone pain, fatigue, and aches at multiple sites [1]. Typically diagnosed in individuals aged 65–75 years with a male predominance [2], plasma cell neoplasms may be overlooked in younger female patients, where symptoms might be mistaken for aging or other conditions. Indications for testing include incidental findings of elevated total protein, unexplained anemia or cytopenias, hypercalcemia, renal impairment, lytic bone lesions, or unexplained osteopenia in patients with bone pain or fractures [3].

Breast cancer, the most common cancer among women globally, has seen reduced mortality rates due to advances in screening and treatment. However, its association with secondary malignancies, such as plasma cell neoplasms, is rare [4]. We present a case of a 44-year-old woman with invasive ductal carcinoma of the right breast, treated with chemotherapy, modified radical mastectomy, and radiotherapy. One-year post-treatment, she developed back pain, anemia, acute kidney injury, and bone lesions, initially suggestive of metastatic tumor deposits. Further evaluation revealed a plasma cell neoplasm, highlighting the diagnostic challenges of distinguishing between metastatic disease and primary bone disorders.

Case Report

A 44-year-old woman presented with a right breast nodule and was evaluated at our hospital. Examination revealed a retroareolar nodule with a peau d'orange appearance and enlarged right axillary lymph nodes. Biopsy confirmed grade 3 invasive ductal carcinoma, with immunohistochemistry indicating ER-negative, PR-low positive, and HER2/neu-positive status. A bone scan showed no lytic or metastatic lesions. The final diagnosis was carcinoma of the right breast, staged as cT4bN2M0. The patient received neoadjuvant chemotherapy with four cycles of Adriamycin and Cyclophosphamide, followed by four cycles of Paclitaxel. She then underwent right modified radical mastectomy and adjuvant radiotherapy over five weeks, followed by hormonal therapy with Tamoxifen.

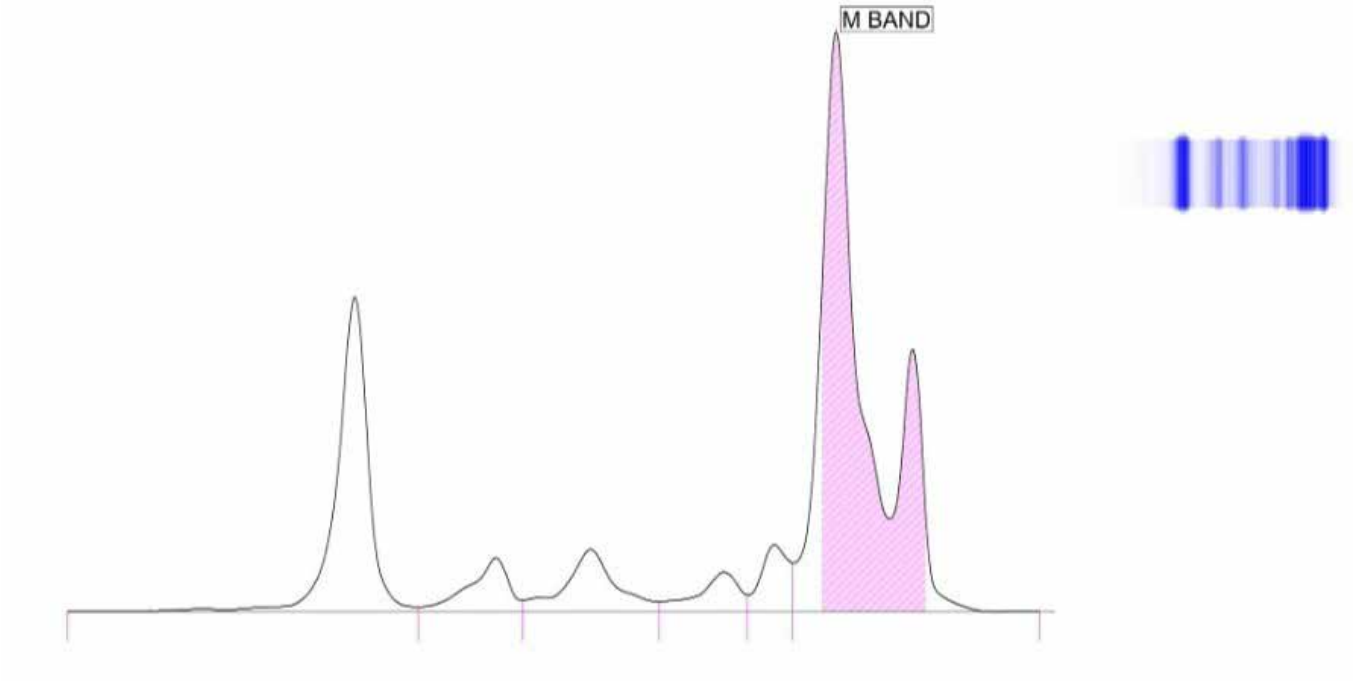
One year later, the patient presented with lower back pain, vomiting, and constipation, managed with supportive care. Routine investigations revealed severe normocytic normochromic anemia, treated with blood transfusion and hematinics. One month later, she was readmitted for persistent anemia. Abdominal ultrasound showed bilateral bulky kidneys with increased cortical echogenicity, prompting kidney function, liver function, and electrolyte tests. These revealed acute kidney injury and hypokalemia. High-resolution CT of the chest and MRI of the lumbar spine identified multiple lytic lesions in the axial and appendicular skeleton, initially suggestive of metastatic bone disease.

Table 1: Investigations Performed During the First Visit and One Year Later.

Investigations	Initial Findings	One Year Later	Biological Reference Interval
Serum Creatinine (µmol/L)	41.5	176.8	45.9–91.9
Total Bilirubin (µmol/L)	5.1	11.9	3.42–22.2
Total Protein (g/L)	79	119	63–82
Serum Albumin (g/L)	40	35	35–50
Serum Globulin (g/L)	39	84	19–37
Albumin/Globulin Ratio	1	0.4	1.0–2.5
Aspartate Transaminase (AST) (U/L)	30	23	14–36
Alanine Transaminase (ALT) (U/L)	17	11	0–35
Alkaline Phosphatase (ALP) (U/L)	101	105	38–126
Serum Sodium (mmol/L)	141	145	137–145
Serum Potassium (mmol/L)	4.1	3	3.5–5.1
Serum Chloride (mmol/L)	108	104	98–107
Hemoglobin (g/L)	117	67	120–160

Given the elevated total protein, globulins, and altered albumin-to-globulin ratio, serum protein electrophoresis (SPE) was performed using the Sebia Minicap system. The electrophoretogram (Figure 1) revealed a distinct M band in the gamma region and reduced albumin levels.

Figure 1: The electrophoretogram revealed a distinct M band in the gamma region and reduced albumin levels.



This finding prompted further evaluation for plasma cell neoplasm, including serum free light chain estimation, bone marrow aspiration and biopsy, and serum calcium measurement. Results are shown in Table 2.

Table 2: Investigations for Plasma Cell Neoplasm Evaluation.

Investigations	Findings	Biological Reference Interval
Free Kappa (mg/L)	113	3.3–19.4
Free Lambda (mg/L)	19.4	5.71–26.3
Kappa:Lambda Ratio	5.8	0.26–1.65
Bone Marrow Aspiration & Biopsy	80% plasma cells	Normocellular
Serum Calcium (mmol/L)	2.87	2.10–2.55

Bone Marrow Biopsy was showing increased plasma cells clusters focal admixed with myeloid cells in the available intertrabecular spaces. Bone marrow aspiration was showing particulate and hypercellular marrow with reduced trilineage hematopoiesis and replaced by sheets of plasma cells. Imprint smears were showing variable cellularity with myeloid cells and increased plasma cells.

The patient was diagnosed with IgG kappa-type multiple myeloma. Palliative radiotherapy to the pelvis provided moderate symptom relief, followed by chemotherapy with the Lenalidomide, Bortezomib, and Dexamethasone (RVD) regimen.

Discussion

Bone lesions in breast cancer patients are commonly attributed to skeletal metastases, which may present with intractable bone pain, hypercalcemia, pathological fractures, nerve compression, spinal cord compression, or bone marrow suppression [5]. In this case, the patient's back pain and bone lesions were initially suggestive of metastatic disease. However, the presence of acute kidney injury, elevated serum total protein, and globulins was atypical.

Elevated globulins are associated with acute infections, chronic inflammatory conditions, and plasma cell neoplasms [6]. As the patient had no signs of infection, SPE was performed, revealing a distinct M band (5.6 g/dL), suggestive of a plasma cell neoplasm. Further tests confirmed an altered kappa:lambda ratio (>100 mg/L kappa chains) and bone marrow with 80% plasma cells, confirming the diagnosis.

Studies, such as Mitchell et al., have reported a 4% increased detection rate of monoclonal gammopathies in patients with elevated globulins, with gamma globulins >4 g/dL associated with a 76% incidence of monoclonal gammopathies [7]. The hallmark features of multiple myeloma, encapsulated by the CRAB acronym (hypercalcemia, renal impairment, anemia, and bone lesions), were all present in this patient. Anemia occurs in ~70% of newly diagnosed myeloma cases, renal impairment in 20–40%, and lytic bone lesions in up to 80% [8].

Distinguishing between metastatic disease and plasma cell neoplasms is critical, as symptoms like hypercalcemia and

anemia overlap. The elevated globulins, altered albumin-to-globulin ratio, and M band on SPE were pivotal in identifying plasma cell neoplasm in this case. Literature reports rare coexistence of breast cancer and plasma cell neoplasms, with Levi et al. noting five cases of multiple myeloma among 443 second neoplasms in breast cancer patients [9]. A French study reported a slightly increased incidence of multiple myeloma (SIRR, 1.5; 95% CI, 1.3–1.7) among breast cancer survivors, though leukaemia and myelodysplastic syndrome were more common [10]. Clayer and Duncan identified multiple myeloma in two of four breast cancer patients with new bone lesions [11]. A case report by Timon et al had a similar finding of development of multiple myeloma in a treated breast cancer patient suggesting that clinicians should consider multiple myeloma as a cause of lytic bone lesions without extra skeletal metastases [12].

Although haematological malignancies like leukaemia and lymphoma are more frequently reported in breast cancer survivors, plasma cell neoplasms mimicking bone metastases are rare [13, 14]. The etiology of this association remains unclear, but treatment-related factors, such as chemotherapy, or genetic predispositions may contribute. Further research is needed to explore these mechanisms and the role of cumulative therapies in secondary malignancies.

Conclusion

Bone lesions in breast cancer patients are typically attributed to skeletal metastases. However, rare primary bone disorders, such as multiple myeloma, should be considered, particularly when accompanied by elevated total protein, globulins, and myeloma-defining events like CRAB symptoms. This case highlights the importance of a high index of suspicion and targeted investigations, such as serum protein electrophoresis, to differentiate plasma cell neoplasms from metastatic disease in breast cancer patients with bone lesions.

Declaration of Conflict of Interest

The authors declare no conflicts of interest that could influence the research or its findings. No financial or personal relationships with individuals or organizations that could bias this work exist.

Ethical Approval

This case report complies with the ethical principles for medical research involving human subjects as outlined in the Declaration of Helsinki. The patient provided informed consent for the use of her de-identified medical data for research and publication purposes.

Credit Author Statement

The corresponding author confirms that all listed authors have reviewed and agreed upon the descriptions of their contributions. The roles of the authors are as follows:

Author 1 (Corresponding Author): Conceptualization, data collection, patient management, manuscript drafting, and final approval.

Author 2: Data analysis, laboratory investigations, and critical revision of the manuscript.

Author 3: Treatment planning, clinical follow-up, interpretation of diagnostic tests, and manuscript review.

Authors contributed to multiple roles as needed to ensure the accuracy and integrity of the case report.

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Data Availability Statement

The data supporting the findings of this case report are included within the article, specifically in Tables 1 and 2 and Figure 1. Additional de-identified patient data are available from the corresponding author upon reasonable request, subject to ethical and institutional approval.

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Case Report

Ulcerative colitis initially misdiagnosed as irritable bowel syndrome: A case report

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Keywords

Inflammatory Bowel Disease (IBD), Ulcerative Colitis (UC), Irritable Bowel Syndrome (IBS), Remission

Abstract

Background: Irritable bowel syndrome (IBS) and early ulcerative colitis (UC) share overlapping gastrointestinal symptoms, which may lead to misdiagnosis and delay in appropriate treatment.

Case presentation: The patient presenting with abdominal pain and altered bowel habits was initially diagnosed with IBS based on symptoms, physical examination, and past medical history, in the absence of alarm features. However, despite one week of symptomatic treatment, the patient's condition worsened.

Diagnostic assessment: Further evaluation including complete blood profile, abdominal ultrasonography, colonoscopy, and colonic biopsy was subsequently performed, which confirmed a diagnosis of inflammatory bowel disease consistent with ulcerative colitis.

Conclusion: This case highlights the diagnostic pitfalls in differentiating IBS from early UC based solely on clinical presentation. Clinicians should maintain a high index of suspicion and pursue appropriate laboratory and endoscopic investigations when symptoms persist or worsen, to avoid misdiagnosis, treatment delay, and disease progression.

Introduction

Inflammatory bowel disease (IBD) is a chronic inflammation and immune-associated illness involving dysbiosis in the intestinal microenvironment. It is a lifelong progressive disorder characterized by unpredictable inflammation and an overactive immune system [1-2].

Case presentation

A 22-year-old male presented with a six-month history of pain during defecation, abdominal pain, bloating, nausea, intermittent blood in stools, and unintentional weight loss. There was no history of fever or acute gastrointestinal infection.

Past medical and surgical history

The patient had a past medical history of irritable bowel syndrome (IBS) diagnosed three years earlier, for which he was treated with chlordiazepoxide and clidinium bromide for 20 days, with symptomatic relief. He also had a history of right-sided inguinal hernioplasty performed at the age of seven years. Six months before the current presentation, the patient developed pain during defecation and hematochezia and was evaluated at a local hospital, where an anal fistula was suspected. He was managed conservatively with symptomatic treatment and advised increased oral fluid intake, following which the anal symptoms improved.

Family History and Allergies

Family history was significant for inguinal hernia, appendicitis, and hypertension in the father, and inguinal hernia in the brother. The patient reported an allergy to fluoroquinolones.

Clinical Examination

On physical examination, the patient appeared anxious. Vital signs revealed a blood pressure of 140/75 mmHg, pulse rate of 74 beats per minute, and he was afebrile. Abdominal examination showed mild tenderness without guarding or rigidity. Further laboratory and diagnostic investigations were subsequently performed.

Table 1: Complete Blood Picture.

Hematology	Observed Values	Normal Values
Hemoglobin	11.8 gm%	12-18 gm%
RBC Count	4.34 m/cmm	3.6-6.0 m/cmm
Total WBC Count	5000 cells/cmm	4,000-11,000 cells/cmm
Neutrophils	73%	45-75%
Lymphocytes	22%	20-45%
Eosinophils	2%	1-6%
Monocytes	3%	1-9%
Basophils	0%	0-1%
Total Platelets count	3.8 L/cmm	1.5-4.5 L/cmm

Table 2: Thyroid Profile.

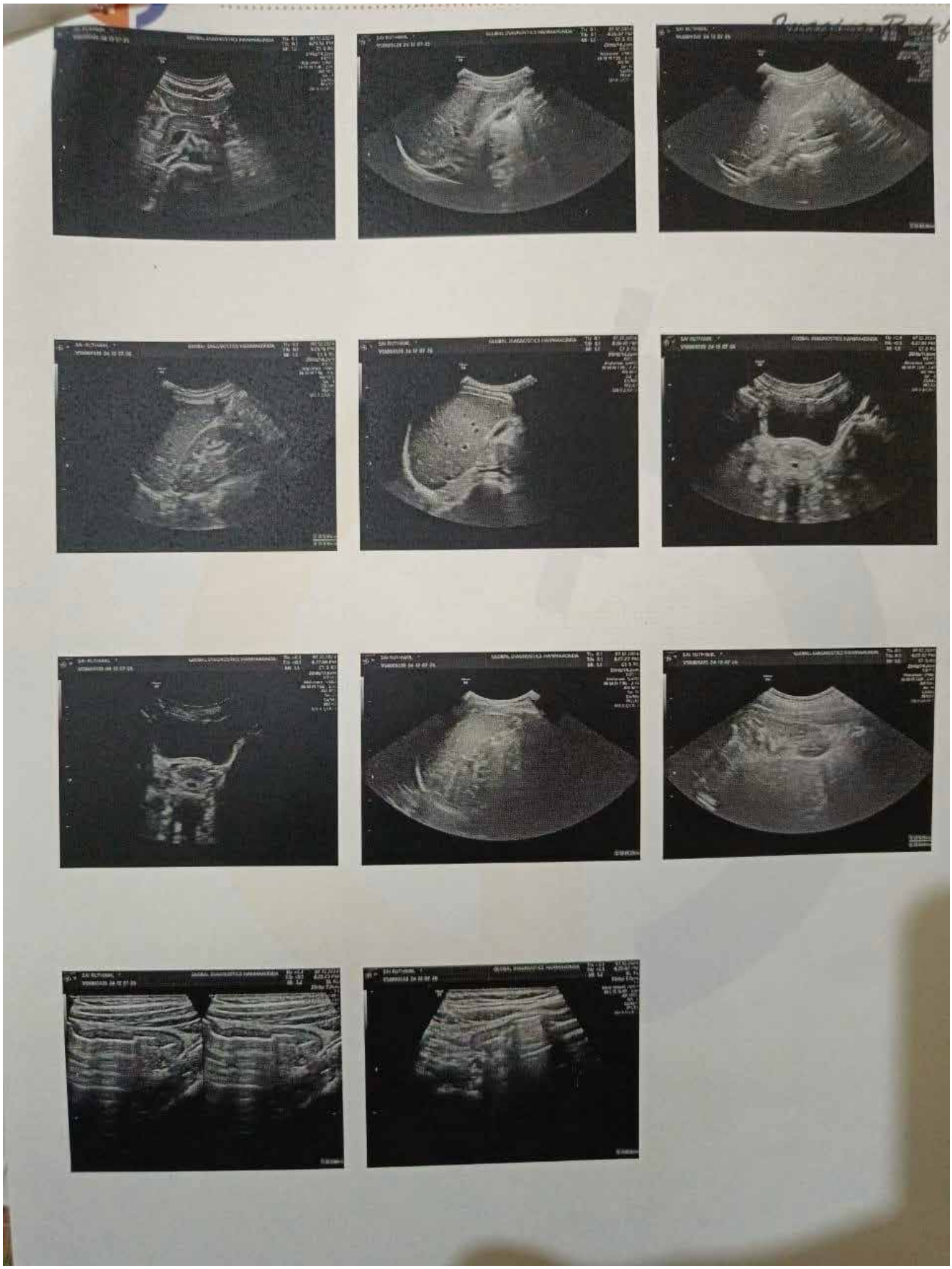
Thyroid Profile	Observed Values	Normal Values
Triiodothyronine (T3)	154.9 ng/dL	60-181 ng/dL
Thyroxine (T4)	10.66 ug/dL	4.6-12.5 ug/dL
Thyroid Stimulating Hormone (TSH)	0.869 uIU/mL	0.35-5.50 uiU/mL

Ultrasound

Impression

Descending colon, sigmoid colon appears minimally thickened and edematous (Maximum
thickness up to 5.0 mm)-? Colitis (Figure 1).

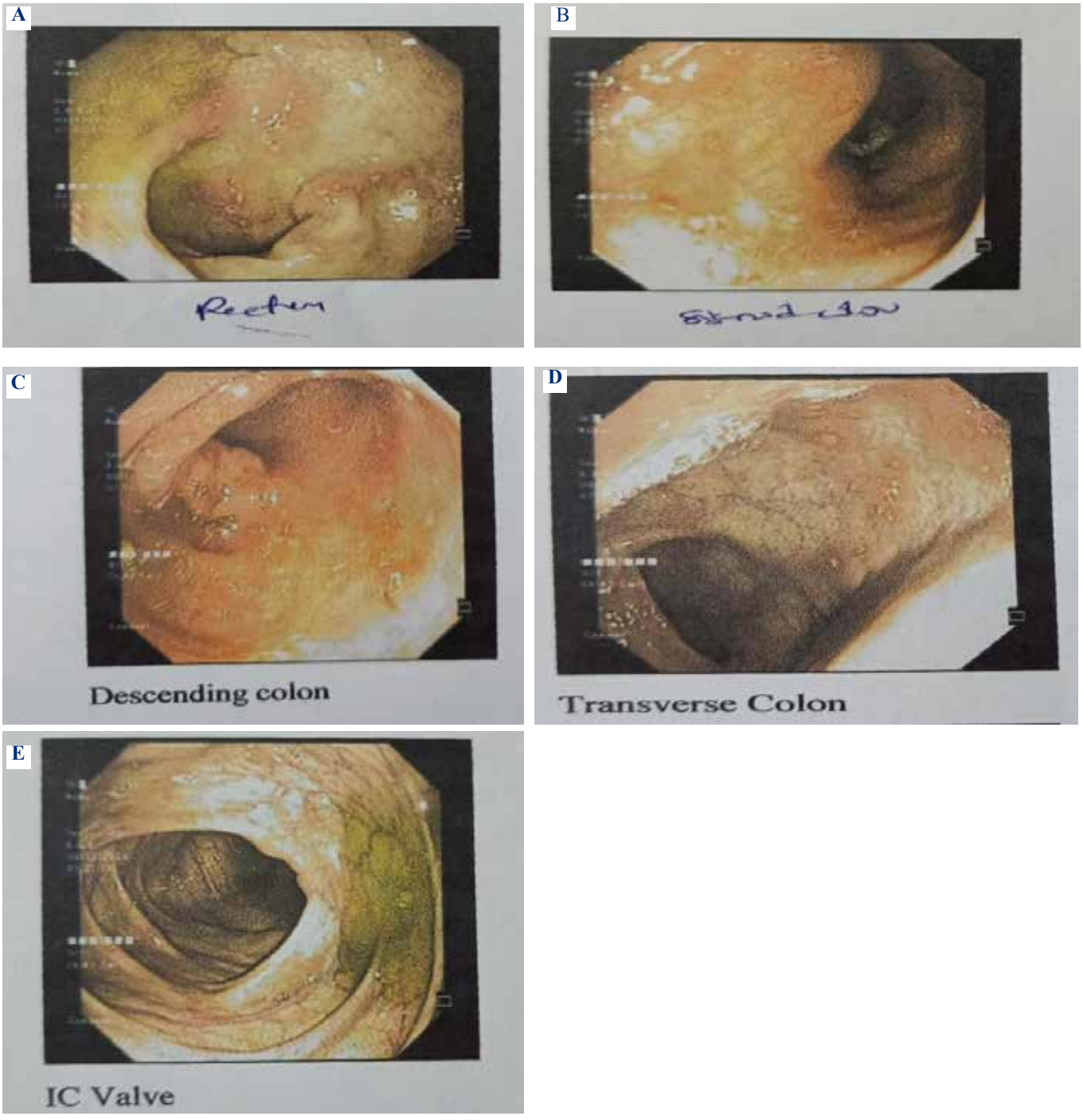
Figure 1: USG Abdomen.



Colonoscopy report
P/R: Hemorrhoids+
Rectum: Mucosal ulcerations with edema, erythema+ (Figure 2A)
Sigmoid Colon: Mucosal ulcerations with edema, erythema+ (Figure 2B)
Descending Colon: Mucosal ulcerations with edema, erythema+ (Figure 2C)
Transverse Colon: Mucosal ulcerations with edema, erythema+ (Figure 2D)
Ascending Colon: Mucosal ulcerations with edema, erythema+
IC Valve: Normal (Figure 2E)
Cecum: Mucosal ulcerations with edema, erythema+
Terminal Ileum: Normal

Impression
Colitis to R/O IBD-UC/Infective (HPE-A waited).

Figure 2: Colonoscopy of the entire colon starting from rectum (A) with thickening mucosa passing through the sigmoid (B), descending (C) and transverse (D) colon. IC Valve is normal (E).



Biopsy report

The biopsy report shows the microscopic examination with multiple fragments of colonic mucosa with focal erosion of the surface epithelium. It shows evidence of cryptitis, crypt abscesses, and depletion of goblet cells in a focal area. Lamina propria shows dense inflammation comprising neutrophils, lymphocytes, and many plasma cells. Focal areas also show basal plasmacytosis. No evidence of granulomas, amoebiasis, dysplasia/malignancy.

Impression

Feature suggestive of chronic active colitis.

Treatment

Table 3: Treatment plan for IBS and IBD.

Drug Name	Generic Name	Dose	ROA	DOA	DOS	Frequency
T. Sitcom Forte	Euphorbia prostrate extract+ calcium dobesilate	100 mg+ 500 mg	PO	29/11/2024	6/12/2024	OD
Cap. Lopisoz-D	Esomeprazole+ Domperidone	40mg + 30mg	PO	29/11/2024	6/12/2024	OD
T. Benizep	Mebeverine HCl+ Chlorodiazepoxide	13 mg+ 5 mg	PO	29/11/2024	6/12/2024	BD
Oint. Pilorute EP	Euphorbia Prostrate dry extract ethanol	1% w/w	PR	29/11/2024	6/12/2024	TID
T. Hyocimax	Hyoscyamine	0.125 mg	PO	30/11/2024	6/12/2024	OD
T. Rifaximin	Rifaximin	400 mg	PO	7/12/2024	14/12/2024	BD
T. Dolopar	Paracetamol + Caffeine	500 mg + 25 mg	PO	7/12/2024	14/12/2024	BD
T. Mesahenz	Mesalamine	1200 mg	PO	9/12/2024	05/02/2024	BD
Cap. Nexpro RD	Esomeprazole + Domperidone	40mg + 30 mg	PO	9/12/2024	14/12/2024	OD
Cap. Vizylac	Lactic acid Bacillus	120 million spores	PO	9/12/2024	14/12/2024	BD
Cap. Rekool D	Rabeprazole + Domperidone	20 mg + 30 mg	PO	14/12/2024	05/02/2024	OD
T. Folvite	Folic acid	5 mg	PO	14/12/2024	03/01/2024	OD
T. Azilide	Azithromycin	500 mg	PO	14/12/2024	19/12/2024	OD
Syp. Ventryl LS1	Levosalbutamol, Ambroxol HCl & Guaipesinhen	10 ml	PO	14/12/2024	25/12/2024	TID

Clinical summary

A 22-year-old male initially presented with chronic abdominal pain, altered bowel habits, intermittent rectal bleeding, bloating, nausea, and weight loss, without features of acute gastrointestinal infection. Based on the clinical presentation, physical examination, and a prior history of irritable bowel syndrome, a provisional diagnosis of IBS was made, and the patient was managed conservatively. As part of the initial evaluation and follow-up plan, a complete blood picture was advised to assess for anemia, and a thyroid profile was requested in view of unexplained weight loss. However, within one week, the patient returned with worsening abdominal pain, pain during defecation, fever, generalized weakness, and persistent symptoms, raising concern for an underlying organic pathology.

Further investigations were therefore undertaken, including abdominal ultrasonography, which suggested large bowel inflammation, prompting colonoscopic evaluation. Colonoscopy revealed features of chronic colonic mucosal inflammation, and tissue samples were obtained for histopathological examination. Biopsy findings confirmed a diagnosis of chronic ulcerative colitis.

Based on disease severity, treatment with mesalamine 1200 mg twice daily was initiated, leading to progressive clinical improvement, resolution of gastrointestinal symptoms, and steady weight gain on follow-up. Mild upper respiratory symptoms developed during treatment and were managed symptomatically, without objective evidence to establish a drug-related adverse effect. At one-month follow-up, the patient was asymptomatic, and the mesalamine dose was reduced to

1200 mg once daily for maintenance therapy.
This clinical course underscores the importance of reassessment and timely laboratory and endoscopic evaluation in patients initially diagnosed with IBS when symptoms persist or worsen.

Final diagnosis
IBD-Chronic Ulcerative Colitis.

Discussion
Our case presentation illustrates several key aspects. Firstly, it shows that functional diseases (IBS) may occur simultaneously with severe organic diseases (IBD) and that differentiation can be challenging and may be overlooked. Gastroenterologists may have overly focused on physical examination and past medical history. While psychosomatic specialists might have been uncertain about their ability to assist this patient. Secondly, it exemplary shows the bidirectional interrelation between IBS and IBD. There can be some overlap, particularly in symptoms presentation but IBS does not automatically progress into IBD. However, research suggests that potentially a higher risk of developing IBD if people with IBS are compared with the general population [3, 4]. The organic disease (IBD) and IBS presented first, then the patient developed a cough after the initiation of Mesalamine therapy. The physician has chosen the rechallenge method, without any alterations in the dose or medication. A symptomatic treatment has been prescribed for cough. By the next follow up the cough has been disappeared. Few studies suggest that the initiated and long-term use of Mesalamine may cause respiratory disease and immediate withdrawal of the drug and starting with the corticosteroids [5-7]. Before discontinuing Mesalamine due to adverse effects, the prescriber needs to choose a rechallenge and dechallenge technique. The drug needs to be discontinued based on the severity of the adverse drug reaction. The treatment needs to be prescribed to cure the adverse effect and follow up with the patient. Thirdly, the case report demonstrates the importance of understanding and explaining the patient's symptoms. Psycho-neuro-immunological connections proved instrumental in explaining these symptoms. The significance of elucidating functional relationships such as the gut-brain axis is emphasized in the German S3 Guideline on IBS (-). However, the German curriculum for medical students does not incorporate the knowledge necessary to explain these linkages. Even in severe cases of IBS that endure for decades, a clear explanation of symptoms and efficient symptom treatment can result in a quick and long-lasting improvement. Treatment guidelines for IBS benefit from self-management measures such as scheduled physical exercise, relaxation techniques, structured daily routines, and dietary alterations. These, however, are insufficient to account for the quick improvement. The patient's drive, ability to comprehend explanation, proactive coping mechanisms, and faith in the attending specialist and the therapeutic process are all factors that contribute to the positive outcome. Finally, integrating probiotics with antibiotic therapy can effectively reduce the incidence and severity of antibiotic-associated diarrhea (AAD) across various age groups. Clinical studies support the use of specific strains, such as Lactobacillus rhamnosus GG and Saccharomyces boulardii, highlighting their potential benefits when administered appropriately [9, 10].
Recent studies suggest adopting a Mediterranean diet, which focuses on fresh fruits, vegetables, monounsaturated fats, complex carbohydrates, and lean proteins, while limiting ultra-processed foods, added sugars, and salt. Reducing the intake of red and processed meat may help manage UC flares. Using Exclusive Enteral Nutrition (EEN) is an effective therapy for inducing clinical remission and endoscopic response in Crohn's disease and may be considered a steroid-sparing bridge therapy for UC patients. Regular screening for vitamin D and iron deficiency is recommended for all patients with IBD, and those with extensive ileal disease or prior ileal surgery should also be monitored for vitamin B12 deficiency. Co-management with a registered dietitian is advised, especially for patients with malnutrition or those requiring complex nutrition therapies [10,11].

Conclusion
The case study emphasizes the difficulties in differentiating between severe organic disorders (IBD) and functional diseases (IBS), as well as the reciprocal relationship between the two conditions. It emphasizes psycho-neuro-immunological links and stresses the significance of comprehending and elucidating symptoms. The importance of clarifying functional links is noted in the German S3 Guideline on IBS, nevertheless, the German medical curriculum does not include the information required to describe these interactions. Self-management practices including planned exercise, relaxation methods, organized daily schedules, and dietary changes can produce immediate and sustained improvement. Positive results are influenced by the patient's motivation, understanding of the explanation, proactive coping strategies, and trust in the attending professional and the therapeutic process.

Abbreviations

5-ASAs	5-aminosalicylates
AAD	Antibiotic-associated diarrhea
AIG	Asian Institute of Gastroenterology
BD	Twice daily
BP	Blood Pressure
CBP	Complete blood picture
CD	Crohn's disease
EEN	Exclusive Enteral Nutrition
GIT	Gastrointestinal Tract
HPE-A	Histopathological Examination
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IMs	Immunomodulators

OD	Once daily
OP	Outpatient
P/A	Per Abdomen
P/R	Per Rectum
PO	Per Oral
PR	Per Rectum
R/O	Rule out
RBC	Red Blood Cells
TID	Three times a day
UC	Ulcerative colitis
WBC	White Blood Cells

Ethical approval and consent to participate
Not Applicable.

Consent for publication
Applicable and uploaded as supplementary file.

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Case Report

Hypophosphatasia with Coexisting Endocrinopathies: A Diagnostic Dilemma

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Keywords

Hypophosphatasia, Alkaline Phosphatase, Urine Phosphoethanolamine, Inborn Errors of Metabolism

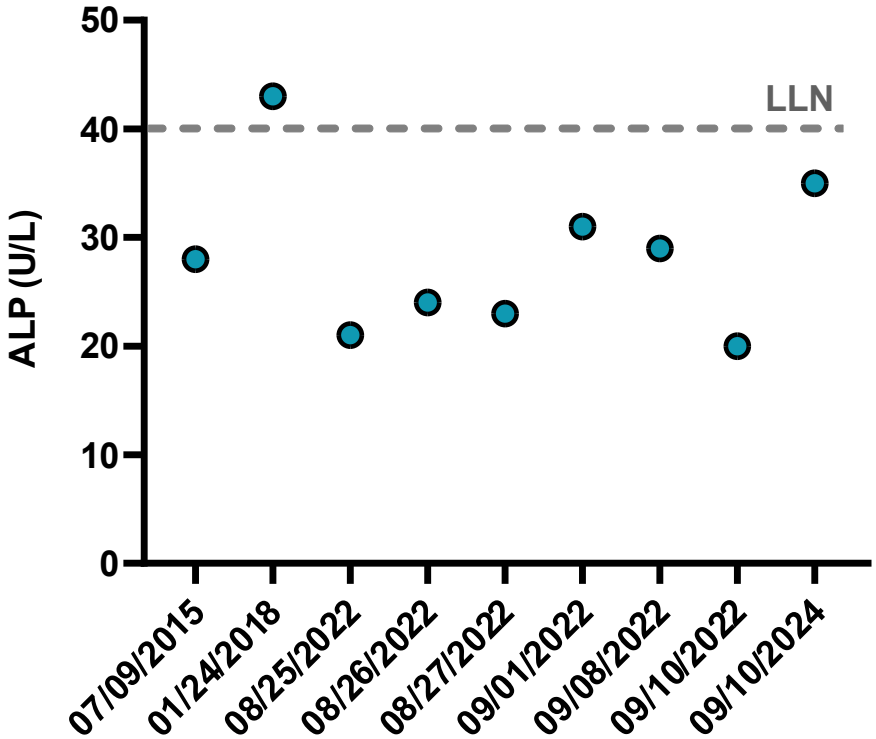
Abstract

Adult-onset hypophosphatasia presents a diagnostic challenge due to confounding clinical and analytical factors. Although rare, the consequence of missed diagnosis is significant, as it can potentiate skeletal mineralization defects. Despite the recent development of diagnostic criteria, integration into routine clinical practice remains limited, partly due to the variable course of disease progression. Effective management often requires a multidisciplinary team, including rheumatologists, orthopedic surgeons, endocrinologists, medical geneticists, dentists, physical and occupational therapists, pain specialists and clinical biochemists. Here, we present a case of adult-onset autosomal dominant hypophosphatasia, where diagnosis was complicated by coexisting endocrine disorders, Addison's disease and primary hypothyroidism. Persistently decreased alkaline phosphatase activity had been observed for over a decade and were initially attributed to hypothyroidism. However, an endocrinologist's clinical suspicion led to genetic testing, confirming hypophosphatasia. Although the patient exhibited no additional symptoms such as premature tooth loss, osteopenia, or osteoporosis, this incidental finding prompted a referral to medical genetics, carrier screening to support family planning, and cascade testing for family members.

Case Description

A 30-year-old female presents to the endocrinology clinic for follow-up and management of primary hypothyroidism and Addison's disease. At the time of initial diagnosis, thyroid-stimulating hormone increased at 15.08 µIU/mL (reference interval: 0.35-4.54 µIU/mL), free thyroxine decreased at 0.6 ng/dL (reference interval: 0.7-1.5 ng/dL), AM cortisol was undetectable at <1µg/dL (reference interval: 3.7-19.4 µg/dL), adrenocorticotropic hormone increased at 2050 pg/mL (reference interval: 7.2-63 pg/mL) and 21-hydroxylase antibodies were positive. Patient continues treatment with hydrocortisone 20 mg, fludrocortisone 0.1 mg and levothyroxine 75 µg. Notably, alkaline phosphatase (ALP) activity has been consistently decreased for past 10 years in the context of normal calcium, 25-hydroxy vitamin D and parathyroid hormone concentration (Figure 1). On average, ALP activity is decreased by 30% below the lower limit of normal. ALP activity is measured using the Abbott Alinity ci system between 2021-2024 and the Abbott Architect c4000 system between 2015-2021. In both assays, ALP catalyzes the hydrolysis of colorless p-nitrophenyl phosphate under alkaline conditions, producing p-nitrophenol (in its yellow phenoxide form) and inorganic phosphate and activity is quantified by measuring the increase in absorbance at 404 nm. Decreased ALP activity is known to be associated with hypothyroidism, predominantly due to impaired production of ALP (Paula Hoff, BMJ Open, 2025). However, ALP activity is expected to normalize after levothyroxine therapy. Despite normal free thyroxine (1 ng/dL) and thyroid-stimulating hormone concentrations (4.94 µIU/mL) post-treatment, her ALP activity remained decreased. ALP isoform testing was unsuccessful due to insufficient activity. Interestingly, bone density studies performed by dual-energy X-ray absorptiometry (DEXA) were unremarkable with z-scores of 1.2 for lumbar spine and 0.6 for right hip. Vitamin B12 concentrations were also normal at 695 pg/mL (reference interval: 213-816 pg/mL). Genetic testing identified a heterozygous pathogenic variant, c.517G>A(p.Glu191Lys), in the alkaline phosphatase (ALPL) gene confirming a diagnosis of adult-onset autosomal dominant hypophosphatasia (HPP). Patient denied symptoms of hypophosphatasia such as bone pain, abnormal dentition, short stature, muscle weakness, muscle pain, impaired mobility or abnormal gait. Due to lack of functional changes associated with HPP, treatment was deferred and patient was referred to medical genetics for further follow-up. A repeat DEXA scan is recommended in 4-5 years due to insidious nature of adult-onset HPP [4]. As the patient prepares for conception, education was provided on the significance of carrier screening to avoid autosomal recessive inheritance of severe forms of infantile or neonatal HPP. Additionally, genetic testing results were shared with family members to allow for cascade testing. To our knowledge, this is the first case report of a patient with HPP in the setting of two endocrine co-morbidities, Addison's disease and primary hypothyroidism.

Figure 1: ALP activity is consistently below the lower limit of normal reference interval (LLN).



Introduction

Hypophosphatasia (HPP) is a rare inborn error of metabolism caused by loss of function mutation in the ALPL gene encoding tissue non-specific ALP, which is predominantly expressed in bone, liver and kidneys [1]. Prevalence is estimated to be ~1 in 100,000 individuals, and >480 ALPL variants are characterized [2]. Hypomineralization is the pathological hallmark of HPP, driven by the accumulation of inorganic pyrophosphate, a natural substrate of ALP and a potent inhibitor of hydroxyapatite crystal formation [3]. Clinical severity is determined by the age of onset and is highly variable ranging from mild tooth loss or periodontal disease to severe bone demineralization, pulmonary hypoplasia, respiratory failure and vitamin B6-responsive seizures [4]. Perinatal and infantile HPP often display severe phenotypes, whereas adult-onset HPP shows mild-to-moderate phenotype [5].

Diagnosis of HPP is made based on combination of signs and symptoms, biochemical findings (persistently decreased ALP activity, elevation of ALP natural substrates such as pyridoxal-5′-phosphate, phosphoethanolamine, or inorganic pyrophosphate), imaging (DEXA) and genetic studies (ALPL variant analysis) [6]. In 2023, the international working group on HPP comprised of experts from Europe and North America provided recommendations for clinical diagnosis in adults and children using 2 major or 1 major and 2 minor criteria [5]. For adults, major criteria include pathogenic or likely pathogenic ALPL variants, elevation of natural ALP substrates, atypical femoral fractures, recurrent metatarsal fractures, whereas minor criteria include poor healing fractures, chronic musculoskeletal pain, early traumatic loss of teeth, chondrocalcinosis and nephrocalcinosis [5]. For children, major criteria include pathogenic or likely pathogenic ALPL variants, elevation of natural ALP substrates, early nontraumatic loss of primary teeth, presence of rickets, whereas minor criteria include short stature or linear growth failure over time, craniosynostosis, nephrocalcinosis, B6-responsive seizures and delayed motor milestones [5]. Currently, human recombinant enzyme replacement therapy for ALP called Asfotase Alpha is the only FDA approved treatment for patients with perinatal/infantile and juvenile-onset HPP but not adult-onset HPP in the United States [7].

Discussion

Decreased ALP activity is seen in a plethora of clinical settings, including anti-resorptive drug therapy, endocrine disorders such as hypothyroidism, hypoparathyroidism, hypercortisolism and renal osteodystrophy, hematological conditions such as pernicious anemia and myeloproliferative disorders, and nutritional deficiencies such as magnesium, zinc, copper, vitamin C, vitamin B6 and vitamin B12 [5] . Due to this overlap, HPP diagnosis is estimated to be delayed by ~5.7 years after onset of symptoms [8]. For instance, the current patient exhibited decreased ALP activity as early as 2015, but a definitive diagnosis was only established in 2024 following genetic confirmation. This delay is partly attributed to co-existing primary hypothyroidism.

In the absence of clinical symptoms, persistently decreased ALP below the lower limit of normal reference interval served as the primary trigger for initiating genetic testing in our case. It is essential to consider preanalytical, analytical, and post-analytical factors that may influence ALP results. Pre-analytically, the use of an incorrect collection tube containing EDTA preservative can lead to chelation of magnesium and zinc ions, resulting in falsely decreased ALP activity [9]. Analytically, ALP test is prone to instrument errors due to insufficient absorbance reads and can lead to misinterpretation of falsely decreased value [10]. ALP assay from multiple vendors were confirmed to detect Asfotase Alpha in the patient samples upon dilution [10]. Hence, it is important to troubleshoot the absorbance errors rather than reporting as undetectable [10]. Post-analytically, it is crucial to interpret ALP activity using the age and sex-partitioned reference intervals. Our current assay has a single reference interval of 40-150 U/L for adults based on the manufacturer's package insert. Before opting for expensive genetic testing, one could assess the concentration of tissue nonspecific ALP natural substrates such a pyridoxal-5′-phosphate, phosphoethanolamine, or inorganic pyrophosphate. However, access to these tests within our geographical location is limited. Both clinical and analytical factors can complicate the diagnosis of HPP. Given the broad spectrum of phenotypes across the HPP disease continuum, genetic testing plays a critical role in confirming the diagnosis. Patients diagnosed with HPP benefit from a multidisciplinary care team, an approach that remains limited in rural healthcare settings worldwide.

Ethical approval

Patient consent submitted to Sanford Health Information Management.

Declaration of conflicts

The authors declare no conflict of interest.

Funding and Data Availability

Not applicable.

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