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Letter to the Editor

# POCT in Cardiovascular Operating Room: A Game Changer?

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**Keywords**

POCT, Clinical Chemistry, Cardiac operating room, advantages

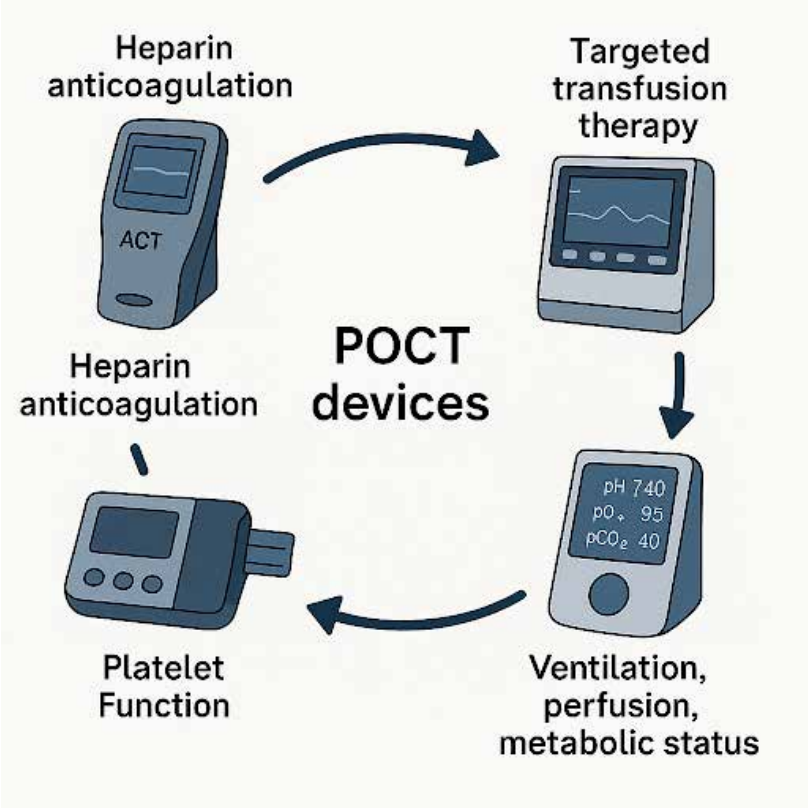
**Abstract**

Point of care testing (POCT) refers to diagnostic tests performed near the patient, rather than in a laboratory, It has been an essential component of operating rooms (ORs) since it was first introduced in the 1980s as it offers rapid diagnostic results that help physicians and surgeons to take critical care decisions, affecting the treatment pathway of a patient. This is particularly crucial in cardiovascular surgeries, where swift and accurate assessments of coagulation status, blood gases and other critical parameters are vital for patient safety and improved patient outcomes [1,2].

The main selling point of POCTs are their ability to deliver immediate and accurate results, enabling surgeons to make prompt, informed decisions in tense situations during surgery [1,3]. Traditional central laboratories often involve huge delays due to sample transportation and processing times, which can be fatal in the fast paced OR environment. POCT devices that are located near the OR eliminate these delays, allowing for real time monitoring of the patient's physiological status. For instance, during cardiopulmonary bypass (CPB) procedures, maintaining appropriate anticoagulation is critical to prevent thrombosis and hemorrhage. ACT, ROTEM, Sonoclot, Clotpro,i-STAT,PFA-100 etc are commonly used to asses hypercoagulability and hypocoagulability[4]. Implementing these technologies has been shown to improve precision and reduce the time required for repeat testing, thereby enhancing overall efficiency in the Cardiovascular Operating room [5,6].

ACT is essential for monitoring heparin anticoagulation during CPB. POCT devices help to provide rapid ACT results, facilitating timely adjustments to heparin dosing [5,7] The function of all POCT devices are listed in the table below.

| Technology | Function | Clinical Use |
|---|---|---|
| ROTEM (rotational thromboelastometry) [8] | Assesses coagulation dynamics,detects fibrinolysis and guides transfusions | Clotting assessment and transfusion guidance |
| TEG(Thromboelastography) [9] | Measures Clot formation, strength, and breakdown, including platelet contribution | Guides uses of antifibrinolytics |
| Quantra | Ultrasound-Based viscoelastic testing, fully automated | Faster and simpler than ROTEM/TEG, best for ICU/post op care. |
| Sonoclot Analyzer | Measures clot formation, fibrin interaction, and platelet function | Combines coagulation & platelet function testing in one device. |
| ClotPro | Cartridge based testing with multiple assays | Faster and more flexible alternative to ROTEM/TEG. |
| TAS-TEG | Compact, cartridge-based TEG, rapid clot assessment. | Trauma and emergency use in the OR. |
| HMS Plus | Precise heparin & protamine dosing during CPB. | Optimizing heparin reversal, reducing complications. |
| Hemochron Signature Elite | Measures Activated Clotting Time (ACT) for heparin monitoring. | Routine ACT monitoring during CPB. |
| i-STAT [4] | Portable blood gas [10], ACT, APTT, INR, electrolytes [11], and troponins. | Multi-function analyzer for ICU & intraoperative monitoring. |
| VerifyNow | Measures platelet function and response to antiplatelet drugs (Aspirin, Clopidogrel) | Assessing antiplatelet therapy before/after surgery. |
| PFA-100 | Shear-stress-induced platelet function testing, detects von Willebrand disease. | Diagnosing platelet dysfunction in surgical patients. |



The selection of POCT depends on clinical needs: ROTEM for speed, TEG for detail, Quantra for automation, HMS Plus for precise anticoagulation management, and VerifyNow for platelet function testing.This ensures tailor made and effective intraoperative and postoperative hemostatic management for all patients [4].

By providing precise coagulation profiles,POCT enables targeted therapy,reducing unnecessary blood product administration. Studies have demonstrated that implementing POCT guided transfusion algorithms in surgery leads to a reduction in blood product use.Timely correction of coagulopathies and metabolic imbalances during surgery can reduce the incidence of complications such as excessive bleeding or acidosis [12,13].

Reduced result times because of POCT has been shown to contribute to shorter operative times,improved workflow and lower mortality rates as decisions can be made swiftly without having to wait for central laboratory results [2].

While POCT offers numerous advantages,its challenges must also be addressed.Stringent quality control measures and regular maintenance are necessary to maintain the accuracy, reliability and integrity of test results [3,10]. OR personnel must be adequately trained to perform and interpret these results to ensure proper utilization of the machinery [7]. Additionally, seamless integration of POCT results into the patient's electronic health record (EHR) is crucial for comprehensive documentation and continuity of care [11].

The development of smaller and more portable devices increases the accessibility and convenience of POCT in various surgical settings, helping to broaden the scope of POCTs, providing surgeons with a more comprehensive and coordinated care plan [2,3,8,10].

The future of Point-of-Care Testing (POCT) in cardiovascular surgery is moving toward AI-driven automation, miniaturization, and personalized diagnostics. Next-generation viscoelastic analyzers like Quantra and ClotPro will be faster and fully automated, while lab-on-a-chip and wearable POCT devices will enable real-time coagulation monitoring for high-risk patients. AI-powered interpretation will enhance decision-making, reducing human error and improving precision in transfusion and anticoagulation management. Multiparameter handheld analyzers will integrate coagulation, blood gases, and cardiac biomarkers into a single test, optimizing patient care in the OR, ICU, and even remote settings. The shift toward cloud-connected, predictive, and personalized POCT will revolutionize perioperative and critical care medicine, ensuring faster interventions and better outcomes.

**Conflict of interests**

None.

**Ethical Approval**

Not required as study does not involve human subjects or data.

**Author Statement**

Sibtain Ahmed conceived the idea and wrote the paper. Raif Jafri conducted the literature review and contributed to writing. Amal Mahmood assisted with the literature search and formatting. All authors reviewed and approved the final manuscript.

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Review Article

# Recommendations on the collection of comparator measurement data in the performance evaluation of continuous glucose monitoring systems

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**Keywords**

Clinical performance evaluation, comparator data requirements, continuous glucose monitoring, standardization, traceability

**Abstract**

While current systems for continuous glucose monitoring (CGM) are safe and effective, there is a high degree of variability between readings within and across CGM systems. In current CGM performance studies, device readings are compared to glucose concentrations obtained with a comparator ("reference") measurement procedure (usually capillary or venous glucose). However, glucose concentrations from capillary and venous samples can systematically differ, often by as much as 5 to 10%. Different comparator methods have shown biases of up to 8%, and comparator devices of the same brand can systematically differ by more than 5%. To address these issues, the Working Group on CGM of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC WG-CGM) recommends standardizing study procedures and the comparator measurement process in CGM performance studies. The majority of IFCC WG-CGM members recommend the use of capillary samples as reference, mainly because CGM readings will then be aligned better with results from self-monitoring of blood glucose (SMBG). Even with factory-calibrated CGM systems, manufacturers require CGM users to perform SMBG in some situations, e.g., manual calibration, confirmation of extreme readings, discordance between CGM readings and symptoms of hyper- or hypoglycemia, or intermittent signal loss.

Comparator devices should meet defined analytical performance specifications for bias and imprecision. Comparator bias can be reduced by retrospective correction of comparator values based on measurements with a method or materials of higher metrological order. Once manufacturers align CGM readings of their systems with comparator results using standardized procedures, variability across CGM systems will be reduced.

## Introduction

Continuous glucose monitoring (CGM) is an essential tool for many people with diabetes. Studies have shown the clinical benefit of CGM systems in persons with type 1 diabetes, independent of being used as a stand-alone device or integrated with sensor-augmented pumps or systems for automated insulin delivery, and there is growing evidence for potential benefit in persons with type 2 diabetes [1,2].

CGM systems can display markedly different readings when used in parallel, even if they show good performance results in their pivotal study [3-10]. As a result, variability between CGM systems can be substantial enough that two systems may give sufficiently different results to affect clinical decisions.

The Working Group on CGM of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC WG-CGM) aims to standardize procedures for assessing the performance of CGM systems. A standardized study design with harmonized measurement procedures will allow better direct comparisons of CGM performance, and readings from different CGM systems will be better aligned. The IFCC WG-CGM acknowledges that procedures may have to be modified for specific populations (e.g., people <18 years of age) or for specific indications (e.g., intensive care units).

Specific characteristics for comparator data have been recommended before:[11] Comparator data should be obtained at 15-minute intervals for 6 to 8 hours during the beginning, the middle, and the end of sensor lifetime. Furthermore, the comparator data distribution should cover the clinically relevant concentration ranges and rates of change, for example, by inducing glucose changes after meal challenges.

To ensure comparability, the procedure for comparator ("reference") measurements must be standardized. A recent review indicates that this has not been the case in the past [12]. The complete description of a comparator measurement procedure comprises the type of comparator device (e.g., laboratory analyzer, blood gas analyzer, handheld analyzer), the brand of the device, the device's analytical methodology (e.g., glucose oxidase, hexokinase), its traceability to a higher order reference method (e.g., isotope dilution mass spectrometry [IDMS]), the specific consumable batches/reagent lots used, and the type of sample (sample origin and pre-analytical handling). The choice of sample type and comparator method can affect each other, as not all methods are intended to be used with all sample types.

This article reports recommendations by the IFCC WG-CGM for standardized study procedures. With these recommendations, the IFCC WG-CGM intends to initiate scientific discourse regarding design of and procedures in CGM performance studies. Ultimately, the IFCC WG-CGM aims to support the establishment of an international standard regarding the performance evaluation of CGM systems.

## Sample origin and handling

*Background information on sample origin*

In CGM performance studies, three blood sample types are commonly used for comparator measurements: capillary, venous, and "arterialized-venous" [12]. Glucose concentrations are physiologically different between these venous and capillary samples, with past studies showing differences between capillary and venous glucose concentrations of, on average, 5% to 10% that can vary depending on the glucose rate of change. While arterialized-venous samples are also drawn from the vein, they undergo different pre-analytical handling by warming the arm or hand where the blood is drawn. In a recent study [13], the difference between capillary and venous glucose concentrations was +5.9%, which could be reduced to +4.2% by applying heat to the arm. As a result, the observed CGM performance was different when using venous, arterialized-venous, or capillary glucose concentrations [13]. The bias of the investigated CGM system was approximately +2% when comparing against venous comparator samples, +0% against arterialized-venous comparator samples, and -4% against capillary comparator samples. This resulted in mean absolute relative difference (MARD) results of approximately 7.5% (venous comparator samples), 7% (arterialized-venous comparator samples), and 8% (capillary comparator samples). It is, therefore, imperative to standardize the origin of samples for comparator measurements in CGM performance studies. Table 1 summarizes advantages and disadvantages of the different sample origins. It should be noted that the selection of sample origin and the selection of comparator measurement method affect each other.

**Table 1**: Summary of sample origins with associated advantages and disadvantages.

| | Advantages | Disadvantages |
|---|---|---|
| Capillary | • Relied on by users to verify CGM accuracy<br>• Used when CGM data is unavailable or implausible<br>• Used for manual CGM calibration<br>• Sampling has low risk of adverse event<br>• Immediate display of results for glucose manipulation procedure if BGMS is used<br>• Considered acceptable in all age groups, including children ≤6 years of age<br>• Does not require sampling by trained health care professional<br>• Alignment of in-clinic and free-living data[3] | • Repeated fingersticks can be painful and burdensome<br>• Sample volume may be too small for some glucose analyzers accepted as comparator by the FDA[1]<br>• Compliance with analytical performance specifications and retrospective correction may have to be performed in two steps (vs. lab analyzer and lab analyzer vs. higher-order material) if higher-order material cannot be used with the comparator device[2] |
| Venous | • Sample volume sufficient for glucose analyzer accepted as comparator by the FDA<br>• Usually, only a single stick is required to place a peripheral or central intravenous line.<br>• Clinical diagnostics are typically based on venous glucose concentrations due to ease of access.<br>• In a situation requiring an urgent result, venous blood from an indwelling intravenous line is the most reliably obtained type of specimen. | • Sampling and sample handling require trained healthcare professionals.<br>• Increased risk of adverse events due to invasiveness<br>• Total blood loss might be an issued<br>• Sampling technique and sample handling can impact measurement results (inadvertent hemolysis, glycolysis)<br>• Immediate display of results for monitoring of glucose manipulation procedure is challenginge<br>• Not feasible in a free-living setting<br>• Not feasible for minors ≤6 years of age |
| Arterialized-venous | • See venous advantages<br>• Better alignment with capillary glucose levels than venous glucose levels, i.e., results of in-clinic and free-living procedures are more comparable[3] | • See venous disadvantages<br>• **Heating procedure currently is not standardized**[7]<br>• Need for additional resources to ensure sustained levels of heat application[8]<br>• Immobilization of participants<br>• Additional burden on participants due to prolonged heat application |

CGM: continuous glucose monitoring system, FDA: Food and Drug Administration, BGMS: blood glucose monitoring system

Information is based on the most common measurement schedule,[11] which is also recommended by the IFCC WG-CGM, which requires blood sampling in 15-minute intervals over a period of 6 to 8 hours, i.e., 25 to 33 consecutive blood draws.

[1]Many laboratory-grade analyzers need at least 25 µL of sample volume. While whole blood samples of this volume might be obtained with the proposed sampling frequency and duration of sampling periods, plasma samples would need additional whole blood to be collected, which cannot be achieved reliably.

[2]Analytical performance specifications and retrospective correction are addressed in section "Analytical performance of comparator methods". It may be more difficult for POC-BGMS or SMBG systems to fulfill C-APS than for laboratory analyzers.

[3]Manufacturers likely base their algorithms on both in-clinic and free-living or real-world data. Alignment leads to better comparability between these data.

[4]The Clinical and Laboratory Standards Institute's (CLSI) guideline POCT[5][14] recommends collecting no more than 5 mL/kg body weight of blood within any 24-hour period or 7 mL/kg in any eight-week period. These limits are lower in protected populations, like pregnant or pediatric participants. It should be noted that the actual blood volume drawn depends on the sampling technique, e.g., loss due to line flushing, and sample container size.
[5]Venous samples have to be collected and centrifuged before measurements can be performed. There will be a delay before display of results.
[6]The arterialization effect likely depends on aspects of the specific heating procedure, e.g., arm or hand to be heated, type of heating pad or chamber, temperature setting. It would, therefore, be necessary to describe the heating procedure in much more detail and to investigate how the arterialization effect depends on the heating procedure.
[7]Additional resources may include temperature monitoring to minimize the risk of excessive temperatures and restarting the heating pad, which are typically automatically switched off after some time for safety reasons. In addition, and this overlaps with the immobilization of participants, participants need help removing and reapplying the heating pad around restroom breaks.

While glucose concentrations also depend on the sample matrix (i.e., whole blood, plasma, serum), whole-blood glucose concentrations can be converted to plasma-equivalent values so that any remaining differences are negligible [15]. However, it must be noted that a conversion factor is only an approximation and the individual association between whole-blood and plasma glucose may be influenced by other factors, like hematocrit and illness [16,17]. Measurement of glucose concentrations in serum is inadequate in the context of comparator data in CGM performance studies due to glycolysis.

*Recommendation for sample origin*

The majority of WG-CGM members recommend capillary samples to be the preferred sample origin in CGM performance studies. Since capillary self-monitoring is supplemented by adjunctive CGM systems and the fallback/verification option for nonadjunctive CGM systems, using capillary comparator samples would optimize the clinical alignment of CGM readings and self-monitoring of blood glucose (SMBG) concentrations. The IFCC WG-CGM viewed this alignment as more important for end-users than the alignment of CGM readings to venous glucose concentrations, which are used in the diagnosis of diabetes and for diabetes management in healthcare settings. However, the IFCC WG-CGM acknowledges that during the development of a future international standard, which is an aim of the IFCC WG-CGM, the sample type must be discussed with different stakeholders like diabetologists, regulatory authorities, and CGM manufacturers.

**Analytical performance of comparator methods**

*Background information*

The consistent analytical performance of comparator methods is essential in CGM performance studies not only to optimize comparability of results between studies, but also because the comparator is assumed to be free from error. For an accuracy metric like measurement bias between a CGM system and a comparator to be meaningful, there is an assumption that the comparator indicates the "true" glucose value. In reality, however, any measurements, including comparator measurements, are affected by different sources of error. Untangling comparator and CGM error is not feasible. Any inaccuracies of the comparator will affect the performance of a CGM system as observed in a study, and so will any differences in comparator method across different studies.

Of course, comparators must be used in compliance with device labeling and applicable legal requirements. Still, comparators can exhibit measurement bias of up to 8% depending on the measurement method [18,19]. In addition, more than 5% bias between individual devices of the same brand can exist [20,21]. Analytical performance of comparators can be optimized by requiring that comparator methods have established metrological traceability with defined analytical performance specifications. These specifications are called "analytical performance specifications for comparators" (C-APS) here to avoid confusion with minimum requirements for CGM performance. C-APS comprise bias and imprecision. C-APS can be defined based on biological variation [22,23]. It may be more difficult for POC-BGMS or SMBG systems to fulfill C-APS than for laboratory analyzers, although it has been shown that they can satisfy the C-APS [20,24]. Furthermore, they may not be recognized by all regulatory bodies. The FDA, for example, stated in the past that they only recognize "laboratory-based methods" [25]. In the European Union, however, a CGM system gained CE marking based on capillary comparator data obtained with an SMBG system [26]. As long as the comparator is used within the intended use and performs within C-APS, the specific brand or type of device (laboratory analyzer, POC-BGMS or SMBG system) is irrelevant, especially if bias is minimized through retrospective correction. It should be noted that compliance with C-APS does not reflect potentially interfering substances. The effect of interfering substances on laboratory analyzers, POC-BGMS, and SMBG systems typically increases in that order, although it depends on the specific brand. This could limit the choice of the comparator in CGM performance studies.

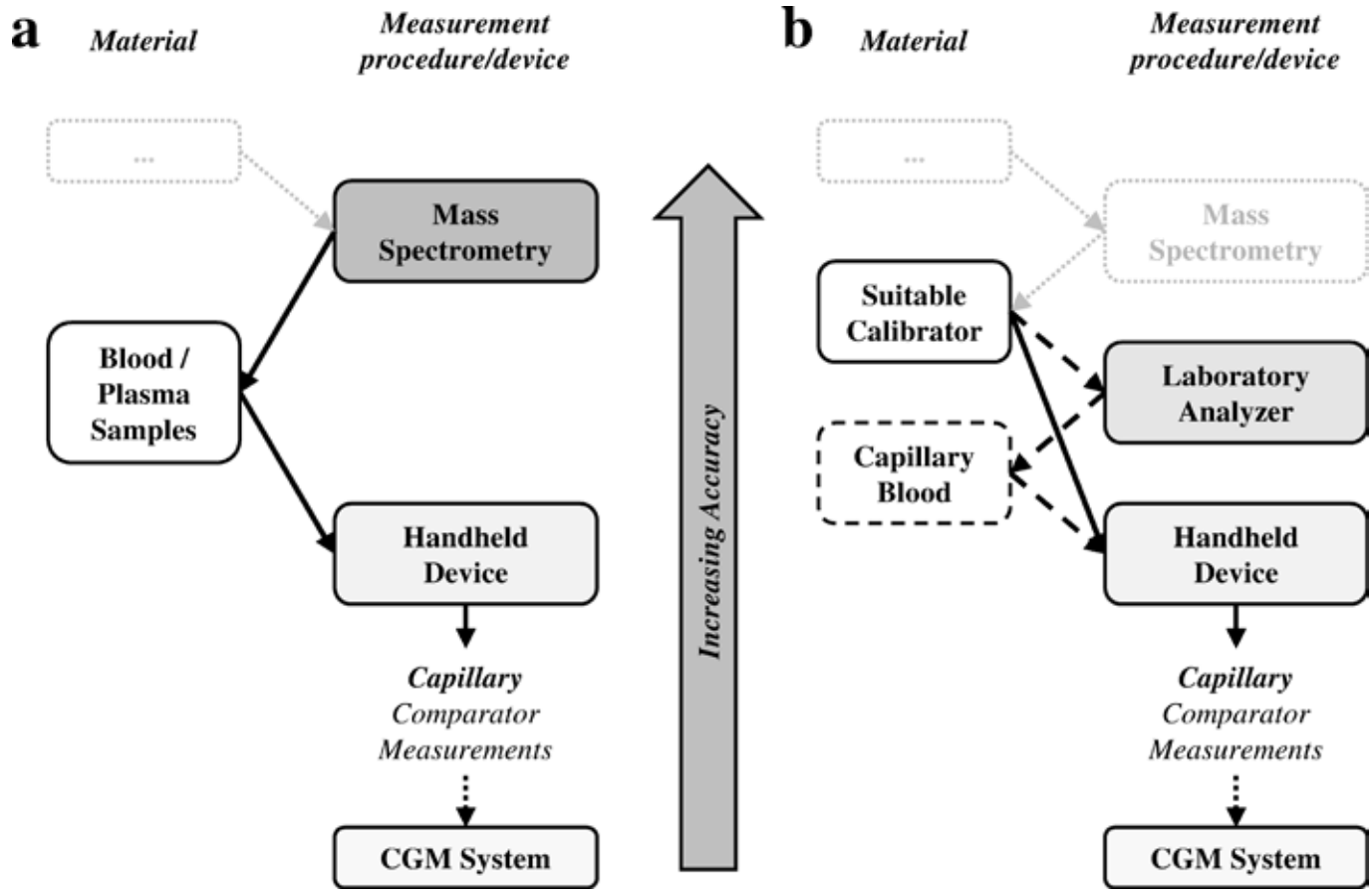
*Assessment of measurement bias*

Bias is defined as the estimate of a systematic difference between methods. In the context of comparators, bias should be assessed using a method or materials of higher metrological order. The higher metrological order associated with these methods and materials implies that corresponding measurement results and assigned target values, respectively, are closer to the "true" glucose concentration than the measurement results obtained with the comparator. An IDMS-based method could constitute a method of higher metrological order if a laboratory analyzer is selected as comparator, and bias could

be verified with a split-sample approach, where comparator samples are split into two aliquots, one for the comparator and one for the higher-order method. If professional-use point-of-care (POC) blood glucose monitoring systems (BGMS) or "over the counter" systems for SMBG are used as comparator, then an additional step by way of, for example, a laboratory analyzer may be required if materials of higher order are used to assess bias, but do not fall within the intended use of the POC-BGMS or SMBG system. In case higher-order materials are used, they are ideally independent from the manufacturer of the selected device. For example, the serum-based Standard Reference Material 965 (National Institute of Standards and Technology, Gaithersburg, MD) could serve as a material of higher metrological order if the samples are commutable when measured with the specific comparator. The steps associated with bias verification are shown in Figure 1.

Commutability of the samples used for bias verification is important. In lay-person's terms, commutability means that the higher-order materials behave like participant samples (whole blood or plasma) in both the method used to assign the target concentrations and the comparator method used in the performance study. Therefore, participant samples in a split-sample approach are commutable by definition, whereas commutability of other samples may have to be verified in advance.

**Figure 1**: Example of a traceability chain when estimating bias.
Panel a) Estimation based on methods of higher metrological order.
Panel b) Estimation based on materials of higher metrological order ("suitable calibrator").




If participant blood/plasma samples are used to verify the comparator method bias, potential commutability issues can be avoided. When using capillary comparator measurements, an additional step (indicated by dashed lines and boxes) by way of, for example, a laboratory analyzer (or other suitable analyzers) may be required if the comparator device is not labeled for use with the sample type of the higher-order materials. The "suitable calibrator" is any material with sufficiently well-characterized assigned target concentrations, e.g., secondary certified reference materials as defined by ISO 17511. Light grey boxes and dotted lines indicate parts of the traceability chain that are outside of the purview of bias estimation in a CGM performance study. Device types in the columns "measurement procedure/ device" are based on what is often used in CGM performance studies [12]. Note that the step between the comparator and the CGM systems is missing because CGM systems measure glucose concentrations in interstitial fluid, where comparator or reference measurements are currently not feasible. Similar traceability chains can be established for other sample origins.

*Recommendations for the assessment and correction of bias*

Bias towards the higher-order method's measurement results or assigned concentrations of the higher-order materials is used to verify the C-APS. Due to potential variation of bias over time, measurements with higher-order methods or materials should be performed sufficiently often, e.g., on every in-clinic day of a CGM performance study. They should cover the relevant glucose concentration range. Calibrator or reagent batch changes should be scheduled outside of the performance study's timeframe; otherwise, bias should be assessed using a sufficient amount of data for each combination of calibrator and reagent batch. The C-APS requirement for bias is defined as bias ≤2.1% [23]. Retrospective correction of comparator bias (sometimes also called "recalibration") should be used if the bias criterion is not met [20]. The IFCC WG-CGM recommends retrospective correction even if the bias criterion is met to further minimize differences between individual studies and between individual devices, if more than one comparator device is used in a study (e.g., in a multi-center study).

*Assessment of measurement imprecision*

Imprecision can, for example, be measured as the coefficient of variation (CV) of replicates from the same sample and pooled across all samples when there is no device drift, there are no changes in the device (like reagent or calibrator batch changes), and when the samples do not contain varying levels of interfering substances. In contrast to bias, it is not possible to minimize CV retrospectively. Imprecision of a potential comparator should, therefore, be assessed beforehand to minimize the risk of using an inadequate comparator. Imprecision of the comparator in a CGM performance study can be estimated from measuring replicates, e.g., duplicates, in each participant sample, and the average of these replicates can be used as comparator value. For a series of duplicate measurements, calculation of SD or CV is outlined using the "Dahlberg" formula by Kallner and Theodorsson [27]. The Dahlberg formula calculates the repeatability within series. Repeatability is different from the reproducibility between series, which is obtained by repeated measurements over many days of the same sample. Staying with the example of duplicate measurements, the effective imprecision of the comparator results when using averages of these duplicates can then be estimated from the standard error of the mean, i.e., CV is divided by the square root of the number of replicates.
For duplicate measurements, the effective imprecision, expressed as effective CV, can be estimated as

$$CV_{eff} = \sqrt{\frac{2 \times \sum_{i=1}^{N} \left( \frac{x_{i,1} - x_{i,2}}{x_{i,1} + x_{i,2}} \right)^2}{2N}}$$

where $x_{i,1}$ and $x_{i,2}$ are the measurement results of the first and second replicate, respectively, and N is the total number of duplicates.
Imprecision can also be estimated from measurements with higher-order materials. If a split-sample comparison with a higher-order method is performed, multiple replicates are required.

*Recommendations for the assessment of imprecision*

The IFCC WG-CGM recommends that participant samples in the CGM performance study are measured in at least duplicates to allow estimation of imprecision as well as outlier detection [20,27]. The average of these (at least) duplicates should be used as comparator value to reduce the impact of imprecision. The C-APS requirement for imprecision is CV ≤2.4% [23]. Ideally, imprecision is characterized in advance to minimize the risk of inadequate imprecision in a CGM performance study.

*Practical application of analytical performance specifications for comparators*

Recently, a CGM performance study (German Clinical Trials Register, ID DRKS 00033697) was conducted. Here, both capillary and venous blood samples were collected for comparator measurements during frequent sampling periods, i.e., 7-hour periods where blood was sampled every 15±5 min. Cobas Integra 400 plus (INT; Roche Diagnostics GmbH, Mannheim, Germany) and, in total, three different YSI 2300 STAT Plus (YSI; YSI Incorporated, Yellow Springs, OH, USA) devices were used to obtain venous comparator data, and Contour Next (CNX; Ascensia Diabetes Care Holdings AG, Basel, Switzerland) was used to obtain capillary comparator data. Additional capillary measurements were performed twice per frequent sampling period with YSI and INT. Results from YSI devices were pooled since measurements from any given sample were only performed on one YSI device. Multiple YSI devices were used for feasibility reasons, as there were more venous samples on each in-clinic day than a single YSI device could have processed.
To assess and to allow for retrospective correction of bias of INT and YSI, 184 participant samples were sent in a split-sample approach for measurements with a spectrophotometric hexokinase-based method (Cobas pro [CBP]; Roche Diagnostics GmbH). The CBP was calibrated with the manufacturer's master calibrator, thus being of higher metrological order than INT and YSI, which were calibrated with marketed, i.e., end user, product calibrators. CBP's expanded measurement uncertainty (k=2) was reported as 3.5%, with negligible bias when compared regularly against IDMS. Bias of CNX was assessed based on the capillary measurements with INT and YSI and retrospectively corrected based on corrected INT and YSI results, respectively. There were slight differences in CNX measurement results depending on whether CNX was corrected retrospectively based on INT or YSI results. However, these were acceptably small and CNX's

bias was in both cases substantially lower after retrospective correction. If venous samples had not been collected in this study, then CBP could have been used as easily with capillary samples so that the traceability chain of Figure 1A would apply. Imprecision was estimated from the duplicate glucose measurements for all comparators [20,27]. Compliance with C-APS is shown in Table 2.

**Table 2**: Adherence with analytical performance specifications for comparators (C-APS).

| Sample | Bias | | | Imprecision | | C-APS met[2] | |
|---|---|---|---|---|---|---|---|
| **Device** | n | original | Corrected[1] | n | CV | original | final |
| **Venous** | | | | | | | |
| INT | 184 | +3.0% | +0.0% | 2059 | 0.3% | No | Yes |
| YSI 1 | 64 | -2.3% | +0.1% | 706 | 0.2% | No | Yes |
| YSI 2 | 55 | -1.5% | +0.0% | 630 | 0.3% | Yes | Yes |
| YSI 3 | 65 | -1.7% | +0.0% | 724 | 0.6% | Yes | Yes |
| **Capillary** | | | | | | | |
| CNX (vs. INT) | 142 | +2.8% | +0.1% | 2061 | 1.6% | No | Yes |
| CNX (vs. YSI) | 142 | +3.8% | +0.2% | 2061 | 1.6% | No | Yes |

As bias was corrected retrospectively, compliance with C-APS is given before and after retrospective correction.

CGM: continuous glucose monitoring system, FDA: Food and Drug Administration, BGMS: blood glucose monitoring system

CV: coefficient of variation, INT: Cobas Integra 400 plus, YSI: YSI 2300 STAT Plus, CNX: Contour Next

[1]For venous samples on INT and the three YSIs, bias was retrospectively corrected based on a split-sample approach with a higher-order method. For capillary CNX samples, bias correction was implemented using a split-sample approach using corrected capillary INT or YSI results (as indicated in the table).

[2]"Original" indicates application of C-APS to original bias and imprecision, whereas "final" indicates application of C-APS to retrospectively corrected bias and imprecision.

To illustrate the impact of bias on observed CGM performance, MARD results, 20%/20 agreement rates (ARs), i.e., percentages of CGM readings found within ±20% of paired comparator results ≥100 mg/dL and within ±20 mg/dL of paired comparator results <100 mg/dL, and bias (mean relative difference) were calculated for one of the CGM systems investigated in the study mentioned above before and after recalibration (Table 3).

**Table 3**: Mean absolute relative difference (MARD), 20%/20 agreement rates (AR), i.e., percentages of CGM readings found within ±20% of paired comparator results ≥100 mg/dL and within ±20 mg/dL of paired comparator results <100 mg/dL, and bias (mean relative difference) for a current-generation CGM system.

| | | Before comparator bias correction | | | After comparator bias correction | | |
|---|---|---|---|---|---|---|---|
| **Comparator** | **n** | **MARD** | **20%/20 AR** | **Bias** | **MARD** | **20%/20 AR** | **Bias** |
| INT (venous) | 2001 | 9.5% | 93.2% | +3.1% | 10.5% | 91.8% | +6.1% |
| YSI (venous) | 2002 | 11.6% | 88.9% | +8.4% | 10.5% | 91.2% | +6.3% |
| CNX (capillary) | 2005 | 9.7% | 92.4% | -1.1% | 9.7% [a] | 92.4% [a] | +1.6%[1] |

AR: Agreement rate, MARD: mean absolute relative difference, INT: Cobas Integra 400 plus, YSI: YSI 2300 STAT Plus, CNX: Contour Next

[1]CNX results retrospectively corrected based on corrected INT results were used as comparator.

Before retrospective correction of comparator bias, there were substantial differences in the observed CGM performance depending on the venous comparator (INT vs. YSI). Correction of comparator bias eliminated the 2.1% MARD difference, reduced the approximately 4% difference in 20%/20 agreement rate, and reduced the difference in bias between the CGM system and the comparator from 5.3% to 0.2%. The differences in the original data could be viewed as relevant from both clinical and marketing perspectives despite being caused by the selection of the comparator and not being a property of the CGM system itself. When using the capillary CNX measurements as comparator, marked differences compared with venous results were found after retrospective correction of comparator bias, whose order of magnitude matches the capillary-to-venous differences in comparator glucose concentrations found in a recent study [13].

**Summary and Outlook**

In CGM performance studies, CGM readings are compared to values obtained with a comparator measurement procedure, whose description comprises comparator device, measurement method, and sample type. Different comparator methods have demonstrated biases of up to 8%, [18,19] different comparator devices of the same brand can systematically differ, on average, by more than 5%, [20,21] and concentrations in capillary and venous samples are physiologically different (often reported as 5 to 10% difference, sometimes more) [13].

The IFCC WG-CGM, therefore, recommends standardizing the comparator measurement procedure (Table 4). Previous recommendations were adopted [11]. Specifically, comparator measurements should be performed every 15 minutes for 6 to 8 hours during in-clinic sessions, and comparator data should exhibit certain characteristics, i.e., distribution of glucose concentrations and rates of change [11]. Furthermore, the use of capillary samples is recommended, mainly because CGM readings should align better with results from SMBG, which must be performed in some situations by people with diabetes:

1. some CGM systems require periodic manual calibration,
2. CGM systems typically require confirmation of very high or very low CGM readings,
3. SMBG is advised if symptoms do not match CGM readings, and
4. SMBG is the fallback option if CGM readings are not available, e.g., during warm-up or malfunction. For the comparator device and method, C-APS should be met; specifically, bias ≤2.1% and imprecision ≤2.4% [23] Bias between comparators can be reduced by retrospective correction of comparator values based on analysis with a method or materials of higher metrological order,[20] and correction is encouraged even if the bias criterion is met. Comparator samples should be measured in duplicate, and the averages should be used as the comparator result to improve imprecision of comparator results as well as to enable outlier detection.

A common reference and use of standardized procedures in CGM performance studies will minimize discrepancies and ensure alignment between readings across CGM systems.

**Table 4**: Recommendations of the IFCC WG-CGM regarding the comparator measurement procedure in CGM performance evaluations.

| Category | Recommendation |
|---|---|
| Comparator sampling frequency | Every 15 minutes for 6 to 8 hours [11] |
| Comparator glucose concentration distribution | At least 7.5% of all values each covering the following situations: [11]<br>a) BG < 70 mg/dL b) BG > 300 mg/dL, c) BG ≥ 70 mg/dL, RoC < -1 mg/dL/min, BG < 70 mg/dL within 30 min at current RoC, and d) BG ≤ 300 mg/dL, RoC > +1.5 mg/dL/min, BG > 250 mg/dL within 30 min at current RoC |
| Comparator sample type | Capillary samples |
| Comparator bias | Bias ≤2.1% across measuring range vs. method (or material) of higher metrological order,[23] retrospective correction encouraged even if criterion is met |
| Comparator imprecision | CV ≤2.4% across measuring range [23] |
| Comparator replicates | ≥2, to assess and reduce imprecision [27] and to detect outliers in participant samples |

BG: blood glucose concentration, CGM: continuous glucose monitoring, CV: coefficient of Variation, IFCC WG-CGM: Working Group on Continuous Glucose Monitoring of the International Federation of Clinical Chemistry and Laboratory Medicine, RoC: rate of change

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**Abbreviations**

| | |
|---|---|
| AR | Agreement rate |
| BGMS | Blood glucose monitoring system |
| C-APS | Analytical performance specifications for comparators |
| CBP | Cobas pro |
| CGM | Continuous glucose monitoring |
| CLSI | Clinical and Laboratory Standards Institute |
| CNX | Contour Next |
| CV | Coefficient of variation |
| FDA | Food and Drug Administration |
| IDMS | Isotope dilution-mass spectrometry |
| IFCC WG-CGM | Working Group on Continuous Glucose Monitoring of the International Federation of Clinical Chemistry and Laboratory Medicine |
| INT | Cobas Integra 400 plus |
| MARD | Mean absolute relative difference |
| POC | Point-of-care |
| SD | Standard deviation |
| SMBG | Self-monitoring of blood glucose |
| YSI | YSI 300 STAT Plus |

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Review Article

# Inflammation: The Mother of All Diseases Meets the Mother of All Therapies

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**Keywords**

Incretins, GLP-1, peptide therapeutics, weight loss, Type-2 diabetes, inflammation, GLP-1 side effects, obesity

**Abstract**

**Introduction:** Incretins are small peptides secreted by the gastrointestinal tract. These peptides exert their action by binding to G-protein-coupled receptors that are widely distributed in the pancreas, throughout the gastrointestinal tract, and the brain. The physiological role of incretins (such as GLP-1) is to regulate glucose levels by increasing insulin secretion, delaying gastric emptying, and decreasing appetite, leading to weight loss.

**Method**: In this review, we aimed to report the effects of inflammation on human health and how GLP-1 and GLP-1 receptor agonists, which are now being used as first-line agents to control obesity, can have a broader effect on human diseases.

**Results**: The literature shows the benefits of these drugs in diseases other than obesity, including in diseases of many organs such as heart, kidneys, liver, blood vessels, and in neurodegenerative and psychiatric conditions. These diverse beneficial effects are attributed to the anti-inflammatory activities of these new drugs.

**Conclusions**: The physiological actions of incretins have recently been better understood. The surprisingly diverse therapeutic activities of this class of new drugs suggest that they will likely play central roles not only in the management of type-2 diabetes but for the treatment of obesity and a wide spectrum of diseases for which inflammation is a major factor.

## Introduction

Inflammation is an immune response to harmful stimuli, such as pathogens, damaged cells, or irritants. Normally, it is a protective mechanism that aims to eliminate the cause of injury, clear out dead cells, and initiate tissue repair [1–3]. However, when acute inflammation becomes chronic, it can cause severe organ dysfunction. Inflammation has been known to humans for about 2 millennia through basic observation; some of the most frequent signs and symptoms (such as pain, heat, redness, and swelling) are usually visible on the skin [1].

Among the 10 leading causes of death of US citizens, 9 of them are associated with chronic inflammation [2]. Chronic inflammation is also responsible for significant patient morbidity, due to malfunction or loss of function of vital organs. Table 1 summarizes the 10 most common causes of death in the US [2]. All these diseases have been previously linked to chronic inflammation (accidents are not related to inflammation or disease).

**Table 1**: Leading causes of death of US citizens. Modified from Ahmad et al., 2023.

| Cause of death in the US in Year 2022 | Number of deaths annually 2022 |
|---|---|
| Heart disease | 702,880 |
| Cancer | 608,371 |
| Accidents (unintentional injury) | 227,039 |
| COVID-19 | 186,552 |
| Stroke (cerebrovascular disease) | 165,393 |
| Chronic lower respiratory disease | 147,382 |
| Alzheimer's disease | 120,122 |
| Diabetes | 101,209 |
| Nephritis and related diseases | 57,937 |
| Chronic liver disease and cirrhosis | 54,803 |

During an injury of any cause to the body, an immediate response takes place that involves immune cells, blood vessels, and molecular mediators. Key immune cells include neutrophils and macrophages, and small molecules include cytokines (e.g. TNF-alpha, IL-1, IL-6), chemokines (e.g. CXCL-8), prostaglandins, leukotrienes, and histamine [3]. Among the most important cells in inflammation are macrophages, whose role in the tissues is to clean up debris and produce growth factors under physiological conditions [3]. During tissue inflammation, tissue-resident macrophages secrete cytokines and chemokines and recruit other cells, such as monocytes, from the general circulation. More details on the pathobiology of inflammation can be found in these reviews [3–5].

The prototype of known and extensively studied chronic inflammatory diseases are autoimmune diseases. These are conditions where the immune system mistakenly attacks the body's own tissues, leading to multi-organ symptoms [6]. Some of the best-known chronic inflammatory diseases are shown in Table 2.

**Table 2:** Examples of chronic inflammatory diseases due to autoimmunity.

| Autoimmune Disease | Cardinal Features | Inflammatory markers/autoantibodies |
|---|---|---|
| Rheumatoid Arthritis | Chronic inflammation of the joints leading to pain, swelling, and eventual joint destruction. | Rheumatoid factor, Anti-citrullinated protein antibodies, anti-carbamylated protein antibodies [7]. |
| Systemic Lupus Erythematosus | A multisystem disease with symptoms affecting the skin, joints, kidneys, and other organs. | Anti-double-stranded DNA antibodies, anti-Smith antibodies, anti-ribonucleoprotein, anti-Ro/SSA, anti-La/SSB, antinuclear antibodies [8]. |
| Multiple Sclerosis | Involves inflammation and damage to the myelin sheath of nerve fibers. | Increased Immunoglobulin G antibodies, pro-inflammatory cytokines [9]. |
| Inflammatory Bowel Diseases | These are chronic inflammatory conditions of the gastrointestinal tract such as Crohn's Disease which can affect any part of the gastrointestinal tract causing deep ulcers and inflammation. Ulcerative Colitis primarily affects the colon and rectum, leading to superficial inflammation and ulcers. | Perinuclear anti-neutrophil cytoplasmic antibodies,anti-*Saccharomyces cerevisiae antibodies*, anti-chitobioside carbohydrate antibodies, pancreatic antibodies, anti-glycan antibodies etc. [10]. |
| Vascular inflammatory disease | These are associated with inflammation affecting blood vessels such as Vasculitis. The latter causes inflammation of blood vessels that can lead to organ damage. | Anti-neutrophil cytoplasmic antibodies, anti-proteinase-3 antibodies, C-reactive protein, interleukin-6, white blood cell count, erythrocyte sedimentation rate etc. [11,12]. |

Diagnosis of inflammatory diseases involves a combination of clinical evaluation, laboratory tests (e.g. C-reactive protein, acute-phase reactants, erythrocyte sedimentation rate, white cell counts), and more recently, imaging studies (e.g., MRI, CT scans), and sometimes tissue biopsies. Acute inflammation (lasting hours or days) is usually managed with anti-inflammatory medications (e.g., NSAIDs, (nonsteroidal anti-inflammatory drugs such as ibuprofen), antibiotics for infections, and other supportive measures).

The treatment of chronic inflammation (lasting weeks to years) focuses on controlling inflammation and its underlying causes with medications such as corticosteroids, disease-modifying antirheumatic drugs (DMARDs), and more recently with an ever-expanding list of biologics such as monoclonal antibodies against TNF (e.g. https://www.humira.com/) and cytokines. Lifestyle changes and physical therapy may also be recommended. Among the lifestyle and preventive measures are anti-inflammatory diets rich in omega-3 fatty acids, antioxidants, and fiber. Additional measures include exercise, stress management, and avoidance of irritants such as environmental toxins, and smoking cessation. Managing chronic infections can help mitigate inflammation.
The most common human organs that are affected by inflammation include the heart, pancreas, liver, kidney, lung, brain, intestinal tract, and the reproductive system [13]. If the chronic inflammation persists for long periods, the afflicted organs are damaged and eventually become non-functional.

**Neuroinflammation**

For many years it was believed that the brain is immune-privileged [14]. When the brain is challenged by non-self antigens or other stimuli, its response is attenuated. It has now been realized that the central nervous system (CNS) does show local inflammation in response to various stimuli, including infections, neurodegeneration, trauma, environmental toxins, and metabolic disturbances [15]. Neuroinflammation is a complex biological response of the CNS to various forms of injury, infection, or disease. It involves the activation of glial cells, (microglia and astrocytes), which play critical roles in maintaining homeostasis and responding to pathological changes, as well as leukocytes [16,17]. While neuroinflammation is a protective mechanism, chronic or excessive inflammation can lead to detrimental effects on neuronal function and health, contributing to a range of neurological and psychiatric disorders [18,19]. The consequences of prolonged neuroinflammation include neuronal damage, Blood-Brain Barrier disruption and cognitive decline, potentially through mechanisms such as synaptic loss, neuronal death and altered neurotransmitter signaling [17].

**Therapeutic Implications**

Given the role of neuroinflammation in many harmful processes, targeting neuroinflammatory pathways presents a potential therapeutic strategy. Approaches include anti-inflammatory agents, immunomodulation (representing therapies that modulate the immune response, such as monoclonal antibodies targeting specific cytokines and receptors, including agonists

and blockers), neuroprotective agents such as neurotropic factors, and lifestyle interventions such as exercise and stress management.

Our motivation for preparing this review originated from the development of a relatively new class of therapeutics, generally known as incretins, and their analogs, which were initially intended to treat type-2 diabetes (T2D) [20]. It was quickly realized that these drugs cause marked reduction in body weight and were thus also deployed for weight loss. Clinical trials worldwide suggest that these drugs can show efficacy in a surprisingly long list of seemingly unrelated diseases (see Table 3). These small peptides interact with receptors that are found throughout the body, including the brain, suggesting that they could target diverse tissues, promoting, among other activities, glycemic control, loss of appetite, and slow gastric emptying [21]. It has been speculated that the pleiotropic activities of these therapeutics are due to their ability to decrease inflammation [20]. While the pathophysiological aspects of these drugs are undoubtedly very interesting, we will briefly describe below a long list of diseases that seem to phenomenologically respond favorably and at various degrees, to these agents. These drugs may represent a new class of compounds with potent anti-inflammatory action, by extrapolation, this could be beneficial in diseases where chronic inflammation is a key characteristic.

**Table 3:** List of human diseases that have been studied for potential treatment using incretins as therapeutic agents.

| Condition | Incretin-like drug (dose, mode, participants) | Study name, Duration | Findings |
|---|---|---|---|
| **Obesity** | Semaglutide (2.4mg/w[1], SC[3], n=803) Liraglutide (3mg/d[2], SC, n=338) Tirzepatide (10mg or 15mg/w, SC, n=670) | STEP 4 (20w), STEP 8 (68w), SURMOUNT-4 (36w + 52w), | Reduction of weight [22–24] |
| **Obstructive sleep apnea** | Tirzepatide (10mg or 15mg/w, SC, n=469) | SURMOUNT-OSA (52w) | Reduced severity, improved sleep-related outcomes [25] |
| **Adverse cardiovascular outcomes** | Semaglutide (3mg, 7mg, 14mg/d [0-4w, 4-8w, 9-82w], oral, n=3183) | PIONEER 6 (82w) | Reduced risk of cardiovascular outcomes [26] |
| **Non-alcoholic streatohepatitis** | Semaglutide (0.1, 0.2 or 0.4mg/d, SC, n=320) Tirzepatide (5mg, 10mg or 15mg/w, SC, n=190) | NCT02970942 (72w) SYNERGY-NASH (52w) | Disease resolution [27,28] |
| **Liver cirrhosis** | Exenatide, Dulaglutide, Liraglutide, Semaglutide (variable doses, unknown, n=16058) | In silico study, data: VHA database (2006-2022) | Reduced risk of cirrhosis [29] |
| **Myocardial infarction** | Semaglutide (2.4mg/w, SC, n= 17604) | SELECT (240w) | Reduced risk of death from disease [30] |
| **Opioid use disorder** | Semaglutide (variable doses, unknown, n=33006) | In silico study, data: TriNetX Analytics Platform (49w) | Reduced craving and opioid overdose [31] |
| **Depression** | Exenatide (2mg/w, SC, n=491) | DURATION-2(26w) | Reduced feelings of depression [32] |
| **Stroke** | Semaglutide (2.4mg/w, SC, n= 17604) | SELECT (240w) | Reduced risk of death from stroke [30,33] |
| **Atherosclerosis** | Tirzepatide (5mg,10mg or 15mg/w, SC, n=2539) | SURMOUNT-1 (72w) | Reduced risk of disease [34] |
| **Addiction (alcohol, smoking)** | Semaglutide (variable doses, unknown, n=222942) Exenatide (2mg/w, SC, n=127) | In silico study (2017-2023) NCT03232112 (26w) | Reduced risk of addiction, improved abstinence [33,35,36] |

| Condition | Incretin-like drug (dose, mode, participants) | Study name, Duration | Findings |
|---|---|---|---|
| **Suicidal tendencies** | Semaglutide (variable doses, oral & SC, n=65176) | In silico study, data: TriNetX US Collaborative Network (2017-2021) | Reduced risk of suicidal ideation [33] |
| **Alzheimer's disease** | Liraglutide (1.8mg/d, SC, n=206)<br>Semaglutide (14mg/d, oral, n=1840) | ELAD (52w)<br>EVOKE (173w)- Ongoing | Slowed cognitive decline, reduced brain shrinkage [37,38] |
| **Dementia** | Semaglutide (variable doses, oral & SC, n=65176) | In silico study, data: TriNetX US Collaborative Network (2017-2021) | Reduced risk of dementia [33] |
| **Parkinson's disease** | Exenatide (2mg/w, SC, n=62) | NCT01971242 (48w) | Improved motor function [39] |
| **Kidney disease** | Semaglutide (1mg/w, SC, n=3533) | FLOW (156w or 265w) | Reduced risk of disease [40] |
| **Polycystic ovary syndrome** | Exenatide (Varying amounts, unknown, n=785) | Meta-analysis (12w-25w) | Increased pregnancy rate [41] |
| **Female infertility** | Exenatide (Varying amounts, unknown, n=785) | Meta-analysis (12w-25w) | Improved measurements [41] |
| **Male infertility** | Liraglutide (1.20-1.80mg/kg, SC, n=42) | Non-human study (42 days) | Improved sperm characteristics (mice) [42] |

[1] per week

[2] per day

[3] subcutaneous

**Incretins and Their Therapeutic Potential**

Incretins are a group of hormones released by the gut in response to food intake. They play a vital role in regulating glucose metabolism by enhancing insulin secretion, inhibiting glucagon release, and promoting satiety [21]. The two primary incretin hormones are glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP). Understanding the physiological roles of incretins has led to significant advancements in diabetes treatment and weight management. Details about the structure of incretins can be found elsewhere [6].

Incretins exert their effects primarily through the following mechanisms [21]:

1. Insulin secretion: In response to meals, GLP-1 and GIP stimulate pancreatic beta cells to release insulin. This response is glucose-dependent, which means that insulin secretion is enhanced when blood glucose levels are elevated.
2. Glucagon suppression: GLP-1 inhibits glucagon secretion from pancreatic alpha cells, which helps lower blood glucose levels by reducing hepatic glucose production.
3. Gastric emptying: Incretins slow gastric emptying, leading to a more gradual release of glucose into the bloodstream, which helps prevent spikes in blood glucose levels.
4. Satiety and weight management: GLP-1 promotes feelings of fullness, reducing appetite and caloric intake, characteristics that are beneficial for weight management.

**Therapeutic Applications of Incretin-Like Peptides**

The unique properties of incretins have led to the development of several therapeutic agents, primarily for the treatment of T2D and obesity [25]. The diverse therapeutic benefits of incretins are attributed to their anti-inflammatory actions, in addition to optimal management of diabetes and weight loss [21]. The GLP-1 receptor is a G protein-coupled receptor (GPCR) found in beta cells of the pancreas, neurons, stomach, duodenum, lung, and hypothalamus [21].

GLP-1 receptor agonists are synthetic analogs of GLP-1 that mimic its effects and have already been successfully used in the treatment of T2D [26]. Notable examples of GLP-1 agonists include liraglutide (used for both diabetes and weight loss management), and semaglutide (marketed as Ozempic) which demonstrates significant weight reduction and glycemic control, with some formulations approved specifically for weight management (e.g. Wegovy) [23].

Dipeptidyl Peptidase-4 (DPP-4) is an enzyme that inactivates GLP-1-like peptides, thus decreasing their long-term efficacy [20]. DPP-4 inhibitors (e.g., sitagliptin, saxagliptin) enhance endogenous incretin activity, by inhibiting the enzyme that breaks down GLP-1 and GIP [43]. These medications help improve glycemic control with a lower risk of hypoglycemia compared to traditional therapies.

Recent studies are also exploring the broader therapeutic potential of incretin-based therapies. Several combination therapies (more than one incretin peptide; with or without other

diabetes drugs) are now in clinical trials [44]. Ongoing research continues to uncover the full therapeutic potential of incretins, offering hope for improved treatment strategies in metabolic disorders and beyond [45].

Any list of candidate diseases that could benefit from these agents is bound to be incomplete, given the extraordinary number of ongoing clinical trials and the possibility of combining the new agents together or other conventional therapeutics. Additionally, the already reported efficacies and side effects need to be independently verified to avoid false discovery and false hopes, especially for serious diseases that currently do not have effective therapies (such as neurodegeneration).

To recognize the importance of these new agents as therapeutics, the prestigious 2024 Lasker-DeBakey Clinical Medical Research Award has been presented to Svetlana Mojsov, PhD, Joel Habener, MD, and Lotte Bjerre Knudsen, DMSc, for the discovery of GLP-1 for the treatment of obesity [46]. The Lasker Award is considered a reliable prodromal to the Nobel Prize.

## Future Directions

The incretins as pharmacological agents have been highly successful for the treatment of diabetes and weight control. Preliminary evidence from small clinical trials suggests a much broader therapeutic benefit, for diseases that did not seem to be related to the pathogenesis of diabetes or obesity. A hypothesis that could explain this surprising finding evolves around the central role of inflammation in a myriad of diseases and the ability of incretins to reduce it.

The future of GLP-1 and related molecules is undoubtedly very bright. New derivatives and combinations will likely lead to even more effective and safer formulations that will have more lasting effects. This is currently an important limitation since it has been shown that drug withdrawal leads to regaining most of the lost weight, and sustained therapy interventions are needed to maintain benefits [47]. Long-term trials have also reported dropout rates, often due to adverse effects and tolerability issue; guidelines to minimize occurrence and severity have been suggested, as well as ways to mitigate the adverse effects after they have appeared [48]. The expected decrease in production costs will catalyze their even more widespread use for diseases that currently have no treatments. The completion of large, multicenter placebo-controlled prospective clinical trials will shed more light on the optimal use of these agents.

## Declarations

### Authors' disclosures

The authors have no conflicts to report.

### Authors' contributions

MKC drafted and edited the manuscript and prepared Tables 2 and 3. EPD conceptualized, drafted the manuscript, and prepared Table 1.

### Ethical approval and human subject authorization

Not applicable.

### Experimental animals authorization

Not applicable.

### Informed consent

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### Non-standard abbreviations

CNS, central nervous system; DPP-4, Dipeptidyl Peptidase-4; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide 1; GPCR, G protein-coupled receptor; NSAIDs, nonsteroidal anti-inflammatory drugs; DMARDs, disease-modifying antirheumatic drugs; T2D, Type-2 diabetes; VHA, Veterans Health Administration.

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Review Article

# The Role of Adiponectin in Diabetic Peripheral Neuropathy: A Systematic Review and Meta-Analysis

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**Keywords**

Diabetic Peripheral Neuropathy, Adiponectin, Systematic Review, Meta-Analysis, Diabetes Mellitus, Neuropathic complications, Diagnostic markers

**Abstract**

**Background**: Diabetic peripheral neuropathy (DPN) is a prevalent microvascular consequence of diabetes with a complex etiology. Adiponectin, an adipokine with anti-inflammatory and neuroprotective properties, has been implicated in DPN, but its significance remains unclear due to conflicting findings. The objective of this systematic review is to assess the association between circulating adiponectin levels and the risk of DPN in individuals with diabetes.

**Methods**: We did a systematic literature search in PubMed, Scopus, and CINAHL for studies investigating adiponectin levels in diabetes patients with and without DPN. A meta-analysis was done to evaluate the pooled mean difference in adiponectin levels between patients and controls. Study quality was rated using the Joanna Briggs Institute's critical appraisal tool.

**Results**: The systematic review comprised 13 studies with 3,337 participants. Meta-analysis of 4 studies (920 participants) indicated no significant difference in adiponectin levels between DPN patients (n=418) and controls (n=502) (pooled mean difference 0.01, 95% CI: -0.24 to 0.26, p=0.94), with strong heterogeneity ($I^2$=59%). Subgroup analyses were not possible due to inadequate data. Risk of bias was generally low, with 7 studies graded as good quality.

**Conclusions**: Our findings imply that circulating adiponectin levels are not linked with the risk of DPN in diabetes. However, the substantial heterogeneity among studies underscores the need for more well-designed prospective studies to explain the role of adiponectin in DPN etiology.

## Introduction

Diabetes is a debilitating metabolic disorder associated with both macro and microvascular complications. Long-term microvascular complications of diabetes include peripheral neuropathy which is clinically impacted with numbness and lack of sensation thereby resulting in poor quality of life [1].

The global prevalence of diabetes is indeed a significant health concern, which is estimated to be around 9.3% [2]. Furthermore, it is alarming that approximately 40.3% of diabetic patients develop Diabetic Neuropathy (DNP), highlighting the serious impact of diabetes on nerve health and overall well-being [3].
The lack of sensation in Diabetic Neuropathy (DNP) is a significant concern because it can lead to unnoticed peripheral injuries, particularly in the feet, ultimately resulting in foot ulcers. If not properly managed, these ulcers can become severe and may ultimately necessitate amputation.

The various mechanisms involved in the pathogenesis of DM include hyperglycemia, insulin resistance, oxidative stress, and inflammation. Adiponectin is an adipokine involved in various metabolic and physiological function [4]. Insulin sensitivity, inflammation, energy regulation, and lipid metabolism are all impacted by adiponectin [5]. Both beta cells and immune cells express adiponectin receptors, thereby indicating its potential to influence immunological activity in diabetes[6]. The anti-inflammatory role of adiponectin is found to improve insulin sensitivity, which is suggestive of its role in delaying the progression of diabetes.

Moreover, adiponectin exhibits neuroprotective effects in various neurological disorders such as Alzheimer's, depression, and stroke [7]. In DNP, the neurons that are affected are predominantly sensory in nature [8]. Interestingly, adiponectin pathway activation is observed to be neuroprotective for somatosensory neurons in diabetic animal models, suggesting that it may play a role in reducing the neuropathy associated with diabetes [9,10].

The literature presents inconsistencies regarding the significance of adiponectin in relation to its serum level in DNP. Some studies have reported elevated blood adiponectin levels in DNP patients whereas others have shown either reduced or no discernible difference, suggesting a more nuanced relationship that needs additional study. In fact, because of inconsistent research results, the function of adiponectin in diabetic neuropathy (DNP) is still up for discussion. This variation emphasizes the need for a more thorough investigation to determine the specific role of adiponectin in DNP.
The primary objective of this systematic review is to evaluate the association between circulating adiponectin levels and the risk of DNP in diabetic patients. In this study, both systematic review and meta-analysis were performed to establish the association between serum adiponectin levels and the risk of DPN.

## Methods

The systematic review followed the guidelines outlined by PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses). The protocol was registered on PROSPERO (Registration ID: CRD42024504656).

### Selection Procedure

The objective of this systematic review and meta-analysis was to evaluate the association between adiponectin levels and diabetic peripheral neuropathy. The inclusion criteria were as follows: 1. Observational studies (cohort studies, case-control studies, cross-sectional studies) investigating the association between adiponectin levels and diabetic peripheral neuropathy, 2. Studies involving participants diagnosed with diabetic peripheral neuropathy, confirmed through appropriate clinical assessments, neuropathy scoring systems, nerve conduction studies, or other established diagnostic methods as specified in individual studies; 3. Studies reporting adiponectin levels (measured in blood/serum/plasma) in diabetic patients with and without peripheral neuropathy or compared to healthy controls, 4. Studies providing data to calculate measures of effect (e.g. mean differences, odds ratios) and associated measures of variance, 5. Studies published in the English language. The exclusion criteria were: 1. Reviews, case reports, editorials, letters, and commentaries, 2. Animal or preclinical studies, 3. Studies not assessing or reporting adiponectin levels in relation to diabetic peripheral neuropathy; 4. Studies with insufficient data to extract adiponectin levels or effect sizes, 5. Duplicate publications or studies with overlapping datasets.

### Search Strategy

A comprehensive literature search was conducted in PubMed, Scopus, and CINAHL databases from inception to March 2024 to identify relevant studies. Our search strategy utilized a combination of keywords related to "adiponectin" and "diabetic neuropathy" (Table 1). Additionally, bibliographies of included studies were manually screened for any missed relevant citations. Only studies published in the English language were considered.

**Table 1:** Search strategy.

| Database | Search Terms |
|---|---|
| **Pubmed** | (“adiponectin”[MeSH Terms] OR “adiponectin”[All Fields] OR “adiponectin s”[All Fields] OR “adiponectine”[All Fields] OR “adiponectins”[All Fields] OR “AdipoQ”[All Fields] OR “Acrp30”[All Fields]) AND (“diabetic neuropathies”[MeSH Terms] OR (“diabetic”[All Fields] AND “neuropathies”[All Fields]) OR “diabetic neuropathies”[All Fields] OR (“diabetic”[All Fields] AND “neuropathy”[All Fields]) OR “diabetic neuropathy”[All Fields] OR ((“diabete”[All Fields] OR “diabetes mellitus”[MeSH Terms] OR (“diabetes”[All Fields] AND “mellitus”[All Fields]) OR “diabetes mellitus”[All Fields] OR “diabetes”[All Fields] OR “diabetes insipidus”[MeSH Terms] OR (“diabetes”[All Fields] AND “insipidus”[All Fields]) OR “diabetes insipidus”[All Fields] OR “diabetic”[All Fields] OR “diabetics”[All Fields] OR “diabets”[All Fields]) AND (“peripheral nervous system diseases”[MeSH Terms] OR (“peripheral”[All Fields] AND “nervous”[All Fields] AND “system”[All Fields] AND “diseases”[All Fields]) OR “peripheral nervous system diseases”[All Fields] OR (“peripheral”[All Fields] AND “neuropathy”[All Fields]) OR “peripheral neuropathy”[All Fields])))  |
| **Scopus** | ( TITLE-ABS-KEY-AUTH ( adiponectin OR adipoq OR acrp30 ) AND TITLE-ABS-KEY-AUTH ( diabetic AND neuropathy OR diabetic AND peripheral AND neuropathy ) ) |
| **CINAHL** | Boolean/Phrase: ( adiponectin OR adipoq OR acrp30 ) AND ( diabetic AND neuropathy OR diabetic AND peripheral AND neuropathy ) ; Expanders: Apply equivalent subjects; Language: English |

### Screening and Data Extraction

Studies identified from the database searches underwent an initial screening by two independent reviewers based on titles and abstracts. Potentially relevant studies then had their full texts reviewed for eligibility using the CADIMA tool version 2.2.3. Any disagreements were resolved by consultation with a third reviewer.

Data from the included studies was extracted by two independent reviewers into a standardized form, with discrepancies resolved by consensus or a third reviewer. Extracted data included participant demographics, study characteristics, adiponectin levels, and outcomes related to diabetic peripheral neuropathy.

### Quality Assessment

The quality of included studies and risk of bias were evaluated using the Joanna Briggs Institute’s critical appraisal checklist for cross-sectional studies. A risk of bias summary and graph were generated using RevMan 5.4 software.

### Data Analysis

Meta-analysis was performed using RevMan 5.4 software. For dichotomous outcomes, risk ratios (RR) with 95% confidence intervals (CI) were calculated. Continuous outcomes were pooled and expressed as mean differences (MD) using the inverse variance method and a random-effects model with 95% CIs.

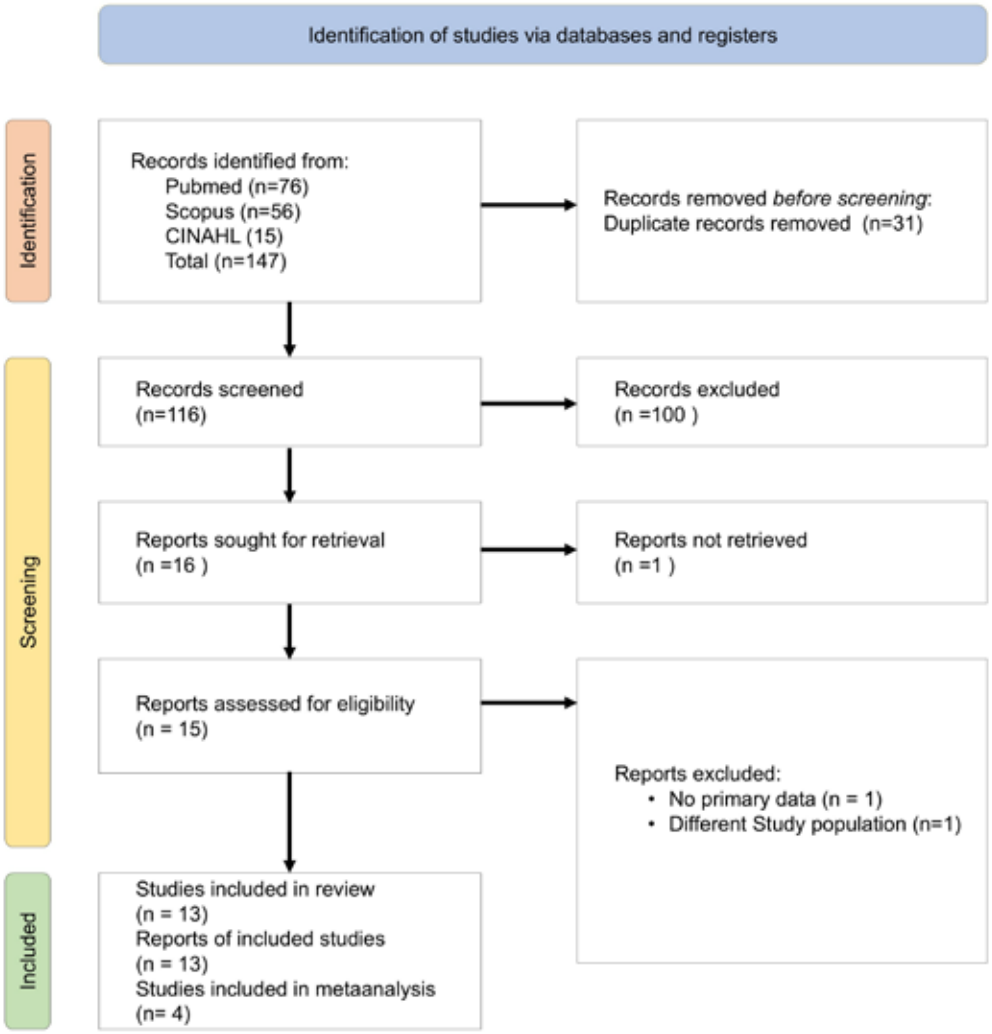
Heterogeneity across studies was assessed using Cochran’s Q statistic and the $I^2$ index. Based on heterogeneity levels, either a fixed-effect or random-effects model was employed. Subgroup analyses were not performed due to lack of sufficient data. Sensitivity analysis was performed using R software.

## Results

### Study Selection

The database searches initially identified 147 records and finally 116 records were screened after removing duplicates. Following screening of titles and abstracts, 15 articles underwent full-text review. Finally, 13 studies which met the eligibility criteria were included in the systematic review and 4 studies were included in meta-analysis. The study selection process is summarized in the PRISMA flow diagram (Figure 1).

**Figure 1:** PRISMA flow chart.




PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses.

**Study Characteristics**

The basic characteristics of the 13 included studies are presented in Table 1. The studies were conducted across various countries including China, India, Japan, Korea, Spain, and Germany. The total sample size was 3,337 participants.

The included studies were cross-sectional (n=10), case-control (n=2), randomized controlled trial (n=1), and an experimental study (n=1). The age of participants in the included studies ranged from 39.7 to 72 years. The duration of diabetes ranged from 1 to 20 years across studies. Diagnosis of diabetic peripheral neuropathy was established using different methods such as neuropathy symptom scores, neurological examinations, nerve conduction studies, current perception threshold testing, and vibratory perception threshold. (Table 2)

**Adiponectin Levels**

Table 3 summarizes the reported adiponectin levels in cases (diabetic peripheral neuropathy) and controls (non-neuropathy or healthy) across the included studies. While some studies found significantly higher adiponectin levels in neuropathy cases compared to controls, others reported no difference or lower levels in cases.

**Table 1**: Basic characteristics of included studies.

| Study | Country | Population | Duration of diabetes | Diabetic Peripheral Neuropathy score/Nerve conduction study |
|---|---|---|---|---|
| Qing Sun et al, 2020 [11] | China | Chinese patients with type 2 diabetes | Median 10 years for non-DPN group, 16 years for DPN group | Not reported |
| Mohanraj PS et al, 2024 [12] | India | Individuals aged 35 to 65 years with type 2 diabetes mellitus | 1 to 20 years | Neuropathy symptom score (NSS), diabetes neuropathy examination (DNE) score, and nerve conduction studies |
| Zhi-Yong Ji et al, 2015 [13] | China | Type 2 diabetes patients with and without diabetic peripheral neuropathy, and healthy controls. | Not reported | Patients with diabetic peripheral neuropathy had clinical manifestations of acroparesthesia or motor nerve involvement, reduced degree of deep and superficial sensation, and reduced sensory nerve conduction velocity (SCV) and motor nerve conduction velocity (MNCV). |
| Ken Satoh et al, 2023 [14] | Japan | Patients with type 2 diabetes | Reported in years (Median values - Non-obese group: 5 years, Obese group: 8 years) | Not reported |
| R. Pradeepa et al., 2014 [15] | India | South Indian type 2 diabetic subjects | Reported in years (Without microvascular complications: 3.5 ± 4.5 years, With microvascular complications: 6.4 ± 6.2 years) | Neuropathy was diagnosed if vibratory perception threshold (VPT) of the great toe using biothesiometry exceeded ≥20 V. |
| Chan-Hee Jung et al, 2014 [16] | Korea | Patients with type 2 diabetes | 6.2 ± 5.2 years | Diabetic peripheral neuropathy was diagnosed using the Michigan Neuropathy Screening Instrument, neurological screening examinations, and current perception threshold test. |

| Study | Country | Population | Duration of diabetes | Diabetic Peripheral Neuropathy score/Nerve conduction study |
|---|---|---|---|---|
| J. M. González-Clemente et al, 2005 [17] | Spain | Subjects with type 1 diabetes mellitus | 14 years | Not reported |
| H. Usta Atmaca et al, 2017 [18] | Not reported | Type 2 diabetes mellitus patients with peripheral neuropathy | Not reported | Patients were diagnosed with diabetic neuropathy using electromyography (EMG) |
| Chan-Hee Jung et al., 2012 [19] | Korea | Type 2 diabetic patients | 6.1 ± 5.0 years (mean ± SD) | Not reported |
| Christophe E.M. De Block et al, 2005 [20] | Not reported | Type 1 diabetic patients without nephropathy | 19 ± 11 years | Electromyographic examination with nerve conduction velocity tests, including motor (peroneal and tibial) and sensory (sural) nerves, H-reflexes, and F-waves. Neuropathy severity was graded. |
| C. Herder et al, 2015 [21] | Germany | People aged 61-82 years with Type 2 diabetes from the population-based KORA F4 study. | Median duration since diagnosis was 3 years for those without polyneuropathy and 8 years for those with polyneuropathy. | Not reported. The presence of clinical diabetic sensorimotor polyneuropathy was defined as bilateral impairment of foot vibration perception and/or foot pressure sensation. |
| Umapathy Dhamodharan et al, 2015 [22] | Not reported | T2DM subjects with and without diabetic foot ulcer | Not reported | Not reported |
| Ágnes Molnár et al, 2022 [23] | Not reported | Type 2 diabetes mellitus patients with distal sensory polyneuropathy | 10.3 ± 3.7 years (patients with neuropathy), 10.9 ± 4.1 years (controls) | Current perception threshold (CPT) measured by Neurometer® |

*Data are presented as mean ± SD or median (range).

**Table 2**: Details of the included Study.

| Study | Sample size | Mean Age, years | Gender distribution | Design |
|---|---|---|---|---|
| Qing Sun et al, 2020 [11] | 219 total, with 98 cases and 121 controls | Median 58 years for non-DPN group, 62.5 years for DPN group | 48.8% male in non-DPN group, 43.9% male in DPN group | Cross-sectional study |
| Mohanraj PS et al, 2024 [12] | 86 participants, with 43 cases and 43 controls | Cases (with neuropathy): 54.5 ± 11 years Controls (without neuropathy): 53.1 ± 8.7 years | Cases: 23 females (53%) Controls: 19 females (44%) | Cross-sectional study |
| Zhi-Yong Ji et al, 2015 [13] | With 90 Cases (DPN group) & 90 Controls (NDPN group), and 90 healthy controls (NC group). | DPN group: 54.1 ± 5.6 years, NDPN group: 54.9 ± 5.1 years, NC group: 53.5 ± 5.0 years. | DPN group: 46 males, 44 females. NDPN group: 50 males, 40 females. NC group: 40 males, 50 females. | Case-control study. |
| Ken Satoh et al, 2023 [14] | 94 patients (197 non-obese, 197 obese) | Not reported (Median age - Non-obese group: 55 years, Obese group: 54 years) | Non-obese group - 98 males, 99 females; Obese group - 104 males, 93 females | Cross-sectional study |
| R. Pradeepa et al., 2014 [15] | Total 487 diabetic subjects (With cases of microvascular complications: 266, Without microvascular complications: 221) | Without microvascular complications: 45.7 ± 9.5 years, With microvascular complications: 54.7 ± 11.1 years | Not explicitly reported | Cross-sectional study |
| Chan-Hee Jung et al, 2014 [16] | With Cases & Controls: 153 total patients, 87 with neuropathy (cases), 66 without neuropathy (controls) | 52.5 ± 10.0 years | 100 men (65.4%), 53 women (34.6%) | Cross-sectional study |
| J. M. González-Clemente et al, 2005 [17] | With Cases = 36, Controls = 84 | With DPN = 28.03 ± 7.15, Without DPN = 27.17 ± 6.35 | 38.9% women in DPN group, 54.8% women in non-DPN group | Cross-sectional study |
| H. Usta Atmaca et al, 2017 [18] | 23 cases with type 2 diabetes and peripheral neuropathy, 21 healthy controls | 56.1 ± 6.6 years for cases, 36.3 ± 7.5 years for controls | Cases - 20 females, 3 males; Controls - 20 females, 1 male | Randomized, double-blind, placebo-controlled, prospective study |

| Study | Sample size | Mean Age, years | Gender distribution | Design |
|---|---|---|---|---|
| Chan-Hee Jung et al., 2012 [19] | Total 142 patients (94 males, 48 females), with cases of cardiac autonomic neuropathy (CAN) | 52.4 ± 10.0 years | 94 males (66.2%), 48 females (33.8%) | Cross-sectional study |
| Christophe E.M. De Block et al, 2005 [20] | 592 (Cases and controls not specified) | 41 ± 12 years | 324 men, 268 women | Cross-sectional study |
| C. Herder et al, 2015 [21] | 47 cases with polyneuropathy, 168 controls without polyneuropathy. | Around 71-72 years in both groups. | 59% male in those without polyneuropathy, 66% male in those with polyneuropathy. | Cross-sectional study. |
| Umapathy Dhamodharan et al, 2015 [22] | Sample Size:<br><br>Total = 515<br>NGT/Control = 106<br>T2DM without DFU = 139<br>T2DM with neuropathic DFU (DFU-DN) = 191<br>T2DM with PVD (DFU-PVD) = 79 | NGT = 39.7 ± 8.4<br>T2DM = 49.0 ± 9.9<br>DFU-DN = 58.6 ± 8.5<br>DFU-PVD = 59.5 ± 8.5 | NGT (M/F) = 56/50<br>T2DM (M/F) = 80/59<br>DFU-DN (M/F) = 125/66<br>DFU-PVD (M/F) = 56/23 | Cross-sectional study |
| Ágnes Molnár et al, 2022 [23] | 30 cases with neuropathy, 32 controls without neuropathy | 61.97 ± 8.09 (cases), 64.37 ± 6.52 (controls) | 9 males/21 females (cases), 10 males/22 females (controls) | Experimental study with 6-week aerobic exercise intervention |

**Table 3**: Details of intervention used and Study outcome.

| Study | Comparison group | Adiponectin level | Outcome | Dropout | Measures of DPN |
|---|---|---|---|---|---|
| Qing Sun et al, 2020 [11] | Non-DPN patients | Median 8.13 mg/ml for non-DPN group, 9.63 mg/ml for DPN group | Serum adiponectin levels were positively associated with diabetic peripheral neuropathy. | Not reported | Presence of common DPN symptoms and abnormal neurological screening tests. |
| Mohanraj PS et al, 2024 [12] | Type 2 diabetes mellitus patients without neuropathy | Cases (with neuropathy): 3.3 ± 1.2 µg/mL<br><br>Controls (without neuropathy): 3.6 ± 1.2 µg/mL | No significant difference in adiponectin levels between cases and controls. Adiponectin showed no significant association with diabetic peripheral neuropathy. | Not reported | Neuropathy symptom score (NSS), diabetes neuropathy examination (DNE) score, nerve conduction studies |
| Zhi-Yong Ji et al, 2015 [13] | Non-diabetic peripheral neuropathy (NDPN) group and healthy normal controls (NC group). | Serum levels of adiponectin were markedly reduced in the DPN group compared to NDPN and NC groups. | The study found that the T allele in +45T/G and +276G/T polymorphisms of the adiponectin gene was associated with an elevated risk of diabetic peripheral neuropathy in type 2 diabetes patients, likely by down-regulating adiponectin serum levels. | Not reported. | Clinical manifestations, reduced sensory and motor nerve conduction velocities. |
| Ken Satoh et al, 2023 [14] | Non-obese (BMI 20-25 kg/m2) vs Obese (BMI ≥32 kg/m2) patients with type 2 diabetes | Reported (Median values - Non-obese group: 3.00 µg/mL, Obese group: 2.36 µg/mL) | Association of adiponectin levels with microvascular complications (retinopathy, nephropathy, neuropathy) | Not reported | Defined as presence of two of the following - diminished Achilles tendon reflex, inability to sense vibration, symptoms of distal neuropathy. |
| R. Pradeepa et al., 2014 [15] | Subjects with and without microvascular complications (retinopathy, nephropathy, neuropathy) | Geometric mean reported<br><br>Without microvascular complications: 5.3 µg/mL<br>With microvascular complications: 6.1 µg/mL<br>With diabetic retinopathy: 6.8 µg/mL<br>Without diabetic retinopathy: 5.5 µg/mL<br>With neuropathy: 6.5 µg/mL<br>Without neuropathy: 5.6 µg/mL | Association of serum adiponectin with diabetic microvascular complications (retinopathy, nephropathy, neuropathy) | Not reported | Vibratory perception threshold using biothesiometry |

| Study | Comparison group | Adiponectin level | Outcome | Dropout | Measures of DPN |
|---|---|---|---|---|---|
| Chan-Hee Jung et al, 2014 [16] | Patients without neuropathy | Not reported specifically | The mean levels of adiponectin were significantly higher in patients with neuropathy compared to those without neuropathy. | Not reported | Michigan Neuropathy Screening Instrument, neurological examinations, current perception threshold test |
| J. M. González-Clemente et al, 2005 [17] | Subjects with type 1 diabetes without diabetic neuropathy | Median (IQR)<br>With DPN = 9.19 (7.61-18.53) mg/l<br>Without DPN = 13.34 (10.01-16.23) mg/l | The study found an association between diabetic neuropathy and increased plasma levels of soluble TNF-α receptors (sTNFR1 and sTNFR2), indicating activation of the TNF-α system, independent of glycemic control and cardiovascular risk factors. | Not reported | Peripheral neuropathy assessed by MNSI questionnaire and neurological examination. Cardiovascular autonomic neuropathy assessed by heart rate variability tests. |
| H. Usta Atmaca et al, 2017 [18] | Healthy controls | 9.3 ± 2.7 ng/mL for cases, 11.6 ± 3.4 ng/mL for controls | 600 mg/day alpha lipoic acid treatment for 6 weeks did not improve metabolic parameters or adiponectin levels in type 2 diabetes patients. | Not reported | Electromyography |
| Chan-Hee Jung et al., 2012 [19] | Patients with CAN compared to patients without CAN | With CAN: 4185 ± 3615 ng/mL, Without CAN: 3138 ± 3010 ng/mL | Higher serum adiponectin levels were associated with increased risk for presence of cardiac autonomic neuropathy (CAN). | Not reported | Cardiac autonomic neuropathy assessed by Ewing's protocol and heart rate variability parameters. |
| Christophe E.M. De Block et al, 2005 [20] | Normal-weight (BMI < 25 kg/m2) vs. overweight (BMI ≥ 25 kg/m2) patients | Reported, but no difference between patients with or without neuropathy | Prevalence of retinopathy and neuropathy in overweight vs. normal-weight patients | Not reported | Electromyography with nerve conduction studies |
| C. Herder et al, 2015 [21] | People with Type 2 diabetes without polyneuropathy. | Median total adiponectin level was 6746 ng/ml in those with polyneuropathy and 7894 ng/ml in those without polyneuropathy. | The study investigated the association between serum omentin levels and polyneuropathy. | Not reported. | Clinical assessment of bilateral foot vibration perception and pressure sensation impairment. |
| Umapathy Dhamodharan et al, 2015 [22] | NGT/Control group | NGT: 536.0 (0.1-1787) ng/mL<br>T2DM: 528.6 (6.2-1255) ng/mL<br>DFU-DN: 524.0 (63.3-1641) ng/mL<br>DFU-PVD: 453.5 (164.9-1078) ng/mL | Association of IL-6, TNF-α and SDF-1 polymorphisms with diabetic foot ulcer | Not reported | Vibration perception threshold |

| Study | Comparison group | Adiponectin level | Outcome | Dropout | Measures of DPN |
|---|---|---|---|---|---|
| Ágnes Molnár et al, 2022 [23] | Age and gender-matched type 2 diabetes patients without neuropathy | 6.91 ± 3.32 µg/mL (cases before exercise), 7.09 ± 3.88 µg/mL (cases after exercise), 6.89 ± 3.32 µg/mL (controls) | Physical activity increased FGF21 levels in cases, which correlated with improvement in CPT values (severity of neuropathy). | Not reported | Current perception threshold (CPT) by Neurometer® |

**Meta-Analysis**

A meta-analysis was conducted to investigate the association between adiponectin levels and diabetic peripheral neuropathy. The meta-analysis included 4 studies with a total of 920 participants, comprising 418 cases and 502 controls. Using a random-effects model, the pooled mean difference in adiponectin levels between cases and controls was 0.01 (95% CI: [-0.24, 0.26]), which was not statistically significant (p = 0.94). However, substantial heterogeneity was observed across the included studies ($I^2$ = 59%, p = 0.06). The random-effects model was chosen due to the moderate level of heterogeneity, as it accounts for potential variability in treatment effects across studies. The overall meta-analysis results suggest that there is no significant difference in adiponectin levels between individuals with diabetic peripheral neuropathy and controls, but the findings should be interpreted with caution given the substantial heterogeneity among the included studies (Figure 2). Both visual inspection of the funnel plot and Egger's test did not indicate potential publication bias (Figure 3).

**Figure 2:** Forest Plot.

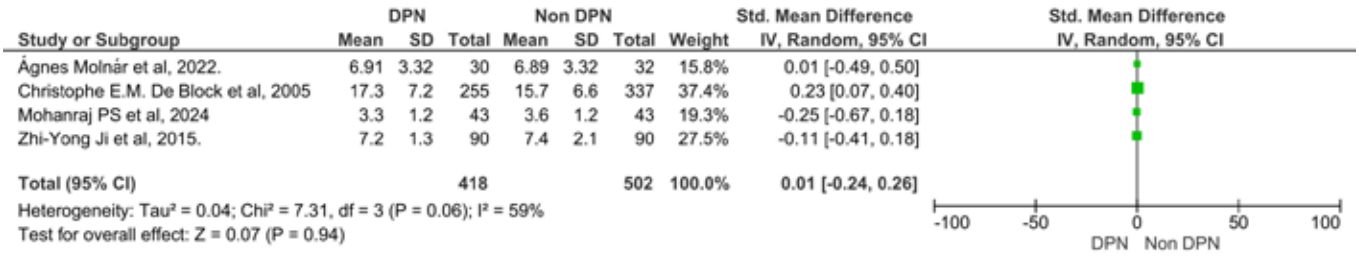
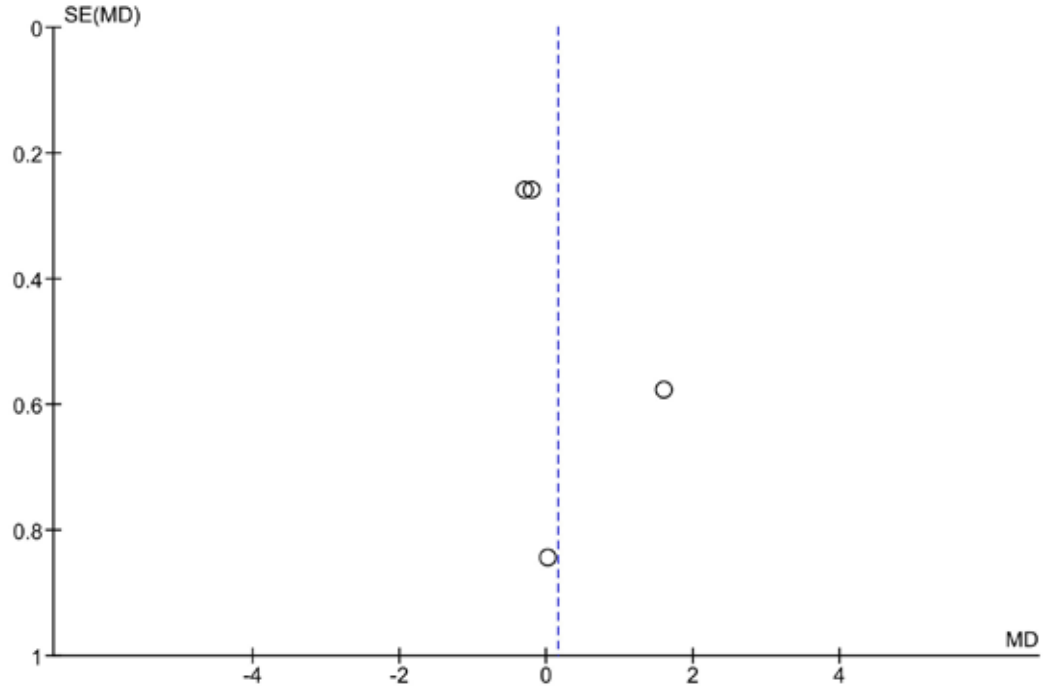



**Figure 3:** Funnel Plot.




Subgroup analyses could not be performed due to the limited number of studies and variability in reporting of data.

### Quality Assessment

The risk of bias assessment for the included studies is summarized in Figure 4 and 5. The main potential sources of bias were related to non-random sampling, lack of blinding of outcome assessors, and unclear handling of missing data in some studies. Overall, 7 studies were judged to be of high quality, while 5 had some concerns regarding risk of bias. Despite the identified potential biases, the majority of the included studies were considered of high quality, enhancing the overall reliability of the study findings.

**Figure 4:** Risk of Bias Graph.

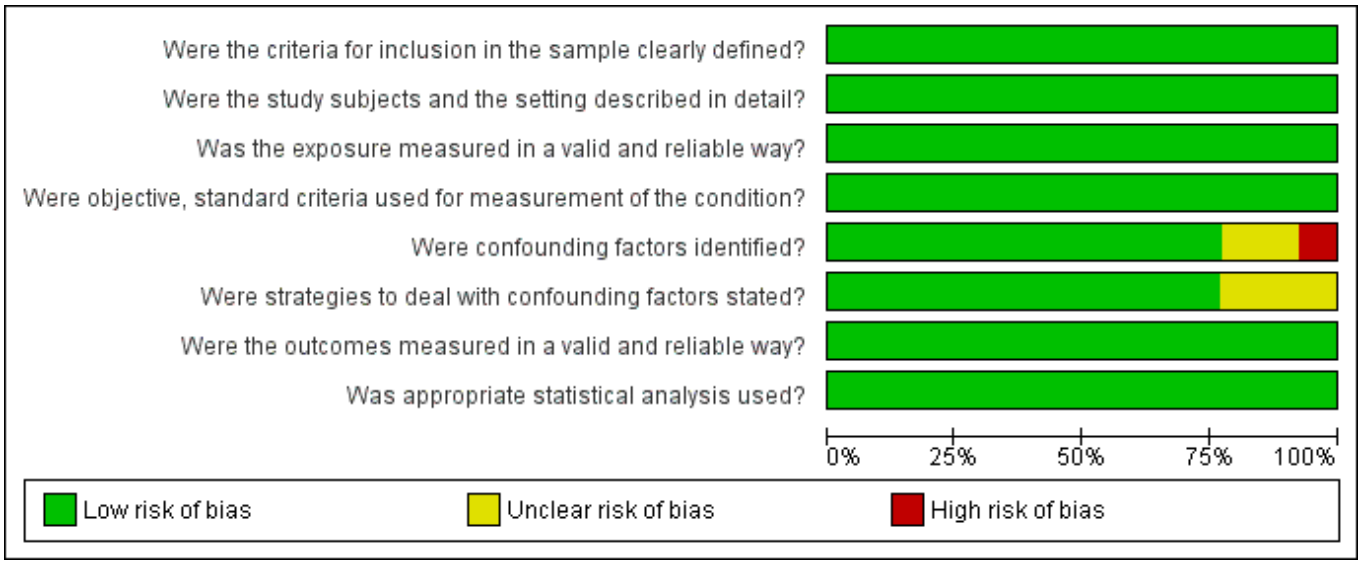
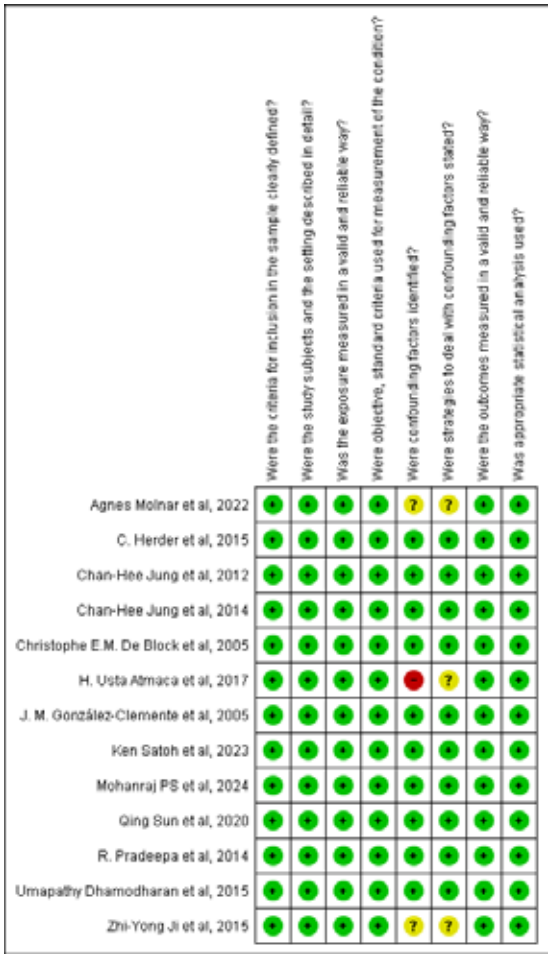



**Figure 5:** Risk of bias Summary.



**Sensitivity Analysis**

During our sensitivity analysis, we deliberately excluded individual studies in a systematic manner to evaluate their influence on the overall pooled effect size and the degree of heterogeneity observed. Despite these exclusions, our findings suggest that there were no significant alterations to the combined estimate or the level of variability. The use of the meta-analytical method, specifically the inverse variance method and the restricted maximum-likelihood estimator for tau^2, ensured the reliability of our findings. Thus, our analysis indicates that the overall conclusion about the effect size and heterogeneity stays consistent irrespective of the various instances of study exclusion.

**Table 4:** Sensitivity analysis.

| Study excluded | SMD | 95%-CI | p-value | tau^2 | I^2 |
|---|---|---|---|---|---|
| Ágnes Molnár et al (2022) | -0.00 | [-0.31, 0.30] | 0.99 | 0.05 | 72% |
| Christophe E.M. De Block et al (2005) | -0.13 | [-0.34, 0.09] | 0.25 | 0.00 | 0% |
| Mohanraj PS et al (2024) | 0.08 | [-0.17, 0.33] | 0.55 | 0.03 | 55% |
| Zhi-Yong Ji et al (2015) | 0.05 | [-0.26, 0.36] | 0.76 | 0.04 | 57% |
| Pooled estimate | 0.01 | [-0.24, 0.26] | 0.94 | 0.04 | 59% |

Meta-analytical method: - Inverse variance method - Restricted maximum-likelihood estimator for tau^2

**Additional Analyses**

Several studies also reported on additional outcomes related to adiponectin levels and diabetic complications. These are summarized below: Qing Sun et al. found that higher adiponectin was associated with increased risk of cardiovascular autonomic neuropathy in addition to peripheral neuropathy. Pradeepa et al. reported higher geometric mean adiponectin levels in patients with diabetic retinopathy (6.8 µg/mL) and nephropathy compared to those without microvascular complications. The study by Dhamodharan et al. found no significant differences in adiponectin across groups with type 2 diabetes, with or without neuropathic diabetic foot ulcers. Molnar et al. showed that a 6-week aerobic exercise intervention increased adiponectin levels in patients with distal sensory polyneuropathy, which correlated with improved current perception threshold (neuropathy severity).

**Discussion**

In this study, systematic review along with metanalysis were performed in order to assess an association between serum adiponectin levels and risk of DPN in diabetes.

On doing a thorough literature search, 13 studies reported adiponectin levels in diabetes, where comparison was done between individuals with and without DPN. Among which, four studies reported an increased level of adiponectin in DPN. However, on the contrary three other studies have reported reduced levels of adiponectin and the remaining six studies have mentioned no significant difference.

When it comes diabetes mellitus, DPN is the most prevalent neuropathic condition associated with it [24]. Lack of peripheral sensation results in unnoticed multiple injuries, intense distressing neuropathic pain, poor wound healing and ulceration, terminating to amputation of the lower limb.

DPN involves multiple aetiology. However, the exact mechanism of its development is still un resolved. The various pathways involved could be summated as disruptions in the pathways associated with hyperglycaemia, dyslipidaemia, oxidative stress-induced microvascular problems, neuronal inflammation, mitochondrial damage, and cell death. Since the pathophysiology of diabetes involves insulin resistant and inflammation, presence of Adiponectin's receptor on beta cells and immune cells is suggestive of a crucial role of adiponectin in its pathogenesis[4]. Furthermore, adiponectin has been linked to ameliorate insulin resistance, subclinical inflammation, and has neuroprotective effects in individuals with diabetes [7]. Adiponectin via AMPK pathway is found to improve diabetic neuropathy by targeting pathways of oxidative stress and anti-oxidants in animal models of diabetes [25]. Therefore, it has a promising role in the pathophysiology of DPN.

One of the most extensively studied pathogenesis of DPN is inflammation. Interestingly, the role of adiponectin is both anti-inflammatory as well as neuroprotective. Other adipokine such as leptin, enhances production of pro-inflammatory cytokines like TNF and IL-6, assisting inflammatory process and stimulating macrophages there by leading to neuropathic pain[26]. Studies where adiponectin levels were increased in DPN are suggestive that this rise in levels could be secondary in nature, in response to reduced inflammation [11,16,17,19].

Understanding the primary function of adiponectin in the pathophysiology of DPN is aided by the evidences in animal models with DPN, knockouts of the protein stimulate the MAPK pathway, resulting in hyperalgesia [27]. Additionally, Adiponectin via AMPK pathway enhances several pathways associated with oxidation, metabolism, insulin sensitivity, inflammatory response and cell survival [28]. Further, AMPK activation is found to be beneficial improving diabetic neuropathy in animal models by targeting oxidative stress [25]. Interestingly, adiponectin receptor agonist is found to be neuroprotective in diabetic neuropathy and has potential future in treatment of DPN [29]. These data support the conclusions of several investigations that have shown that DPN has lower levels of adiponectin [13,14,18].

There have been reports of both elevated and lowered adiponectin levels, which may have an impact on the pathophysiology of DPN. Its precise function is unclear, though. Consequently, it was necessary to carry out a thorough analysis. After doing this meta-analysis, we discovered that, in a diverse population, there were no appreciable differences in adiponectin levels between instances of diabetes with and without diabetic peripheral neuropathy.

Our findings imply that adiponectin might not have a role in the etiology of diabetic peripheral neuropathy. However, given the ethnic heterogeneity, chronicity of the condition (DPN is a long-term consequence of diabetes), age group disparities in the research population, and the impact of standard treatment, these results should be interpreted cautiously.

This study employs a thorough and meticulous methodology to investigate the potential role of adiponectin as a novel biomarker, which may prove useful in the future for diagnostic and prognostic purposes, as well as serving as a therapeutic targeting of diabetic peripheral neuropathy. However, this study comes with certain limitations, with medication being prime factor in almost all the studies. Levels of adiponectin are found to be reduced in newly diagnosed cases of diabetes [30]. Nonetheless, it has been discovered that taking metformin increases adiponectin levels [31]. Given that DPN is a long-term side effect of diabetic drugs, it is possible that it will influence the results of most investigations. Further, this study included both type 1 and type 2 diabetes which can have an impact on generalizability of the study results.

Additionally, study design of the studies included in this meta-analysis were mostly either cross-sectional or case control in nature, which could lead to bias since not all confounding variables were taken into account. Furthermore, the heterogeneity observed across studies, particularly in adiponectin levels, is a notable limitation. Although we addressed this using a random-effects model and sensitivity analysis, it may still affect the robustness of our findings. Lastly, the inclusion of studies focusing on other neuropathy-related complications (e.g., cardiac autonomic neuropathy) may introduce bias. While these studies were included for discussion purposes and excluded from the meta-analysis, their inclusion underscores the need for standardized diagnostic criteria and reporting in future research. Therefore, additional prospective cohort studies can provide more precise relationship between serum adiponectin and the risk of DPN in individuals with diabetes.

## Conclusion

Based on our systematic review and meta-analysis of multiple studies, we find no correlation between adiponectin levels and the heightened risk of diabetic peripheral neuropathy (DPN) in individuals with diabetes. Despite variations across studies, our systematic review provides a comprehensive overview, underscoring the necessity for further investigation to clarify adiponectin's exact role in the development of DPN.

## Conflict of Interests

The authors declare that they have no conflict of interest regarding the publication of this article.

## Ethical Approval

This systematic review and meta-analysis utilized publicly available data and did not involve direct research on human participants or animals. Therefore, no ethical approval was required.

## Credit Author Statements

Aniruddha Sen: Conceptualization, Methodology, Data Collection, Formal Analysis, Writing – Original Draft.

Palani Selvam Mohanraj: Supervision, Validation, Writing – Review & Editing.

Vinoth Rajendran: Data Curation, Statistical Analysis, Writing – Review & Editing.

Sundhar Mohandas: Resources, Investigation, Writing – Review & Editing.

Abhimanyu Vasudeva: Visualization, Writing – Review & Editing.

Akash Bansal: Methodology, Software, Data Validation.

Neha Keshri: Literature Search, Validation.

Srinivas Nagaram: Validation, Writing – Review & Editing.

Anupriya Velu: Conceptualization, Resources, Writing – Review & Editing.

All authors have read and approved the final manuscript.

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Research Article

# TPMT genotyping in 1000 Indian patients: 14-year experience from a tertiary-care hospital

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Thiopurine, TPMT, genetic testing, Indian patients, allele frequency

**Abstract**

Thiopurine methyltransferase (TPMT) enzyme plays a key role in the metabolism of the thiopurine drugs that are used for the treatment of inflammatory diseases. Mutations in the TPMT gene cause abnormal metabolism, resulting in toxicity; therefore, TPMT genetic analysis has been recommended for effective dose management. We retrospectively analysed data to determine the distribution of TPMT genotypes in a western Indian population. TPMT genotyping test was performed on 1000 patients with different inflammatory conditions between January 2009 and October 2023. The common TPMT genotypes *2, *3A, *3B and *3C were detected by amplification refractory mutation system - polymerase chain reaction (ARMS-PCR) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) techniques. TPMT mutants were detected in 36 (3.6%) patients, of whom 14 (39%) had TPMT*1/*3A, 19 (53%) had TPMT*1/*3C, two (5.5%) had TPMT*1/*3B, and one (2.8%) had TPMT*3B/*3B alleles; mutant allele frequencies were 0.7% for *3A, 0.2% for *3B and 1.65% for *3C. A sub-group analysis explained thiopurine toxicity in only 33% patients by TPMT gene polymorphism whereas in 67% the toxicity remained unexplained. The low prevalence of TPMT mutants (3.6%) along with unexplained thiopurine toxicity suggest that TPMT genotyping solely might be clinically less relevant in patients of Indian origin, underscoring the role of other genetic factors that may be involved in thiopurine toxicity in these patients.

## Introduction

Thiopurine methyltransferase (TPMT) enzyme plays a key role in the metabolism of thiopurine drugs such as azathioprine (AZA) and 6-mercaptopurine (6-MP). These drugs are used as antimetabolite cytotoxic and immunosuppressive agents in the treatment of acute lymphoblastic leukaemia (ALL), inflammatory bowel disease (IBD), and autoimmune disorders such as rheumatoid arthritis, systemic lupus erythematosus, autoimmune hepatitis, and generalized eczematous disorders. [1,2,] However, gastrointestinal disturbances, rashes, as well as more serious adverse drug reactions like bone marrow toxicity, hepatotoxicity, and pancreatitis can lead to discontinuation of therapy in some patients.[3] The wide inter-individual variations observed in the efficacy and toxicity of thiopurine drugs have been largely attributed to the presence of genetic polymorphisms in the TPMT gene affecting TPMT enzyme activity.[1,4,5] To date, over 40 TPMT variant alleles have been identified, of which four, i.e., TPMT*2, *3A, *3B and *3C, account for ~85%-95% of intermediate and low TPMT enzyme activity.[6,7]

The prevalence of TPMT variants varies across different populations. TPMT*2 is prevalent in European Caucasian populations, TPMT*3A is predominant in Caucasians and Latin Americans, and TPMT*3C is most common in East and South-East Asians and African populations.[8-14] The Indian population being heterogeneous shows ethnic differences in the distribution of these defective alleles.

Hence, we have retrospectively analysed our data to determine the TPMT allele frequencies as well as to assess the relevance of TPMT genotyping in Indian patients.

## Patients and Methods

TPMT gene polymorphism was analysed in patients attending our hospital who had an indication for immunomodulator therapy. An informed consent was obtained from all the patients who underwent TPMT genotyping test. TPMT genotyping was performed as a test at our Molecular Diagnostic Laboratory that is accredited by the College of American Pathologists (CAP) and National Accreditation Board for testing and laboratories (NABL). 1000 samples for TPMT genotyping have been received over a period of 14 years from January 2009 until October 2023. The institutional ethics committee –II (IEC-II/IRB) had no objection to the publication of the manuscript.

TPMT genotyping assay had been standardized in-house using amplification refractory mutation system - polymerase chain reaction (ARMS-PCR) and polymerase chain reaction - restriction length polymorphism (PCR-RFLP), and further validated by DNA sequencing [15]. The methodology we used has been reported earlier [15] and the quality control measures, method validation (which includes repeatability, accuracy, sensitivity and specificity), inter-laboratory comparison (ILC) testing, and Sanger sequencing confirmation of the control samples have been followed, practised and documented over 14 years of the test performance. The assay included the common TPMT alleles, i.e. TPMT*2 (NM_000367.5:c.238G>C, p.Ala80Pro, rs18004622), TPMT*3B (NM_000367.5:c.460G>A, p.Ala154Thr, rs1800460), TPMT*3C (NM_000367.5:c.719A>G, p.Tyr240Cys, rs1142345), and TPMT*3A (which is characterized by the presence of TPMT*3B and TPMT*3C alleles).

TPMT allele frequencies were calculated using an online calculator (https://www.had2know.org/academics/hardy-weinberg-equilibrium-calculator-2-alleles.html). Diagnostic characteristics of the TPMT genotyping in thiopurine-intolerant and -tolerant patients were calculated using MedCalc® version 20.211 (https://www.medcal.org). Heatmaps were generated for comparative analysis between different populations using Microsoft Excel 2013 (https://www.ablebits.com/office-addins-blog/create-heat-map-excel/).

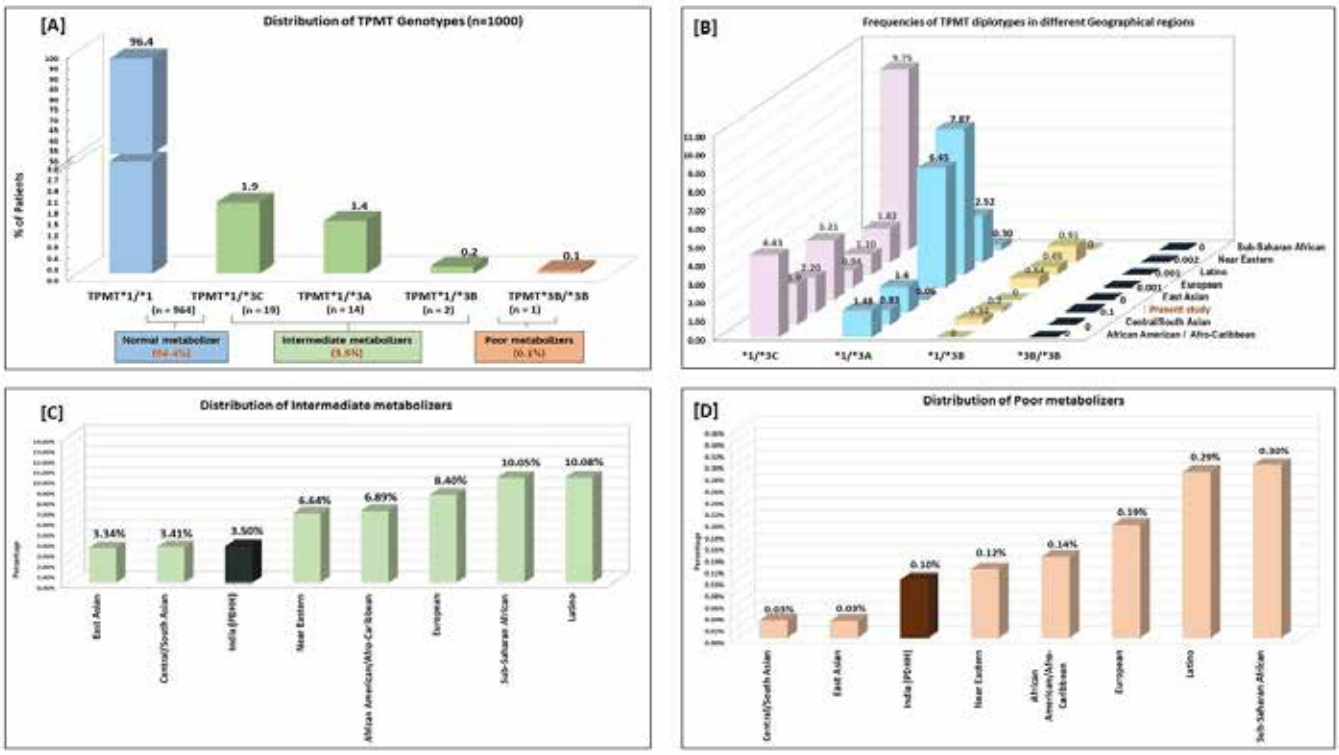
## Results and Discussion

A total of 1000 samples were obtained from patients with mean age 38±17 years (range 03-87 years) of whom 535 patients were male. A majority of these were patients with IBD (76%, n=763), 5% (n=52) had ALL and 19% (n=185) had other indications such as rheumatology, nephrology, dermatology etc.

### TPMT genotypes

TPMT wild-type genotype was present in 96.4% (n=964) of patients, whereas TPMT mutants were detected in only 3.6% (n=36). The distribution of TPMT genotypes (Figure 1A) showed predominance of TPMT*1/*1 genotype (96.4%; n=964), followed by TPMT*1/*3C (1.9%; n=19), TPMT*1/*3A (1.4%; n=14), TPMT*1/*3B (0.2%; n=2), and TPMT*3B/*3B (0.1%; n=1) patients. TPMT*2 variants were not detected in our patient population. The worldwide frequency of TPMT diplotypes as obtained from the updated Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines (Figure 1B) showed TPMT*1/*3C genotype predominance in Sub-Saharan populations (9.75%), followed by African-American and Afro-Caribbean populations (4.43%) [5,6]; TPMT*1/*3A genotype was reported mainly from Latino (7.87%) and European (6.45%) populations.

**Figure 1:** [A] Allele frequencies of TPMT*3A, TPMT*3C, TPMT*3B and TPMT*2. Distribution of TPMT genotypes in total samples along with predicted phenotypes. [B] Frequencies of TPMT diplotypes in different geographical regions. [C] and [D] Comparison of Intermediate and poor metabolisers, respectively, with worldwide populations.




### TPMT predicted metabolisers

TPMT genotype-based predicted metabolisers, according to the CPIC classification, showed 3.5% as intermediate metabolisers (Figure 1C) and 0.1% poor metabolisers (Figure 1D) in our population. The updated CPIC guidelines showed predominance of intermediate metabolisers in Latino (10.08%) and Sub-Saharan African (10.05%) populations followed by Europeans (8.4%) [5,6]. Poor metabolizers (Figure 1D) were detected in Sub-Saharan Africans (0.3%), Latinos (0.29%), and Europeans (0.19%).

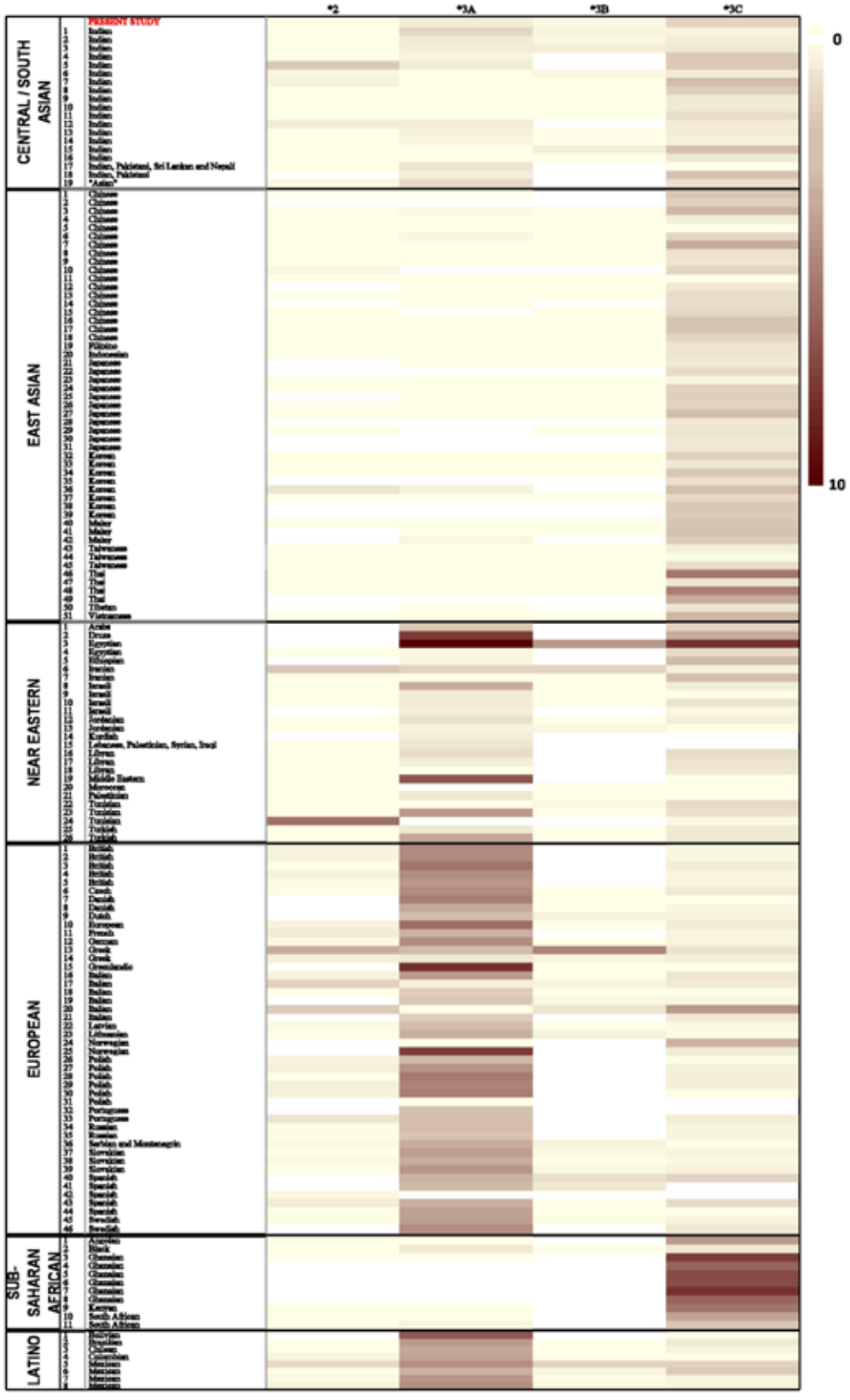
### Comparison of TPMT allele frequencies

We observed TPMT mutant allele frequencies to be 0.7% for TPMT*3A, 0.2% for TPMT*3B, and 1.65% for TPMT*3C. Comparative analysis of allele frequencies with Indian and other geographical populations was performed using heatmap analysis. Data from different worldwide populations were selected from the updated CPIC guidelines [5,6] (Supplementary Table) and other worldwide reported studies.

### TPMT*2 allele

Our data showed absence of TPMT*2 allele in our population. The non-functional TPMT*2 allele has low preponderance in worldwide populations. Low frequency has been reported in Europeans, the highest (3.19%) [5,6] (Supplementary Table) being reported from a Greek population (Figure 2). Its prevalence is low amongst Latinos while it is almost absent in East Asians and infrequent in South/Central Asians. Likewise, it is less frequent in the Indian population except for a study from southern India that reported a frequency of 3.1% [5,6] (Supplementary Table).

**Figure 2:** Heatmaps of allele frequencies (in %) of different geographical populations.



**TPMT*3A allele**

A 0.7% frequency of this allele was observed in our data from a western Indian population. Heatmap (Figure 2) shows characteristically distinctive patterns for TPMT*3A in Europeans and Americans. This allele is predominant in Latinos and Europeans, with frequencies of 3%-6.5% [5,6] (Suppl. Table) and 0%-8.1%, respectively [5,6,16-18] (Supplementary Table). Interestingly, in both these populations TPMT*3B and TPMT*3C alleles were infrequent; however compound variants were predominant. Conversely, this allele was scarcely present in East Asians, Africans, and near-Eastern populations. South/Central Asians showed low frequency and Indian populations also showed low frequencies from 0-1.6% [5,6,19-22] (Supplementary Table).

**TPMT*3B allele**

Our patient population had 0.2% frequency of this allele. The non-functional TPMT*3B allele is less commonly reported in worldwide populations (Figure 2). It is largely absent in East Asians and African populations and scarcely detected in the Middle East and South-Central Asians. Based on Heatmap, the reported frequency amongst Europeans varies from 0% in most populations to 1%, 3% and 4.78%, respectively, in the Italian, Spanish and Greek populations [5,6,18] (Supplementary Table). Studies from India have reported infrequent dissemination (0-0.67%) [5,6,19-22] (Supplementary Table).

**TPMT*3C allele**

Our data shows allele frequency of 1.65%. Heatmap (Figure 2) shows distinctive predominance of this allele in Africans, with highest frequency of 8% reported in the Ghanaian population [5,6] (Suppl. Table). East Asians show variability in frequency ranging from 0%-5.3% [5,6,23] (Suppl. Table). TPMT*3C variant is infrequent in major European populations except for the Norwegian and Italian populations that show frequencies of 3% and 4%, respectively [5,6] (Supplementary Table). Similarly, it is less frequently reported from the near Eastern, Latino and Central/South Asian populations. Amongst Indian populations it is reported in frequency from 0.4-2.6% [5,6,19-22] (Supplementary Table).

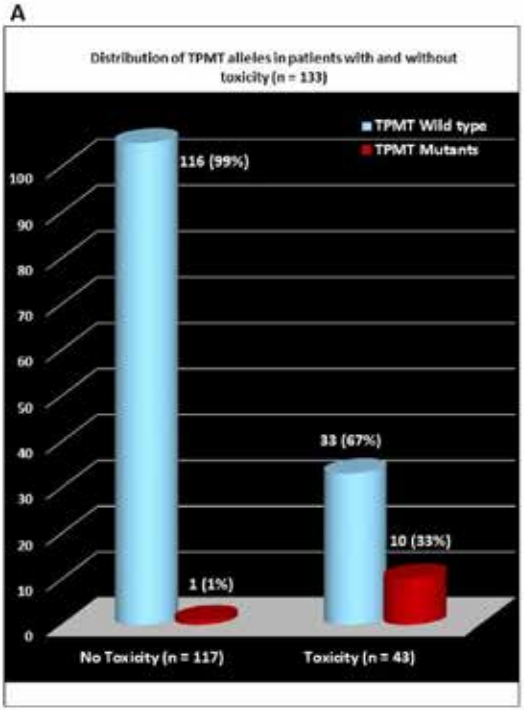
Overall, the comparative heatmap shows that there is low prevalence of TPMT alleles in Indian populations as compared with the European, Near Eastern, Sub-Saharan African and Latino populations. Indian populations shows similarity in the distribution of the TPMT alleles with the East Asian population wherein the TPMT*3C allele has been commonly reported as compared to other alleles. Based on the TPMT genotypes, the predicted intermediate metabolizers were detected in 3.5% patients in the present study (Figure 1A). Overall, the worldwide population shows more prevalence of intermediate metabolizers as compared to poor metabolizers (Figure 1C and 1D). Strong recommendations has been laid down by CPIC for the use of thiopurines in TPMT intermediate metabolizers wherein 30-80% reduction of doses is recommended to minimize the risk of thiopurine-related leukopenia, neutropenia and myelosuppression [5].

**TPMT alleles and thiopurine toxicity**

Genetic polymorphisms for enzymes involved in thiopurine metabolism have been recognised to predict toxicity. Leucopenia has been reported in 9%-27.7% of patients with IBD who were initiated on thiopurine treatment [20,24]. However, the important polymorphisms responsible for causing leucopenia vary amongst different populations: in Western countries, for example, TPMT gene polymorphisms have been recognised as an important cause. Hence, to further assess the role of TPMT gene polymorphisms in thiopurine-induced toxicity, we performed a sub-group analysis in 160 patients. Thiopurine toxicity was classified as reported [25]; based on this, 117 (73%) patients had no toxicity to thiopurine, whereas 43 (27%) patients had thiopurine toxicity (Figure 3A).

**Figure 3:** [A] Sub-group analysis in patient with and without thiopurine toxicity. [B] The table depicts the clinical characteristics of TPMT genotyping test.

A




B

| Statistics | Value | 95% Interval | Significance |
|---|---|---|---|
| Sensitivity | 23.26% | 11.755% to 38.631% | |
| Specificity | 99.15% | 95.33% to 99.98% | |
| AUC | 0.612 | 0.532 to 0.688 | |
| Fischer's exact test | | - | p = 0.000023 |
| Chi-square test | 24.6453 | - | P < 0.00001 |
| Odds ratio | 35.1515 | 4.3403 to 284.686 | z statistic = 3.326; p = 0.0009 |
| Relative risk | 27.2093 | 3.5888 to 206.2914 | z statistic = 3.196; p = 0.0014 |
| Positive Likelihood Ratio | 27.21 | 3.59 to 206.29 | |
| Negative Likelihood Ratio | 0.77 | 0.66 to 0.91 | |
| Disease prevalence | 26.88% | 20.18% to 34.45% | |
| Positive Predictive Value | 90.91% | 56.88% to 98.70% | |
| Negative Predictive Value | 77.85% | 74.87% to 80.57% | |
| Accuracy | 78.75% | 71.59% to 84.81% | |

TPMT wild-type allele was predominant (99%) in the thiopurine-tolerant patient group while only one patient had TPMT mutant allele. TPMT mutant allele accounted for thiopurine toxicity in only 33% (n=10) of patients who exhibited thiopurine intolerance (Figure 3A). Genetic testing for TPMT did not provide any explanation for thiopurine toxicity in the remaining 67% (n=33). Diagnostic characteristics (Figure 3B) showed poor sensitivity (26.7%) and AUC (0.628), suggesting that TPMT genotyping alone is not adequate to predict thiopurine toxicity in Indian patients.

To summarize, the association between gene polymorphisms and thiopurine-related toxicity shows differences in different ethnic populations. It has been reported that Asians has lower TPMT mutation frequency than the Caucasians (~3% versus ~10%) [11,26], however the prevalence of thiopurine-induced toxicity is higher in Asians which is more often associated with the nucleoside diphosphatelinked moiety X-type motif 15 (NUDT15) genotype [27,28]. In the present study, the unexplained toxicity in 67% patients with normal TPMT genotypes could be attributed to other genetic variants (e.g. NUDT15, Inosine triphosphate pyrophosphatase (ITPA) and non-genetic factors such as environmental, drug-drug interactions, dietary and metabolic factors. The current study though limited to the traditional TPMT genetic marker over 14 years on a large sample size of 1000 patients, population-specific genetic markers (such as NUDT15) should be incorporated in the screening of thiopurine induced toxicity.

## Conclusions

Our 14-year data on 1000 patient samples showed low prevalence of TPMT genetic variants in Indian patients. TPMT genotyping could not explain thiopurine toxicity in 67% of patients. Our data suggest that only TPMT genotyping might be clinically less relevant for patients of Indian origin, underscoring the need to include other genetic factors (e.g., NUDT15) to explain thiopurine toxicity in Indian patients.

## Conflict of Interest

All authors have no conflict of interest to declare.

## Supplemental material

One table containing TPMT allele frequency data, taken majorly from CPIC, used for generation of Heatmaps.

## Credit Author Statement

Minal Paradkar: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing-original draft and Visualization. Swarup AV Shah: Methodology, Validation, Formal analysis, Investigation, and Writing-review and editing. Rani Deepak: Methodology, Investigation, Writing-review and editing. Mihika Dave: Investigation and Writing-review and editing. Alpa Dherai and Dhanashri Shetty: Writing-review and editing. Devendra Desai, Philip Abraham, Anand Joshi and Tarun Gupta: Resources and Writing-review and editing. Tester F Ashavaid: Conceptualization, Writing-review and editing, and Supervision.

**Ethics approval and consent**

An informed consent was obtained from all the patients who underwent TPMT genotyping test. The institutional ethics committee –II (IEC-II/IRB) had no objection to the publication of the manuscript. This study is in compliance with the ethical principles for medical research involving human subjects, in accordance with the Declaration of Helsinki.

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**SUPPLEMENTARY TABLE: TPMT ALLELE FREQUENCY IN DIFFERENT WORLDWIDE POPULATIONS**

| No. | Authors | Year | PMID | Population group | Population | Add'l population info | Subject type | N subjects genotyped | Allele frequency | | | | | Allele frequency in % | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | *1 | *2 | *3A | *3B | *3C | *1 | *2 | *3A | *3B | *3C |
| **Central/South Asian** | | | | | | | | | | | | | | | | | | |
| 1 | Davavala Sandeep Kirit | 2014 | 23996738 | Central/South Asian | Indian | Western India | Gastrointestinal disease patients | 126 | 0.976 | 0.0 | 0.016 | 0.004 | 0.004 | 97.6 | 0.0000 | 1.6000 | 0.4000 | 0.4000 |
| 2 | Shah SAV | 2018 | 29470173 | Central/South Asian | Indian | Western India | IBD patients | 370 | 0.9824 | 0 | 0.0081 | 0.0027 | 0.0068 | 98.24 | 0.0000 | 0.8100 | 0.2700 | 0.6800 |
| 3 | Iyer Sandhya N | 2012 | 22580794 | Central/South Asian | Indian | | Healthy individuals | 225 | 0.9777 | 0.0 | 0.0067 | 0.0067 | 0.0089 | 97.77 | 0.0000 | 0.6700 | 0.6700 | 0.8900 |
| 4 | Kapoor Gauri | 2009 | 19675376 | Central/South Asian | Indian | North Indian | Healthy individuals | 120 | 0.975 | 0.0 | 0.004 | - | 0.021 | 97.5 | 0.0000 | 0.4000 | | 2.1000 |
| 5 | Kapoor Gauri | 2010 | 20153897 | Central/South Asian | Indian | North Indian | Pediatric ALL patients | 71 | 0.951 | 0.021 | 0.007 | - | 0.021 | 95.1 | 2.1000 | 0.7000 | | 2.1000 |
| 6 | Murugesan Raju | 2010 | 23136604 | Central/South Asian | Indian | Southern Indian | Healthy individuals | 326 | 0.9862 | 0.0031 | 0.0 | 0.0031 | 0.0076 | 98.62 | 0.3100 | 0.0000 | 0.3100 | 0.7600 |
| 7 | Desire Salamun | 2010 | 19830600 | Central/South Asian | Indian | South Indian | ALL patients | 98 | 0.969 | 0.005 | 0.0 | 0.0 | 0.026 | 96.9 | 0.5000 | 0.0000 | 0.0000 | 2.6000 |
| 8 | Umamaheswaran Gurusamy | 2012 | 22318545 | Central/South Asian | Indian | Andhrite | Healthy individuals | 158 | 0.981 | 0.0 | 0.0 | 0.0 | 0.019 | 98.1 | 0.0000 | 0.0000 | 0.0000 | 1.9000 |
| 9 | Umamaheswaran Gurusamy | 2012 | 22318545 | Central/South Asian | Indian | Kannadiga | Healthy individuals | 161 | 0.991 | 0.0 | 0.0 | 0.0 | 0.009 | 99.1 | 0.0000 | 0.0000 | 0.0000 | 0.9000 |
| 10 | Umamaheswaran Gurusamy | 2012 | 22318545 | Central/South Asian | Indian | Keralite | Healthy individuals | 177 | 0.992 | 0.0 | 0.0 | 0.0 | 0.008 | 99.2 | 0.0000 | 0.0000 | 0.0000 | 0.8000 |
| 11 | Umamaheswaran Gurusamy | 2012 | 22318545 | Central/South Asian | Indian | Tamilian | Healthy individuals | 112 | 0.987 | 0.0 | 0.0 | 0.0 | 0.013 | 98.7 | 0.0000 | 0.0000 | 0.0000 | 1.3000 |
| 12 | Linga Vijay Gandhi | 2014 | 25538405 | Central/South Asian | Indian | South Indian | Pediatric ALL patients | 72 | 0.9793 | 0.0069 | 0.0069 | - | 0.0069 | 97.93 | 0.6900 | 0.6900 | | 0.6900 |
| 13 | Kham S K Y | 2002 | 12142782 | Central/South Asian | Indian | Singaporean | Healthy individuals and pediatric ALL patients | 200 | 0.987 | 0.0 | 0.005 | 0.0 | 0.008 | 98.7 | 0.0000 | 0.5000 | 0.0000 | 0.8000 |
| 14 | Ambar G | 2018 | 30477053 | Central/South Asian | Indian | North Indian | Autoimmune disease patients | 176 | 0.9916 | 0 | 0.0028 | 0 | 0.0056 | 99.16 | 0.0000 | 0.2800 | 0.0000 | 0.5600 |
| 15 | Banerjee R | 2020 | 33111378 | Central/South Asian | Indian | Hyderabad, South India | IBD patients | 81 | 0.9691 | 0 | 0 | 0.0062 | 0.0247 | 96.91 | 0.0000 | 0.0000 | 0.6200 | 2.4690 |
| 16 | Kham Shirley Kow Yin | 2008 | 18193212 | Central/South Asian | Indian | Singaporean | Healthy individuals | 163 | 0.991 | 0.0 | 0.0 | 0.0 | 0.009 | 99.1 | 0.0000 | 0.0000 | 0.0000 | 0.9000 |
| 17 | Collie-Duguid E S | 1999 | 10208641 | Central/South Asian | Indian, Pakistani, Sri Lankan and Nepali | | Healthy individuals | 99 | 0.99 | 0.0 | 0.01 | - | 0.0 | 99 | 0.0000 | 1.0000 | | 0.0000 |
| 18 | Marinaki Anthony M | 2003 | 12563179 | Central/South Asian | Indian and Pakistani | British | Gastroenterology patients | 85 | 0.97 | - | 0.006 | - | 0.024 | 97 | | 0.6000 | | 2.4000 |
| 19 | Lennard Lynne | 2015 | 25940902 | Central/South Asian | "Asian" | from Britain | Pediatric ALL patients | 164 | 0.97 | 0.003 | 0.015 | - | 0.012 | 97 | 0.3000 | 1.5000 | | 1.2000 |
| **East Asian** | | | | | | | | | | | | | | | | | | |
| 1 | Collie-Duguid E S | 1999 | 10208641 | East Asian | Chinese | Han Chinese | Healthy individuals | 192 | 0.977 | 0.0 | 0.0 | - | 0.023 | 97.7 | 0.0000 | 0.0000 | | 2.3000 |
| 2 | Chen ZY | 2021 | 34084143 | East Asian | Chinese | | | 1419 | 0.9821 | - | - | - | 0.0179 | 98.21 | | | | 1.7900 |
| 3 | Kham S K Y | 2002 | 12142782 | East Asian | Chinese | Singaporean | Healthy individuals and pediatric ALL patients | 271 | 0.97 | 0.0 | 0.002 | 0.0 | 0.028 | 97 | 0.0000 | 0.2000 | 0.0000 | 2.8000 |
| 4 | Zhang Jian-ping | 2004 | 15255798 | East Asian | Chinese | Han Chinese | Healthy individuals | 87 | 0.994 | 0.0 | 0.0 | 0.0 | 0.006 | 99.4 | 0.0000 | 0.0000 | 0.0000 | 0.6000 |
| 5 | Zhang Jian-ping | 2004 | 15255798 | East Asian | Chinese | Yao Chinese | Healthy individuals | 126 | 1 | 0.0 | 0.0 | 0.0 | 0.0 | 100 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| 6 | Zhang Jian-Ping | 2004 | 14985891 | East Asian | Chinese | Uygur Chinese | Healthy individuals | 160 | 0.981 | 0.0 | 0.003 | 0.0 | 0.016 | 98.1 | 0.0000 | 0.3000 | 0.0000 | 1.6000 |
| 7 | Song D-K | 2006 | 17176368 | East Asian | Chinese | Han Chinese | Healthy individuals and renal transplant recipients | 332 | 0.968 | 0.0 | 0.0 | 0.0 | 0.032 | 96.8 | 0.0000 | 0.0000 | 0.0000 | 3.2000 |
| 8 | Zhang Jian-Ping | 2006 | 16223474 | East Asian | Chinese | Han Chinese | Healthy individuals | 312 | 0.989 | 0.0 | 0.0 | 0.0 | 0.011 | 98.9 | 0.0000 | 0.0000 | 0.0000 | 1.1000 |
| 9 | Zhang Jian-Ping | 2006 | 16223474 | East Asian | Chinese | Jing Chinese | Healthy individuals | 103 | 0.9903 | 0.0 | 0.0 | 0.0 | 0.0097 | 99.03 | 0.0000 | 0.0000 | 0.0000 | 0.9700 |
| 10 | Zhang Jian-Ping | 2006 | 16223474 | East Asian | Chinese | Uygur Chinese | Healthy individuals | 160 | 0.981 | 0.003 | - | - | 0.016 | 98.1 | 0.3000 | | | 1.6000 |
| 11 | Zhang Jian-Ping | 2006 | 16223474 | East Asian | Chinese | Yao Chinese | Healthy individuals | 126 | 1 | 0.0 | 0.0 | 0.0 | 0.0 | 100 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| 12 | Lu Yi | 2007 | 17113562 | East Asian | Chinese | Singaporean | Healthy individuals | 166 | 0.991 | - | 0.0 | 0.0 | 0.009 | 99.1 | | 0.0000 | 0.0000 | 0.9000 |
| 13 | Zhang Li-Rong | 2007 | 16952345 | East Asian | Chinese | Han Chinese | Renal failure patients and healthy individuals | 278 | 0.987 | 0.0 | 0.0 | 0.0 | 0.013 | 98.7 | 0.0000 | 0.0000 | 0.0000 | 1.3000 |
| 14 | Kham Shirley Kow Yin | 2008 | 18193212 | East Asian | Chinese | Singaporean | Healthy individuals | 153 | 0.987 | - | 0.0 | - | 0.013 | 98.7 | | 0.0000 | | 1.3000 |
| 15 | Cao Qian | 2009 | 19252404 | East Asian | Chinese | Han Chinese | Healthy individuals and inflammatory bowel disease patients | 462 | 0.985 | 0.0 | - | 0.0 | 0.015 | 98.5 | 0.0000 | | 0.0000 | 1.5000 |

**SUPPLEMENTARY TABLE: TPMT ALLELE FREQUENCY IN DIFFERENT WORLDWIDE POPULATIONS**

| No. | Authors | Year | PMID | Population group | Population | Add'l population info | Subject type | N subjects genotyped | Allele frequency | | | | | Allele frequency in % | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | *1 | *2 | *3A | *3B | *3C | *1 | *2 | *3A | *3B | *3C |
| 16 | Xin Hua-Wen | 2009 | 19048245 | East Asian | Chinese | | Renal transplant recipients | 150 | 0.977 | 0.0 | 0.0 | 0.0 | 0.023 | 97.7 | 0.0000 | 0.0000 | 0.0000 | 2.3000 |
| 17 | Xiong Hui | 2010 | 19682085 | East Asian | Chinese | | Renal transplant recipients | 155 | 0.977 | 0.0 | 0.0 | 0.0 | 0.023 | 97.7 | 0.0000 | 0.0000 | 0.0000 | 2.3000 |
| 18 | Chen Dongying | 2014 | 24322830 | East Asian | Chinese | Han Chinese | Lupus patients | 126 | 0.984 | 0.0 | 0.0 | 0.0 | 0.016 | 98.4 | 0.0000 | 0.0000 | 0.0000 | 1.6000 |
| 19 | Chang Jan-Gowth | 2002 | 11927834 | East Asian | Filipino | | Healthy individuals | 100 | 0.99 | 0.0 | 0.0 | 0.0 | 0.01 | 99 | 0.0000 | 0.0000 | 0.0000 | 1.0000 |
| 20 | Chang Jan-Gowth | 2002 | 11927834 | East Asian | Indonesian | | Healthy individuals | 100 | 0.99 | 0.0 | 0.0 | 0.0 | 0.01 | 99 | 0.0000 | 0.0000 | 0.0000 | 1.0000 |
| 21 | Hiratsuka M | 2000 | 10751626 | East Asian | Japanese | | Healthy individuals | 192 | 0.992 | - | 0.0 | 0.0 | 0.008 | 99.2 | | 0.0000 | 0.0000 | 0.8000 |
| 22 | Ando M | 2001 | 11337943 | East Asian | Japanese | | Pediatric ALL patients | 71 | 0.979 | - | - | - | 0.014 | 97.9 | | | | 1.4000 |
| 23 | Kubota T | 2001 | 11422006 | East Asian | Japanese | | Healthy individuals | 151 | 0.997 | 0.0 | 0.0 | 0.0 | 0.003 | 99.7 | 0.0000 | 0.0000 | 0.0000 | 0.3000 |
| 24 | Kumagai K | 2001 | 11337944 | East Asian | Japanese | | Rheumatic disease patients and healthy individuals | 507 | 0.983 | 0.0 | 0.0 | 0.0 | 0.017 | 98.3 | 0.0000 | 0.0000 | 0.0000 | 1.7000 |
| 25 | Kubota Takahiro | 2004 | 15167635 | East Asian | Japanese | | Healthy individuals | 157 | 0.981 | - | 0.0 | 0.0 | 0.019 | 98.1 | | 0.0000 | 0.0000 | 1.9000 |
| 26 | Okada Yuko | 2005 | 16272700 | East Asian | Japanese | | Healthy individuals and lupus patients | 242 | 0.983 | 0.0 | 0.0 | 0.0 | 0.017 | 98.3 | 0.0000 | 0.0000 | 0.0000 | 1.7000 |
| 27 | Tamori Akihiro | 2007 | 17241387 | East Asian | Japanese | | Liver disease patients | 236 | 0.975 | 0.0 | 0.0 | 0.0 | 0.025 | 97.5 | 0.0000 | 0.0000 | 0.0000 | 2.5000 |
| 28 | Ban Hiromitsu | 2008 | 18827410 | East Asian | Japanese | | Healthy individuals and inflammatory bowel disease patients | 111 | 0.991 | - | - | - | 0.009 | 99.1 | | | | 0.9000 |
| 29 | Takatsu Noritaka | 2009 | 19682195 | East Asian | Japanese | | Inflammatory bowel disease patients | 147 | 0.99 | 0.0 | - | 0.0 | 0.01 | 99 | 0.0000 | | 0.0000 | 1.0000 |
| 30 | Ban Hiromistu | 2010 | 20393862 | East Asian | Japanese | | Healthy individuals and inflammatory bowel disease patients | 279 | 0.991 | - | - | - | 0.009 | 99.1 | | | | 0.9000 |
| 31 | Osaki Rie | 2011 | 22977575 | East Asian | Japanese | | Healthy individuals and inflammatory bowel disease patients | 279 | 0.991 | - | - | - | 0.009 | 99.1 | | | | 0.9000 |
| 32 | Jun J B | 2005 | 16396707 | East Asian | Korean | | Lupus patients | 342 | 0.975 | 0.0 | 0.0 | 0.0 | 0.018 | 97.5 | 0.0000 | 0.0000 | 0.0000 | 1.8000 |
| 33 | Lee Sang Seop | 2008 | 18775689 | East Asian | Korean | | Healthy individuals | 400 | 0.988 | 0.0 | 0.0 | 0.0 | 0.009 | 98.8 | 0.0000 | 0.0000 | 0.0000 | 0.9000 |
| 34 | Jung Yoon Suk | 2010 | 19960028 | East Asian | Korean | | Inflammatory bowel disease patients | 204 | 0.978 | 0.0 | 0.0 | 0.0 | 0.022 | 97.8 | 0.0000 | 0.0000 | 0.0000 | 2.2000 |
| 35 | Kim Jae Hak | 2010 | 20308917 | East Asian | Korean | | Inflammatory bowel disease patients | 286 | 0.988 | - | - | - | 0.012 | 98.8 | | | | 1.2000 |
| 36 | Kim Hyery | 2012 | 23029095 | East Asian | Korean | | Pediatric ALL patients | 100 | 0.96 | 0.01 | 0.005 | - | 0.025 | 96 | 1.0000 | 0.5000 | | 2.5000 |
| 37 | Kim Hyun-Young | 2015 | 25564374 | East Asian | Korean | | Hospital patients | 900 | 0.9832 | 0.0 | 0.0 | 0.0 | 0.0144 | 98.32 | 0.0000 | 0.0000 | 0.0000 | 1.4400 |
| 38 | Lee Mi-Na | 2015 | 25851563 | East Asian | Korean | | Pediatric inflammatory bowel disease patients | 137 | 0.97 | - | - | - | 0.022 | 97 | | | | 2.2000 |
| 39 | Lee Minseok | 2017 | 28966507 | East Asian | Korean | | Dermatology patients | 123 | 0.976 | - | - | - | 0.02 | 97.6 | | | | 2.0000 |
| 40 | Kham S K Y | 2002 | 12142782 | East Asian | Malay | Singaporean | Healthy individuals and pediatric ALL patients | 217 | 0.975 | 0.0 | 0.0 | 0.0 | 0.023 | 97.5 | 0.0000 | 0.0000 | 0.0000 | 2.3000 |
| 41 | Lu Yi | 2007 | 17113562 | East Asian | Malay | Singaporean | Healthy individuals | 104 | 0.976 | - | 0.0 | 0.0 | 0.024 | 97.6 | | 0.0000 | 0.0000 | 2.4000 |
| 42 | Kham Shirley Kow Yin | 2008 | 18193212 | East Asian | Malay | Singaporean | Healthy individuals | 163 | 0.977 | - | 0.003 | - | 0.02 | 97.7 | | 0.3000 | | 2.0000 |
| 43 | Chang Jan-Gowth | 2002 | 11927834 | East Asian | Taiwanese | | Healthy individuals | 249 | 0.994 | 0.0 | 0.0 | 0.0 | 0.006 | 99.4 | 0.0000 | 0.0000 | 0.0000 | 0.6000 |
| 44 | Lu H-F | 2006 | 16476125 | East Asian | Taiwanese | Han Taiwanese | Healthy individuals | 117 | 0.9988 | 0.0 | 0.0 | 0.0 | 0.0012 | 99.88 | 0.0000 | 0.0000 | 0.0000 | 0.1200 |
| 45 | Lu H-F | 2006 | 16476125 | East Asian | Taiwanese | Taiwan aborigines | Healthy individuals | 409 | 0.9872 | 0.0 | 0.0 | 0.0 | 0.0128 | 98.72 | 0.0000 | 0.0000 | 0.0000 | 1.2800 |
| 46 | Hongeng S | 2000 | 11025471 | East Asian | Thai | | Pediatric ALL patients | 75 | 0.947 | 0.0 | 0.0 | 0.0 | 0.053 | 94.7 | 0.0000 | 0.0000 | 0.0000 | 5.3000 |
| 47 | Chang Jan-Gowth | 2002 | 11927834 | East Asian | Thai | | Healthy individuals | 100 | 0.99 | 0.0 | 0.0 | 0.0 | 0.01 | 99 | 0.0000 | 0.0000 | 0.0000 | 1.0000 |
| 48 | Srimartpirom Somrudee | 2004 | 15206995 | East Asian | Thai | Northeastern Thai | Healthy individuals | 200 | 0.95 | 0.0 | 0.0 | 0.0 | 0.05 | 95 | 0.0000 | 0.0000 | 0.0000 | 5.0000 |
| 49 | Vannaprasaht Suda | 2009 | 19695401 | East Asian | Thai | | Renal transplant recipients | 139 | 0.968 | - | - | - | 0.032 | 96.8 | | | | 3.2000 |
| 50 | Lu H-F | 2005 | 16164497 | East Asian | Tibetan | | Healthy individuals | 50 | 0.99 | - | 0.0 | - | 0.01 | 99 | | 0.0000 | | 1.0000 |
| 51 | Lee Sang Seop | 2008 | 18775689 | East Asian | Vietnamese | Viet Kinh | Healthy individuals | 159 | 0.972 | 0.0 | 0.0 | 0.0 | 0.028 | 97.2 | 0.0000 | 0.0000 | 0.0000 | 2.8000 |

**SUPPLEMENTARY TABLE: TPMT ALLELE FREQUENCY IN DIFFERENT WORLDWIDE POPULATIONS**

| No. | Authors | Year | PMID | Population group | Population | Add'l population info | Subject type | N subjects genotyped | Allele frequency | | | | | Allele frequency in % | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | *1 | *2 | *3A | *3B | *3C | *1 | *2 | *3A | *3B | *3C |
| **Near Eastern** | | | | | | | | | | | | | | | | | | |
| 1 | Ronen Ofri | 2010 | 21348397 | Near Eastern | Arabs | Israeli | Healthy individuals | 118 | 0.962 | - | 0.021 | - | 0.017 | 96.2 | | 2.1000 | | 1.7000 |
| 2 | Ronen Ofri | 2010 | 21348397 | Near Eastern | Druze | Israeli | Healthy individuals | 46 | 0.891 | - | 0.076 | - | 0.033 | 89.1 | | 7.6000 | | 3.3000 |
| 3 | El-Rashedy Farida H | 2015 | 26811598 | Near Eastern | Egyptian | | Pediatric ALL patients | 25 | 0.44 | - | 0.44 | 0.04 | 0.08 | 44 | | 44.0000 | 4.0000 | 8.0000 |
| 4 | Hamdy Samar I | 2003 | 12814450 | Near Eastern | Egyptian | | Healthy individuals | 200 | 0.984 | 0.0 | 0.003 | - | 0.013 | 98.4 | 0.0000 | 0.3000 | | 1.3000 |
| 5 | Ronen Ofri | 2010 | 21348397 | Near Eastern | Ethiopian | Jewish/Israeli | Healthy individuals | 169 | 0.97 | - | 0.003 | - | 0.027 | 97 | | 0.3000 | | 2.7000 |
| 6 | Bahari Ali | 2010 | 20408054 | Near Eastern | Iranian | Southeastern Iranian | Healthy individuals | 832 | 0.94 | 0.0216 | 0.0168 | 0.0162 | 0.0054 | 94 | 2.1600 | 1.6800 | 1.6200 | 0.5400 |
| 7 | Moini Maryam | 2012 | 21938428 | Near Eastern | Iranian | Southern Iran | Healthy individuals | 500 | 0.974 | 0.001 | 0.0 | 0.0 | 0.025 | 97.4 | 0.1000 | 0.0000 | 0.0000 | 2.5000 |
| 8 | Efrati Edna | 2009 | 19048244 | Near Eastern | Israeli | Druze | Healthy individuals | 156 | 0.9606 | 0.0 | 0.0319 | 0.0 | 0.0075 | 96.06 | 0.0000 | 3.1900 | 0.0000 | 0.7500 |
| 9 | Efrati Edna | 2009 | 19048244 | Near Eastern | Israeli | Jewish | Healthy individuals | 531 | 0.9927 | 0.0 | 0.0073 | 0.0 | 0.0 | 99.27 | 0.0000 | 0.7300 | 0.0000 | 0.0000 |
| 10 | Efrati Edna | 2009 | 19048244 | Near Eastern | Israeli | Moslem | Healthy individuals | 194 | 0.9816 | 0.0 | 0.0079 | 0.0 | 0.0105 | 98.16 | 0.0000 | 0.7900 | 0.0000 | 1.0500 |
| 11 | Ronen Ofri | 2010 | 21348397 | Near Eastern | Israeli | Jewish | Healthy individuals | 164 | 0.991 | - | 0.006 | - | 0.003 | 99.1 | | 0.6000 | | 0.3000 |
| 12 | Hakooz Nancy | 2010 | 20521035 | Near Eastern | Jordanian | | Healthy individuals | 169 | 0.982 | 0.0 | 0.012 | 0.0 | 0.006 | 98.2 | 0.0000 | 1.2000 | 0.0000 | 0.6000 |
| 13 | Elawi Asma M | 2013 | 23398787 | Near Eastern | Jordanian | | Healthy individuals and rheumatoid arthritis patients | 360 | 0.993 | 0.0 | 0.0042 | 0.0028 | 0.0 | 99.3 | 0.0000 | 0.4200 | 0.2800 | 0.0000 |
| 14 | Ronen Ofri | 2010 | 21348397 | Near Eastern | Kurdish | Israeli | Healthy individuals | 73 | 0.993 | - | 0.007 | - | - | 99.3 | | 0.7000 | | |
| 15 | Phillips Paul H | 2007 | 17577869 | Near Eastern | Lebanese, Palestinian, Syrian, Iraqi | | Pediatric ALL patients | 143 | 0.99 | 0.0 | 0.01 | - | - | 99 | 0.0000 | 1.0000 | | |
| 16 | Zeglam Hamza Ben | 2015 | 25819542 | Near Eastern | Libyan | Tawargha | Healthy individuals | 38 | 0.974 | 0.0 | 0.013 | - | 0.013 | 97.4 | 0.0000 | 1.3000 | | 1.3000 |
| 17 | Zeglam Hamza Ben | 2015 | 25819542 | Near Eastern | Libyan | Tripoli | Healthy individuals | 154 | 0.984 | 0.0 | 0.006 | - | 0.01 | 98.4 | 0.0000 | 0.6000 | | 1.0000 |
| 18 | Zeglam Hamza Ben | 2015 | 25819542 | Near Eastern | Libyan | Yefren | Healthy individuals | 54 | 0.991 | 0.0 | 0.0 | - | 0.009 | 99.1 | 0.0000 | 0.0000 | | 0.9000 |
| 19 | Lennard Lynne | 2015 | 25940902 | Near Eastern | Middle Eastern | from Britain | Pediatric ALL patients | 15 | 0.933 | 0.0 | 0.067 | - | 0.0 | 93.3 | 0.0000 | 6.7000 | | 0.0000 |
| 20 | Janati Idrissi Meryem | 2015 | 25573490 | Near Eastern | Moroccan | | Healthy individuals | 103 | 1 | 0.0 | | 0.0 | 0.0 | 100 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| 21 | Ayesh Basim Mohammad | 2013 | 24499706 | Near Eastern | Palestinian | | Pediatric ALL patients | 56 | 0.9911 | 0.0 | 0.0089 | 0.0 | 0.0 | 99.11 | 0.0000 | 0.8900 | 0.0000 | 0.0000 |
| 22 | Melaouhia Salma | 2012 | 22225964 | Near Eastern | Tunisian | | Crohn's disease patients | 208 | 0.9832 | 0.0 | 0.0 | 0.0024 | 0.0144 | 98.32 | 0.0000 | 0.0000 | 0.2400 | 1.4400 |
| 23 | Ben Salah Lynda | 2013 | 23553048 | Near Eastern | Tunisian | | Crohn's disease patients | 88 | 0.949 | 0.0 | 0.0397 | 0.0 | 0.0113 | 94.9 | 0.0000 | 3.9700 | 0.0000 | 1.1300 |
| 24 | Ouerhani Slah | 2013 | 23065291 | Near Eastern | Tunisian | | Healthy individuals and ALL patients | 206 | 0.942 | 0.056 | - | - | 0.002 | 94.2 | 5.6000 | | | 0.2000 |
| 25 | Tumer Tugba Boyunegmez | 2007 | 17617792 | Near Eastern | Turkish | | Pediatric ALL patients | 106 | 0.982 | 0.0 | 0.009 | 0.0 | 0.009 | 98.2 | 0.0000 | 0.9000 | 0.0000 | 0.9000 |
| 26 | Albayrak Meryem | 2011 | 21400026 | Near Eastern | Turkish | | Pediatric ALL patients | 58 | 0.957 | 0.0 | 0.034 | 0.0 | 0.009 | 95.7 | 0.0000 | 3.4000 | 0.0000 | 0.9000 |
| **European** | | | | | | | | | | | | | | | | | | |
| 1 | Ameyaw M M | 1999 | 9931345 | European | British | | Healthy individuals | 199 | 0.947 | 0.005 | 0.045 | - | 0.003 | 94.7 | 0.5000 | 4.5000 | | 0.3000 |
| 2 | Collie-Duguid E S | 1999 | 10208641 | European | British | | Healthy individuals | 199 | 0.947 | 0.005 | 0.045 | - | 0.003 | 94.7 | 0.5000 | 4.5000 | | 0.3000 |
| 3 | McLeod H L | 1999 | 10354134 | European | British | | Pediatric ALL patients | 147 | 0.939 | 0.0 | 0.054 | - | 0.007 | 93.9 | 0.0000 | 5.4000 | | 0.7000 |
| 4 | McLeod H L | 1999 | 10634140 | European | British | | Healthy individuals | 199 | 0.947 | 0.005 | 0.045 | - | 0.003 | 94.7 | 0.5000 | 4.5000 | | 0.3000 |
| 5 | Lennard Lynne | 2015 | 25940902 | European | British | White | Pediatric ALL patients | 1965 | 0.9539 | 0.0008 | 0.041 | - | 0.004 | 95.39 | 0.0800 | 4.1000 | | 0.4000 |
| 6 | Slanar Ondrej | 2008 | 18600549 | European | Czech | | Healthy individuals and inflammatory bowel disease patients | 696 | 0.9443 | 0.001 | 0.045 | 0.0007 | 0.009 | 94.43 | 0.1000 | 4.5000 | 0.0700 | 0.9000 |
| 7 | Reuther L O | 2003 | 12492733 | European | Danish | | Crohn's disease patients | 120 | 0.95 | - | 0.05 | 0.0 | 0.0 | 95 | | 5.0000 | 0.0000 | 0.0000 |
| 8 | Toft Nina | 2006 | 17129980 | European | Danish | | Healthy individuals | 200 | 0.964 | - | 0.033 | 0.0 | 0.003 | 96.4 | | 3.3000 | 0.0000 | 0.3000 |
| 9 | Corominas H | 2000 | 11007234 | European | Dutch | | Healthy individuals and ulcerative colitis patients | 190 | 0.964 | - | 0.026 | 0.005 | 0.005 | 96.4 | | 2.6000 | 0.5000 | 0.5000 |
| 10 | Spire-Vayron de la Moureyre C | 1998 | 9831928 | European | European | | Healthy individuals and patients receiving thipurine therapy | 191 | 0.927 | 0.005 | 0.057 | 0.0 | 0.008 | 92.7 | 0.5000 | 5.7000 | 0.0000 | 0.8000 |

**SUPPLEMENTARY TABLE: TPMT ALLELE FREQUENCY IN DIFFERENT WORLDWIDE POPULATIONS**

| No. | Authors | Year | PMID | Population group | Population | Add'l population info | Subject type | N subjects genotyped | Allele frequency | | | | | Allele frequency in % | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | *1 | *2 | *3A | *3B | *3C | *1 | *2 | *3A | *3B | *3C |
| 11 | Ganiere-Monteil Catherine | 2004 | 15022030 | European | French | | | | | | | | | | 0.7500 | 3.0000 | | 0.4000 |
| 12 | Schaeffeler Elke | 2004 | 15226673 | European | German | | Healthy individuals | 1214 | 0.947 | 0.002 | 0.045 | 0.0 | 0.004 | 94.7 | 0.2000 | 4.5000 | 0.0000 | 0.4000 |
| 13 | Gazouli M | 2010 | 20175817 | European | Greek | | Pediatric inflammatory bowel disease patients | 97 | 0.8831 | 0.0319 | 0.0266 | 0.0478 | 0.0106 | 88.31 | 3.1900 | 2.6600 | 4.7800 | 1.0600 |
| 14 | Coucoutsi Constantina | 2017 | 28857898 | European | Greek | Cretan | Healthy individuals and inflammatory bowel disease patients | 342 | 0.975 | 0.006 | 0.009 | 0.004 | 0.006 | 97.5 | 0.6000 | 0.9000 | 0.4000 | 0.6000 |
| 15 | Toft Nina | 2006 | 17129980 | European | Greenlandic | | Outpatient patients | 142 | 0.919 | - | 0.081 | 0.0 | 0.0 | 91.9 | | 8.1000 | 0.0000 | 0.0000 |
| 16 | Rossi A M | 2001 | 11372592 | European | Italian | | Healthy individuals | 103 | 0.9464 | 0.0049 | 0.039 | 0.0 | 0.0097 | 94.64 | 0.4900 | 3.9000 | 0.0000 | 0.9700 |
| 17 | Rossino R | 2006 | 16789994 | European | Italian | Sardinian | Healthy individuals | 259 | 0.9652 | 0.0174 | 0.0058 | 0.0039 | 0.0077 | 96.52 | 1.7400 | 0.5800 | 0.3900 | 0.7700 |
| 18 | Christensen A F | 2009 | 19473573 | European | Italian | | Autoimmune disease patients | 78 | 0.9808 | 0.0 | 0.0192 | 0.0 | 0.0 | 98.08 | 0.0000 | 1.9200 | 0.0000 | 0.0000 |
| 19 | Serpe Loredana | 2009 | 19891552 | European | Italian | | Healthy individuals | 943 | 0.972 | | 0.022 | 0.003 | 0.003 | 97.2 | 0.0000 | 2.2000 | 0.3000 | 0.3000 |
| 20 | Larussa Tiziana | 2012 | 22385887 | European | Italian | | Inflammatory bowel disease patients | 51 | 0.93 | 0.02 | 0.0 | 0.01 | 0.04 | 93 | 2.0000 | 0.0000 | 1.0000 | 4.0000 |
| 21 | Di Salvo Angela | 2016 | 27665263 | European | Italian | Sicilian | Patients with inflammatory bowel disease, autoimmune or hematologic diseases | 184 | 0.973 | - | 0.019 | - | 0.008 | 97.3 | | 1.9000 | | 0.8000 |
| 22 | Zalizko P | 2020 | 32704308 | European | Latvian | | Inflammatory bowel disease patients | 244 | 0.9692 | 0.0021 | 0.0266 | 0 | 0.0021 | 96.92 | 0.2100 | 2.6600 | 0.0000 | 0.2100 |
| 23 | Steponaitiene Ruta | 2016 | 26674571 | European | Lithuanian | | Inflammatory bowel disease patients | 551 | 0.963 | 0.0 | 0.031 | 0.005 | 0.001 | 96.3 | 0.0000 | 3.1000 | 0.5000 | 0.1000 |
| 24 | Loennechen T | 2001 | 11503013 | European | Norwegian | Saami | Cardiology patients | 194 | 0.97 | - | 0.0 | - | 0.03 | 97 | | 0.0000 | | 3.0000 |
| 25 | Loennechen T | 2001 | 11503013 | European | Norwegian | White | Cardiology patients | 66 | 0.9164 | - | 0.076 | - | 0.0076 | 91.64 | | 7.6000 | | 0.7600 |
| 26 | Kurzawski Mateusz | 2004 | 15385838 | European | Polish | | Healthy individuals | 358 | 0.968 | 0.004 | 0.027 | - | 0.001 | 96.8 | 0.4000 | 2.7000 | | 0.1000 |
| 27 | Kurzawski Mateusz | 2005 | 16044099 | European | Polish | | Renal transplant recipients | 180 | 0.946 | 0.006 | 0.042 | - | 0.006 | 94.6 | 0.6000 | 4.2000 | | 0.6000 |
| 28 | Chrzanowska Maria | 2006 | 17220558 | European | Polish | | Hemodialysis patients | 87 | 0.942 | 0.0 | 0.052 | - | 0.006 | 94.2 | 0.0000 | 5.2000 | | 0.6000 |
| 29 | Kurzawski Mateusz | 2009 | 19229528 | European | Polish | | Renal transplant recipients | 157 | 0.94 | 0.006 | 0.048 | - | 0.006 | 94 | 0.6000 | 4.8000 | | 0.6000 |
| 30 | Chrzanowska Maria | 2012 | 22594254 | European | Polish | | Pediatric ALL patients and healthy individuals | 98 | 0.944 | 0.005 | 0.051 | - | 0.0 | 94.4 | 0.5000 | 5.1000 | | 0.0000 |
| 31 | Skrzypczak-Zielinska Marzena | 2013 | 23252704 | European | Polish | | Healthy individuals and inflammatory bowel disease patients | 274 | 0.9982 | - | 0.0 | - | - | 99.82 | | 0.0000 | | |
| 32 | Alves S | 1999 | 10376773 | European | Portuguese | Northern Portuguese | Healthy individuals | 310 | 0.976 | - | 0.024 | - | - | 97.6 | | 2.4000 | | |
| 33 | Alves S | 2001 | 11503011 | European | Portuguese | | Healthy individuals | 143 | 0.958 | 0.01 | 0.025 | - | 0.007 | 95.8 | 1.0000 | 2.5000 | | 0.7000 |
| 34 | Nasedkina Tatyana V | 2006 | 16724002 | European | Russian | | Healthy individuals and pediatric hematology patients | 700 | 0.969 | 0.0014 | 0.026 | - | 0.0036 | 96.9 | 0.1400 | 2.6000 | | 0.3600 |
| 35 | Samochatova Elena V | 2009 | 19034904 | European | Russian | | Pediatric hematology patients, other pediatric patients and healthy adults | 995 | 0.972 | 0.001 | 0.023 | - | 0.004 | 97.2 | 0.1000 | 2.3000 | | 0.4000 |
| 36 | Dokmanovic Lidija | 2006 | 17164697 | European | Serbian and Montenegrin | | Healthy individuals and pediatric ALL patients | 200 | 0.961 | 0.002 | 0.032 | 0.005 | 0.0 | 96.1 | 0.2000 | 3.2000 | 0.5000 | 0.0000 |
| 37 | Chocholova Alica | 2013 | 23581716 | European | Slovakian | | Healthy individuals and pediatric inflammatory bowel disease patients | 395 | 0.956 | 0.0 | 0.038 | 0.002 | 0.004 | 95.6 | 0.0000 | 3.8000 | 0.2000 | 0.4000 |
| 38 | Desatova B | 2013 | 23731044 | European | Slovakian | | Inflammatory bowel disease patients | 330 | 0.964 | 0.002 | 0.032 | 0.0 | 0.002 | 96.4 | 0.2000 | 3.2000 | 0.0000 | 0.2000 |

**SUPPLEMENTARY TABLE: TPMT ALLELE FREQUENCY IN DIFFERENT WORLDWIDE POPULATIONS**

| No. | Authors | Year | PMID | Population group | Population | Add'l population info | Subject type | N subjects genotyped | Allele frequency | | | | | Allele frequency in % | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | *1 | *2 | *3A | *3B | *3C | *1 | *2 | *3A | *3B | *3C |
| 39 | Milek M | 2006 | 16691038 | European | Slovenian | | Healthy individuals | 194 | 0.951 | 0.0 | 0.041 | 0.003 | 0.005 | 95.1 | 0.0000 | 4.1000 | 0.3000 | 0.5000 |
| 40 | Corominas H | 2000 | 11007234 | European | Spanish | | Healthy individuals and ulcerative colitis patients | 169 | 0.941 | - | 0.03 | 0.012 | 0.017 | 94.1 | | 3.0000 | 1.2000 | 1.7000 |
| 41 | Corominas H | 2003 | 12509611 | European | Spanish | | Rheumatoid arthritis patients | 111 | 0.964 | - | 0.027 | 0.009 | - | 96.4 | | 2.7000 | 0.9000 | |
| 42 | Bosó Virginia | 2014 | 24232128 | European | Spanish | | Solid organ transplant donors and recipients | 569 | 0.998 | 0.002 | - | - | - | 99.8 | 0.2000 | | | |
| 43 | Díaz-Villamarín X | 2023 | 37857254 | European | Spanish | | | 149 | 0.9494 | 0.007 | 0.0302 | 0 | 0.0134 | 94.94 | 0.7000 | 3.0200 | 0.0000 | 1.3400 |
| 44 | Casajús A | 2022 | 35192152 | European | Spanish | | | 109 | 0.9633 | 0 | 0.0367 | 0 | 0 | 96.33 | 0.0000 | 3.6700 | 0.0000 | 0.0000 |
| 45 | Haglund Sofie | 2004 | 14656901 | European | Swedish | | Healthy individuals | 800 | 0.95633 | 0.0006 | 0.0375 | 0.0012 | 0.00437 | 95.63 | 0.0600 | 3.7500 | 0.1200 | 0.4370 |
| 46 | Hindorf U | 2004 | 15545169 | European | Swedish | Blekinge County | Inflammatory bowel disease patients | 55 | 0.946 | - | 0.045 | - | 0.009 | 94.6 | | 4.5000 | | 0.9000 |
| **Sub-Saharan African** | | | | | | | | | | | | | | | | | | |
| 1 | Oliveira E | 2007 | 17473918 | Sub-Saharan African | Angolan | Cabinda | Healthy individuals | 103 | 0.937 | 0.0 | 0.0 | - | 0.039 | 93.7 | 0.0000 | 0.0000 | | 3.9000 |
| 2 | Lennard Lynne | 2015 | 25940902 | Sub-Saharan African | Black | from Britain | Pediatric ALL patients | 55 | 0.982 | 0.0 | 0.009 | 0.0 | 0.009 | 98.2 | 0.0000 | 0.9000 | 0.0000 | 0.9000 |
| 3 | Ameyaw M M | 1999 | 9931345 | Sub-Saharan African | Ghanaian | | Healthy individuals | 217 | 0.924 | 0.0 | 0.0 | - | 0.076 | 92.4 | 0.0000 | 0.0000 | | 7.6000 |
| 4 | Yen-Revollo J L | 2009 | 19546880 | Sub-Saharan African | Ghanaian | Akwapim | Healthy individuals | 90 | 0.94 | - | - | - | 0.06 | 94 | | | | 6.0000 |
| 5 | Yen-Revollo J L | 2009 | 19546880 | Sub-Saharan African | Ghanaian | Ashanti | Healthy individuals | 103 | 0.93 | - | - | - | 0.07 | 93 | | | | 7.0000 |
| 6 | Yen-Revollo J L | 2009 | 19546880 | Sub-Saharan African | Ghanaian | Ewe | Healthy individuals | 183 | 0.93 | - | - | - | 0.07 | 93 | | | | 7.0000 |
| 7 | Yen-Revollo J L | 2009 | 19546880 | Sub-Saharan African | Ghanaian | Fanti | Healthy individuals | 160 | 0.92 | - | - | - | 0.08 | 92 | | | | 8.0000 |
| 8 | Yen-Revollo J L | 2009 | 19546880 | Sub-Saharan African | Ghanaian | Ga | Healthy individuals | 183 | 0.94 | - | - | - | 0.06 | 94 | | | | 6.0000 |
| 9 | McLeod H L | 1999 | 10634140 | Sub-Saharan African | Kenyan | | Healthy individuals | 101 | 0.946 | 0.0 | 0.0 | - | 0.054 | 94.6 | 0.0000 | 0.0000 | | 5.4000 |
| 10 | Heckmann Jeannine M | 2005 | 15792824 | Sub-Saharan African | South African | Black | Healthy individuals and neurology patients | 227 | 0.965 | 0.0 | 0.0 | - | 0.035 | 96.5 | 0.0000 | 0.0000 | | 3.5000 |
| 11 | Heckmann Jeannine M | 2005 | 15792824 | Sub-Saharan African | South African | Mixed ancestry | Healthy individuals and neurology patients | 272 | 0.974 | 0.0 | 0.004 | - | 0.022 | 97.4 | 0.0000 | 0.4000 | | 2.2000 |
| **Latino** | | | | | | | | | | | | | | | | | | |
| 1 | Lu H-F | 2005 | 16164497 | Latino | Bolivian | | Healthy individuals | 115 | 0.9348 | - | 0.0652 | - | 0.0 | 93.48 | | 6.5200 | | 0.0000 |
| 2 | Silva Marcilene Rezende | 2008 | 19057372 | Latino | Brazilian | | Pediatric ALL patients | 116 | 0.952 | 0.0 | 0.039 | 0.0 | 0.009 | 95.2 | 0.0000 | 3.9000 | 0.0000 | 0.9000 |
| 3 | Farfan Mauricio J | 2014 | 24774509 | Latino | Chilean | | Pediatric ALL patients | 103 | 0.961 | 0.0 | 0.034 | 0.0 | 0.005 | 96.1 | 0.0000 | 3.4000 | 0.0000 | 0.5000 |
| 4 | Isaza C | 2003 | 12949626 | Latino | Colombian | Mestizo | Healthy individuals | 140 | 0.96 | 0.004 | 0.036 | 0.0 | 0.0 | 96 | 0.4000 | 3.6000 | 0.0000 | 0.0000 |
| 5 | Taja-Chayeb Lucia | 2008 | 18188716 | Latino | Mexican | | Healthy individuals and ALL patients | 147 | 0.908 | 0.014 | 0.044 | 0.017 | 0.017 | 90.8 | 1.4000 | 4.4000 | 1.7000 | 1.7000 |
| 6 | Ramos Marco A | 2011 | 21254844 | Latino | Mexican | Baja California | Healthy individuals | 150 | 0.947 | 0.0 | 0.03 | 0.003 | 0.02 | 94.7 | 0.0000 | 3.0000 | 0.3000 | 2.0000 |
| 7 | Jiménez-Morales Silvia | 2016 | 28476189 | Latino | Mexican | | Healthy individuals and pediatric ALL patients | 849 | 0.952 | 0.002 | 0.041 | 0.001 | 0.004 | 95.2 | 0.2000 | 4.1000 | 0.1000 | 0.4000 |
| 8 | Ramirez-Florencio Mireya | 2018 | 29264794 | Latino | Mexican | | Lupus patients and rheumatoid arthritis patients | 553 | 0.945 | 0.005 | 0.044 | 0.002 | 0.004 | 94.5 | 0.5000 | 4.4000 | 0.2000 | 0.4000 |

Research Article

# Role of hematic iron and anemia in SARS-CoV-2 pathogenesis

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**Keywords**

Iron, anemia, hemolysis, morbidity, COVID-19

**Abstract**

**Background**: The role of anemia and iron deficit in the pathogenesis of SARS-CoV-2 is not well established. Anemia is a common finding in patients infected with SARS-CoV-2, however few studies analyze the impact of iron metabolism changes in disease progression during SARS-CoV-2 infection. Our study analyses the influence of hemoglobin and red blood cell iron deficit at the time of infection in the prognosis of patients with COVID.

**Materials and Methods**: This observational retrospective study collected and analyzed data from a cohort of unvaccinated patients, collecting data on variables such as erythrocyte indices associated with iron deficiency, hemoglobin and several analytical variables associated with inflammation, and analyzing its correlation with clinical outcome. Patients were classified into three groups: non-anemic, anemic (non-iron deficiency) and iron deficiency anemic (IDA). We looked for the impact of those parameters and classification on disease progression.

**Results**: We collected data of 435 patients with COVID infection, 322 patients with anemia and 113 without anemia as controls. Among patients with anemia, 159 had IDA and 163 were non-IDA patients. As expected, anemic patients had worse clinical evolution compared to non-anemic patients: ward admission 71.7% vs. 42.4%, $p<0.001$; ICU admission 18% vs. 7%, $p=0.03$. Interestingly, patients presenting with IDA at the onset of infection showed a better outcome when compared to non-iron deficiency anemic patients, with lower rate (56.6% vs. 86.5%, $p<0.001$) and duration (8 vs. 15 days, $p<0.001$) of admission to ward, ICU admission (8.1% vs. 27.6%, $p<0.001$) and length of ICU stay (17 vs. 23 days, $p<0.001$). Furthermore, patients with IDA showed less pronounced signs of an inflammatory process, as reflected by lower CRP (114 vs. 168 mg/L, $p<0.001$) and ferritin levels (301 vs. 1026 g/L, $p<0.001$). Other factors as age, sex, presence of comorbidities, ratio lymphocytes/neutrophils and maximum COHb concentration exhibited a significant influence on patient's outcome. Multivariate regression analysis showed that presence of IDA remains an independent prognostic factor that protect patients from admission to ward and/or ICU.

**Conclusion**: Our findings highlight the importance of evaluating the iron status, particularly iron deficiency anemia, in patients with COVID-19, as it is associated with a more favorable prognosis. Patients with iron deficiency anemia exhibit a more favorable outcome compared to other anemic patients. This association remains significant even after adjusting for confounding factors such as age, sex, and the presence of other comorbidities.

## Introduction

The clinical manifestations of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are highly variable, and chronic conditions and comorbidities have a great impact on prognosis. Anemia is a common finding in patients infected with SARS-CoV-2. It may exist as a prior condition, or appear during the infection, possibly due to the hemolytic effect of the coronavirus [1,2]. There is contradictory data about the impact of anemia on the outcome of the disease. Some studies have reported similar hemoglobin (Hb) concentrations in fatal COVID-19 cases or those requiring intensive care admission (ICU) to those with milder clinical presentations [3]. Other studies report a clear association between anemia and prognosis [4]. Anemia may influence mortality through tissue hypoperfusion, or it may indirectly affect elderly, frail patients and impact their quality of life. Iron deficiency is the most common cause of anemia.

Iron is a fundamental element for immune system development and function, specifically for the proliferation and maturation of immune cells. Iron is involved in the inflammatory response. A deficiency of this mineral significantly impairs the body's ability to mount an immune response against infectious agents, causing a lower leukocyte count (particularly lymphocytes), and hinders its capacity to neutralize pathogens. The importance of iron in immunity and infection is evidenced by the fact that mammalian hosts have evolved multiple mechanisms of innate immunity (ex. transferrin and lactoferrin proteins) that limit the availability of the essential nutrient iron to infecting microbes [1].

On the other hand, iron overload may result in uncontrolled inflammation and immune dysfunction. Free unbound iron is very reactive and potentially toxic due to its role in the generation of reactive oxygen species (ROS) [5,6]. Excess iron is toxic to the body's cells as it produces peroxidation of cell membranes and intracellular organelles. Excessive levels of iron can be detrimental to the immune system by promoting oxidative stress and inflammation. Studies have suggested that iron may have different effects on different subsets of T cells, and that its effects on Treg cells may depend on the context and timing of exposure. Indeed, several of the manifestations of COVID-19, such as inflammation, hypercoagulation, hyperferritinemia, and immune dysfunction may arise directly or indirectly from iron overload, which in turn might be derived from virus-dependent dissociation of hemoglobin [7–9]. Many of the more severe clinical manifestations of COVID-19 arise from an exacerbated inflammatory process. In the lungs, uncontrolled inflammation causes alveolar damage, hyaline membrane formation and pulmonary oedema. These results in impaired gas exchange, low blood oxygen levels and facilitates the development of pneumonia [10,11]. Systemic immune dysregulation can lead to an excessive release of pro-inflammatory cytokines in response to an infection, resulting in a cytokine storm [12]. This can lead to damage in various organs and tissues, including the brain, gut, and kidneys.

The aim of the study is to examine the relationship between anemia, iron metabolism, and patient prognosis in COVID-19, and to determine whether these associations vary by age, sex, and the presence of chronic medical conditions.

## Material and methods

This is a single-centre, observational, retrospective cohort study carried out in a third-level public health hospital in northern Spain. The study collected data from patients admitted to the emergency department of our hospital with PCR-confirmed SARS-CoV-2 infection and anaemia during the first wave (from 15 March to 31 December 2020). We recorded the laboratory findings and clinical course of the patients from laboratory and clinical information systems. Main outcomes were admission to ward or UCI and death. The study followed the tenets of the Declaration of Helsinki and was approved by the Ethics Committee of Cantabria. (CEIm of Cantabria; 2023.416).

### Study sample

We selected all the subjects who arrive at the Emergency Unit of the University Hospital "Marqués de Valdecilla" at Santander (Cantabria) between March and June of 2020 who were infected with SARS-CoV$^2$ virus (PCR confirmed) and with concomitant anemia. Presence and classification of anemia were established by previous diagnosis documented in clinical records and/or by data of the hemogram at admission: Hb less than 120 g/L for woman and 130 g/L for man, mean corpuscular volume (MCV) less than 8x$^{10}$-14 L and mean corpuscular haemoglobin concentration (MCHC) less than 30 g/L. We also recorded main risk factors and comorbidities, such as chronic obstructive pulmonary disease (COPD), asthma, cancer, heart disease, diabetes, obesity, smoking, hypertension, and kidney disease. Data of unvaccinated patients with PCR-confirmed SARS-CoV-2 infection and without anemia (Hb greater than 140g/L) were collected as controls

Different biochemical and hematological parameters from routine analysis were collected, including C-reactive protein (CRP), lactate dehydrogenase (LDH), carboxyhaemoglobin, iron profile, D-dimer, hemoglobin and leucocyte count. These values were recorded both at admission and at their peak levels. Hemograms were analyzed using the Beckman Coulter DXH 800 autoanalyzer, coagulation was assessed using the Werfen ACL TOP 700, biochemistry was measured using the Siemens Atellica autoanalyzer, and blood gas analyses were conducted using the Radiometer ABL 800 Flex blood gas analyzer.

The study recorded the duration and nature of hospital admission for all patients included in the analysis. Patients were classified based on whether they were not admitted, admitted to a general ward, admitted to the intensive care unit (ICU), or deceased.

Variables were presented as either n (%) or medians with 25th

to 75th percentiles, as most biomarkers were not normally distributed. Differences between groups were evaluated using the Mann-Whitney-U test and Kruskal-Wallis test. Correlations were assessed using Pearson chi-square tests. The Mann-Whitney-U test was used to compare parameters between non-anemic and anemic patients, as well as between iron-deficient and non-iron-deficient patients within the latter group. Logistic regression was utilized to assess whether comorbidities impacted the disease's progression, with hospital admission, ICU admission, and mortality serving as endpoints. Statistical analysis was performed using SPSS version 26.0

**Role of the Funding Source**
The funding source was the Instituto de Investigación Sanitaria de Valdecilla (IDIVAL), project INNVAL[20]/15. The funders had no role in study design, data collection and analysis, preparation of the manuscript, or the decision to submit the manuscript for publication.

**Results**
In this retrospective study, we gathered and analyzed data of 322 unvaccinated patients with PCR-confirmed SARS-CoV-2 infection and anemia (159 with iron deficiency anemia and 163 with non-iron deficiency anemia) who attended our hospital in the period of the study. Data of 113 unvaccinated patients with PCR-confirmed SARS-CoV-2 infection and without anemia attended in the same period were collected as controls. The subjects included were 206 men and 229 women, with a median age of 69 years (range 19-103 years). Out of all the subjects analyzed, 285 (64.3%) required hospitalization, 71 (16%) were admitted to the ICU, and 52 (11.7%) succumbed to the disease.

Patients with SARS-CoV-2 infection and concurrent anemia had higher rates of hospitalization (71.7% vs. 42.4%, p<0.001) and longer hospital stays (11 vs. 9 days, p < 0.025) than infected patients without anemia. Furthermore, they were more frequently transferred to the ICU (18.0% vs. 7%, p=0.003) and experienced higher mortality (13.0% vs. 6.0%, p=0.031) (Table 1).

Furthermore, Table 1 also reveals that individuals with IDA had a significantly lower likelihood of requiring hospitalization (56.6% vs. 86.5%, p<0.001) and ICU admission (8.1% vs. 27.6%, p<0.001) compared to anemic patients without iron deficiency.

**Table 1**: Frequency of admission and fatal outcome, and length of hospital stays, depending on the presence of anemia and/or iron deficiency.

| | **Anemic n (%)** | | | **Non anemic n (%)** | **p** |
|---|---|---|---|---|---|
| | 322 (74.0%) | | | 113 (26.0%) | |
| | **Non-IDA n (%)** | **IDA n (%)** | **p** | | |
| | 163 (51.0%) | 159 (49.0%) | | | |
| **Age** | 74 [19 - 103] | | | 65 [30-99] | **0.006** |
| | 77 [22-103] | 67 [19-99] | **<0.001** | | |
| **Ward Admission** | 231 (71.7 %) | | | 48 (42.4 %) | **< 0.001** |
| | 141 (86.5 %) | 90 (56.6 %) | **<0.001** | | |
| **Length of Ward stay (days)** | 11 [1 - 151] | | | 9 [3-31] | **0.025** |
| | 15 [2-151] | 8 [1-78] | **<0.001** | | |
| **ICU Admission** | 58 (18.0%) | | | 8 (7.0 %) | **0.003** |
| | 45 (27.6 %) | 13 (8.1%) | **<0.001** | | |
| **Length of ICU stay (days)** | 22 [1 - 80] | | | 14.5[3-54] | 0.216 |
| | 23 [3-80] | 17 [0-57] | **<0.001** | | |
| **Death** | 42 (13.0 %) | | | 7 (6.0 %) | **0.031** |
| | 25 (15.3 %) | 17 (10.7 %) | 0.142 | | |

Furthermore, patients with IDA had shorter hospital stays both in general admission (median of 8.0 vs. 15.0 days, p<0.001) and ICU admission (median of 17.0 vs. 23.0 days, p<0.001).
Women were more likely to be anemic than men (80% vs. 67%, p=0.002). IDA was also more frequent in women than in men (58.0% vs. 38.0%, p=0.001). However, men were more prone to hospitalization than women (68.9% vs. 59.8%; p=0.030) with longer hospital stays (13.0 vs. 9.0 days, p=0.001).

In addition, men were more frequently admitted to the ICU (22.3% vs. 8.7%; p<0.001) with longer ICU stays (6.7 vs. 1.9 days, p<0.001), and higher mortality (15.0% vs. 7.8%; p=0.013) than women (see Table 2). Age did not differ significantly between sexes (70 vs. 67.5 years, p=0.143).

**Table 2**: Distribution of demographic and outcome variables according to sex.

| | Men n (%) | Women n (%) | p |
|---|---|---|---|
| | 206 (47.4%) | 229 (52.6%) | |
| **Age (years)** | 70 | 67.5 | 0.143 |
| **Anemia** | 138 (67.0%) | 184 (80.0%) | **0.002** |
| **IDA*** | 53 (38.0%) | 106 (58.0%) | **0.001** |
| **G. Admission** | 142 (68.9%) | 137 (59.8%) | **0.03** |
| **ICU** | 46 (22.3%) | 20 (8.7%) | **<0.001** |
| **Death** | 31 (15.0%) | 18 (7.8%) | **0.013** |

Tables 3 and 4 present the key biochemical and hematological parameters that have a known impact on the prognosis of COVID-19. As expected, patients with anemia displayed decreased levels of iron and hemoglobin, along with reduced lymphocyte count. Otherwise, these patients exhibited elevated levels of D-dimer, CRP, procalcitonin, and LDH (Table 3).

**Table 3**: Comparison of biochemical and hematological variables between non-anemic patients and those with anemia (all kind).

| | Anaemic patients | Non anaemic patients | p |
|---|---|---|---|
| | Median (IQR) | Median (IQR) | |
| Leukocytes (1x10^9 /L) | 6.6 [0.4 - 79.2] | 6.4 [2.3 - 37.2] | 0.717 |
| Neutrophils (1x10^9 /L) | 4.6 [0.1 - 28.0] | 4.1 [1.1 - 69.9] | 0.166 |
| Lymphocytes (1x10^9 /L) | 0.9 [0.1 - 74.9] | 1.3 [0.2 - 3.8] | **<0.001** |
| Monocytes (1x10^9 /L) | 0.5 [0 - 1.7] | 0.6 [0.1 - 3.0] | **0.023** |
| COHb (%) | 1.6 [0.2 - 4.1] | 1.2 [0 - 6] | 0.309 |
| Hb (g/L) | 112 [60 - 119] | 149 [119 - 191] | **<0.001** |
| MCHC (g/L) | 315 [181 - 395] | 338 [260 - 355] | **<0.001** |
| MCV (1x10^ - 15 L) | 87.3 [59.5 - 122.0] | 90.5 [79.0 - 105.4] | **<0.001** |
| D Dimer max (ng/mL) | 19.90 [2.23 - 1216.23] | 7.05 [0.76 - 530.46] | **<0.001** |
| Procalcitonin Max (ng/mL) | 0.2 [0.02 - 156.4] | 0.1 [0.02 - 3.5] | **<0.001** |
| LDHo (U/L) | 185.0 [5.0 - 612.0] | 165.0 [76.0 - 449.0] | **0.029** |
| LDHmax (U/L) | 317.0 [17.0 - 1072.0] | 250.0 [88.0 - 642.0] | **0.001** |
| CRPo (mg/L) | 74 [0 - 441] | 33 [5.5 - 305] | **<0.001** |
| CRPmax (mg/L) | 14 [7 - 470] | 77 [5 - 305] | **<0.001** |
| Ferritino (µg/L) | 302.5 [1.2 - 4021.0] | 350.0 [10.0 - 2808.0] | 0.305 |
| Ferritinmax (µg/L) | 743.0 [7.0 - 15472.0] | 456.0 [87.0 - 2808.0] | 0.333 |
| Fe (µmol/L) | 3.8 [0 - 30.9] | 6.2 [0.9 - 26.3] | **<0.001** |
| Femin (µmol/L) | 3.0 [0.9 - 11.4] | 3.4 [0.5 - 9.7] | 0.509 |
| Femax (µmol/L) | 12.9 [2.3 - 56.6] | 16.6 [6.8 - 25.9] | 0.077 |
| Transferrin (g/L) | 1.89 [0.83 - 4.56] | 2.02 [1.20 - 3.51] | 0.287 |
| Transferrin Saturation (%) | 7 [1 - 63] | 12 [2 - 41] | **0.001** |

On their part, patients with iron deficiency anemia (IDA) demonstrated even lower levels of iron and hemoglobin, accompanied by decreased erythrocyte count and mean corpuscular hemoglobin concentration (MCHC), along with higher lymphocyte count. Intriguingly, patients with IDA showcased lower levels of D-dimer, higher lymphocyte count, and less pronounced signs of an inflammatory process, as reflected by lower CRP and ferritin levels (Table 4).

**Table 4**: Comparison of biochemical and hematological variables between patients with anemia (IDA vs. non-IDA patients).

| | IDA | Non-IDA | p |
|---|---|---|---|
| | **Median (IQR)** | **Median (IQR)** | |
| Leukocytes (1x10^9 /L) | 6.2 [1.1 - 33.9] | 6.9 [0.4 - 79.2] | 0.053 |
| Neutrophils (1x10^9 /L) | 3.7 [0.3 - 28.0] | 5.2 [0.1 - 18.3] | **0.002** |
| Lymphocytes (1x10^9 /L) | 1.2 [0.1 - 17.9] | 0.8 [0.1 - 74.9] | **<0.001** |
| Monocytes (1x10^9 /L) | 0.5 [0 - 2.1] | 0.5 [0 - 1.7] | 0.853 |
| COHb (%) | 1.3 [0.4 - 3.2] | 1.7 [0.2 - 4.1] | 0.056 |
| Hb (g/L) | 109 [60 - 119] | 114 [68 - 119] | **<0.001** |
| MCHC (g/L) | 248 [181 - 338] | 331 [279 - 395] | **<0.001** |
| MCV (1x10^ - 15 L) | 75.7 [59.5 - 84.8] | 96.2 [83.2 - 122.0] | **<0.001** |
| D Dimer max (ng/mL) | 14.18 [2.23 - 872.28] | 25.45 [2.47 - 1216.23] | **<0.001** |
| Procalcitonin Max (ng/mL) | 0.105 [0.02 - 18.41] | 0.26 [0.03 - 156.47] | **0.056** |
| LDHo (U/L) | 219.0 [81.0 - 612.0] | 165.0 [5.0 - 451.0] | **<0.001** |
| LDHmax (U/L) | 338.0 [132.0 - 1072.0] | 304,5 [17.0 - 780.0] | 0.151 |
| CRPo (mg/L) | 55[0 - 253] | 84 [4 - 441] | **<0.001** |
| CRPmax (mg/L) | 114 [16 - 305] | 168 [7 - 470] | **<0.001** |
| Ferritino (µg/L) | 115.0 [2.0 - 1651.0] | 593.0 [75.0 - 4021.0] | **<0.001** |
| Ferritinmax (µg/L) | 301.0 [7.0 - 4752.0] | 1026.0 [85.0 - 15472.0] | **<0.001** |
| Fe (µmol/L) | 0.29 [0.04 - 1.79] | 0.45 [0.0 - 3.09] | **<0.001** |
| Femin (µmol/L) | 0.29 [0.04 - 1.79] | 0.45 [0.09 - 3.09] | 0.637 |
| Femax (µmol/L) | 1.12 [0.23 - 5.66] | 1.38 [0.34 - 3.85] | 0.058 |
| Transferrin (g/L) | 2.37 [1.06 - 4.18] | 1.70 [0.83 - 4.56] | **<0.001** |
| Transferrin Saturation (%) | 5 [1 - 30] | 10 [1 - 63] | **<0.001** |

In view of the age differences between anemic patients and control subjects, as well as between patients with different types of anemia we performed further analyses to compare homogeneous age groups. In the inspection of subjects older than 70 years (n=239), no significant differences in age were observed between anemic and non-anemic patients (83 vs 81 years, p=0.166); however, significant differences in length of hospital stay (9 vs 5, p=0.004) and frequency of admission (79% vs 65%, p=0.031) remain. Within the group of anemic patients, a similar analysis was performed, selecting patients over 50 years of age (n=256). No significant differences in age were found between iron-deficient and non-iron-deficient patients (77 vs 78 years, p=0.080), but there were significant differences in the frequency of admission ( 65.4% vs 88.5%) and the length of ward (6 vs 13) and ICU (2 vs 7) stays (p<0.001 for both variables). No significant differences in mortality were observed for any of these groups (p=0.415).

**Regression and Multivariate analysis**

To examine comprehensively the impact of age, comorbidities, as well as other demographic and analytical variables, on disease progression, we performed logistic regression analyses, with ward admission (Table 5) and transfer to the ICU and/or death (Table 6) as outcome variables. Consistent with previous findings, both sex and age were significant factors that influenced disease severity and progression. The presence of comorbidities also had a clear impact on disease evolution, with COPD patients having a 5-fold increased probability of admission to ward (OR=5.098 (95%CI 1.771–14.672); p=0.003), obese patients having around a 3-fold increase (OR=3.006 (95%CI 1.637–6.609); p=0.006), and patients with heart disease (OR=3.121 (95%CI 1.895–5.139); p<0.001) or arterial hypertension (OR=3.166 (95%CI 2.107–4.756); p<0.001) having approximately a 3-fold increase in probability of ward admission (Table 5). In general terms, the presence of any comorbidity implied a 4.5-fold increase in risk. At the analytical level, Hb and MCV values were significant predictors of disease progression (p<0.001), as well as the lymphocyte/neutrophil ratio (LNR) (OR=0.96 (95%CI 0.94–0.98); p<0.001), and the level of inflammatory parameters such as CRP (OR=1.1 (95%CI 1.025–1.194); p=0.01) or ferritin

(OR=1.001 (95%CI 1.000–1.003); p=0.015) at the beginning of the episode. The presence of anemia was a significant negative prognostic factor for hospitalization (OR=3.437 (95%CI 2.203–5.364); p<0.001), whereas among anemic patients, having iron deficiency anemia was a protective factor (OR=0.204 (95%CI 0.118–0.352); p<0.001). (Table 5)

A multivariate analysis was performed to include the most significant variables with an influence on ward admission. Two models were developed, with the first including the presence or lack of anemia and the second exploring the influence of the type of anemia (iron deficiency or not). In Model I, only sex lost its independent significance when anemia was included as a variable (Table 5). However, when iron deficiency was included in the model (Model II), only iron deficiency (OR=0.315 (95%CI 0.173–0.574); p<0.001), the presence of comorbidities, and age maintained their independent statistical significance. The presence of anemia was a significant negative prognostic factor (adjusted OR=3.733 (95%CI 2.270–6.138); p<0.001), while iron deficiency acted as a protective factor (adjusted OR=0.315 (95%CI 0.173–0.574); p<0.001).

**Table 5**: Logistic regression analysis of demographic and analytical variables and comorbidities as determinants of admission to the ward.

| | **Individual logistic regressions** | | |
|---|---|---|---|
| | **OR** | **95%CI** | **p** |
| **Demographic data** | | | |
| Age | 1.041 | 1.029 - 1.054 | <0.001 |
| Sex | 1.49 | 1.003 - 2.214 | 0.048 |
| **Comorbidities** | | | |
| Obesity | 3.006 | 1.367 - 6.609 | 0.006 |
| Diabetes | 2.577 | 1.547 - 4.293 | <0.001 |
| HBP | 3.166 | 2.107 - 4.756 | <0.001 |
| Oncologic | 2.067 | 1.052 - 4.063 | 0.035 |
| CKD | 2.579 | 1.535 - 4.333 | <0.001 |
| COPD | 5.098 | 1.771 - 14.672 | 0.003 |
| Smoker | 2.04 | 1.206 - 3.452 | 0.008 |
| Cardiopathy | 3.121 | 1.895 - 5.139 | <0.001 |
| Comorbidities | 4.504 | 2.780 - 7.296 | <0.001 |
| **Laboratory parameters** | | | |
| Haemoglobin | 0.831 | 0.758 - 0.910 | <0.001 |
| MCV | 1.038 | 1.019 - 1.057 | <0.001 |
| Neutrophils | 1.07 | 1.004 - 1.139 | 0.037 |
| Lymph/Neut Ratio | 0.962 | 0.941 - 0.984 | 0.001 |
| CRP | 1.106 | 1.025 - 1.194 | 0.01 |
| Ferritin | 1.001 | 1.000 - 1.003 | 0.015 |
| Transferrin | 0.987 | 0.982 - 0.992 | <0.001 |
| **Presence and type of anemia** | | | |
| Anaemia | 3.437 | 2.203 - 5.364 | <0.001 |
| Iron deficit anaemia | 0.204 | 0.118 - 0.352 | <0.001 |

*Inflammatory parameters correlated with the L/N Ratio. Erythrocyte parameters are reflected in the anemia and iron deficiency classification.

In cases with a worse clinical course, which required ICU admission or resulted in death (Table 6), anemia remained associated with poor prognosis (OR=2.715 (95%CI 1.447–5.093); p=0.002), while iron deficiency maintained its protective role (OR=0.329 (95%CI 0.193–0.561); p<0.001). Comorbidities continued to play a significant role (OR=2.87 (95%CI 1.43–5.79), p=0.003), although some individual comorbidities (diabetes, cancer, renal failure, and heart disease) show no significance. Age (OR=1.015 (95%CI 1.002–1.028); p=0.027) and, particularly, sex (OR=2.677 (95%CI 1.669–4.293); p<0.001) strongly influenced admission to the ICU and/or death. At the analytical level, the protective role of a high LNR (lymphocyte/neutrophil ratio) (OR=0.904 (95%CI 0.868–0.942); p<0.001) and high Hb values (OR=0.840 (95%CI 0.751–0.940); p=0.002) stood out. Parameters associated with hemolysis during the episode, such as maximum carboxyhemoglobin in non-smokers (COHb) and maximum LDH value, were associated with worse clinical outcomes, with maximum COHb (OR=2.272 (95%CI 1.353–3.813); p=0.002) being particularly noteworthy.

The multivariate analysis included the most significant variables with an influence on ICU admission or death. In Model I, which included the presence of anemia, not statistically significant and independent association was found (OR=2.575 (95%CI 0.900–7.367), p=0.078) for anemia or for the presence of comorbidities on the outcome. Instead, smoking (OR=3.335 (95%CI 1.200–9.266); p=0.021), sex (OR=2.303 (95%CI 1.011–5.246); p=0.047), and high COHb values (OR=2.092 (95%CI 1.179–3.712); p=0.012) were found to be significant risk factors for worse prognosis (Table 6, Model I). Conversely, in Model II, which focused on the role of iron deficiency, patients with elevated COHb values still had an increased risk (OR=2.822 (95%CI 1.352–5.887); p=0.006), while the presence of iron deficiency was independently associated with a protective effect (OR=0.274 (95%CI 0.097–0.769); p=0.014) (Table 6, Model II).

## Discussion

There is still much to learn about the pathophysiology of SARSCoV-2 virus infection and the determinants that govern the progression of COVID-19 disease. While risk factors such as age, comorbidities, and male sex have been explored, there are still numerous aspects left to be discovered and understood. One of the theories that explains the pathophysiology of the disease focuses on the detrimental effects of the virus on erythrocytes and the hemoglobin molecule, which can lead to anemia and tissue hypoperfusion, among other consequences.

Several studies have confirmed the prevalence of anemia among COVID-19 patients and have evaluated the impact of anemia on the prognosis of the disease. Nonetheless, given the crucial role of iron in the immune response and inflammation and the possibility of iron release as protective factor a consequence of hemoglobin destruction, some researchers have hypothesized that this could be an important determinant of cytokine storm and pathological inflammation. Hence, we aimed to investigate the influence of anemia prior to infection on the development of COVID-19 disease, and whether the presence of iron deficiency anemia with low blood iron reserves has a significant impact on the progression of the disease.

Our study demonstrates that patients with iron deficiency anemia who contract the SARS-CoV-2 virus tend to exhibit less severe disease and consequently, have a more favorable prognosis in comparison to other patients with anemia. Presence of IDA was a protective factor (OR=0.204) for hospitalization as well as for ICU admission or death (OR=0.329). This remains an independent protective factor after adjusting for other clinical relevant determinants as sex, age or presence of comorbilities. While numerous studies have been conducted on the prognosis and outcome of COVID-19 patients with anemia, to the best of our knowledge, none have compared the outcome of patients with iron deficiency anemia to that of other causes of anemia.

**Table 6**: Logistic regression analysis of demographic and analytical variables and comorbidities as determinants of ICU admission or death.

| | Individual logistic regressions | | |
|---|---|---|---|
| | **OR** | **95%CI** | **pa** |
| **Demographic data** | | | |
| Age | 1.015 | 1.002 - 1.028 | 0.027 |
| Sex | 2.677 | 1.669 - 4.293 | <0.001 |
| **Comorbidities** | | | |
| HBP | 1.623 | 1.013 - 2.601 | 0.044 |
| COPD | 3.379 | 1.692 - 6.746 | 0.001 |
| Smoker | 3.387 | 2.053 - 5.590 | <0.001 |
| Comorbidities | 2.877 | 1.430 - 5.790 | 0.003 |
| **Laboratory parameters** | | | |
| Haemoglobin | 0.84 | 0.751 - 0.940 | 0.002 |
| MCV | 1.033 | 1.01 - 1.055 | 0.004 |
| Leukocytes0 | 1.035 | 0.997 - 1.074 | 0.068 |
| Lymph/Neut Ratio | 0.904 | 0.868 - 0.942 | <0.001 |
| LDH max | 1.002 | 1.001 - 1.004 | 0.011 |
| CRP max | 1.11 | 1.073 - 1.148 | <0.001 |
| Procalcitonin max | 1.065 | 0.995 - 1.139 | 0.07 |
| COHb max | 2.272 | 1.353 - 3.813 | 0.002 |
| **Anaemia and type** | | | |
| Anaemia | 2.715 | 1.447 - 5.093 | 0.002 |
| Ferropenia | 0.329 | 0.193 - 0.561 | <0.001 |

*Inflammatory parameters correlated with the L/N Ratio. Erythrocyte parameters are reflected in the anemia and iron deficiency classification.

This comparison is particularly noteworthy as iron supplementation is often recommended as a treatment for anemic patients with SARS-CoV-2 pneumonia, which could be detrimental based on our findings [13].

Our results diverge from certain articles that report lower iron levels in severe COVID-19 patients than in those with milder disease [14,15]. A notable limitation of those studies is that they assess baseline iron reserves using parameters, such as ferritin, which are influenced by inflammatory states. Furthermore, they do not distinguish between anemic and non-anemic patients and rely on a limited sample size.

The majority of the pathophysiological mechanisms associated with SARS-CoV-2 infection pertain to the infectious and inflammatory impacts of the coronavirus. The repercussions on pulmonary function, which leads to a decrease in gas exchange capacity, have been of particular interest. However, systemic inflammatory effects have also been documented, implying that the virus's effects are observable in organs such as the liver, gastrointestinal tract, and kidneys. Other pathophysiological pathways, including hemoglobin dysfunction due to viral action and iron overload at the tissue level, have been postulated by some studies [16,17]. Several pieces of evidence indicate that the virus can affect hemoglobin by binding to the net hemoglobin chain, resulting in its denaturation and the dissociation of iron and porphyrin, thereby releasing Fe into circulation [17–19].

Numerous investigations in scientific literature explore the role of iron during infection. The outcomes of such studies display substantial variation, and an overarching consensus appears elusive [4,15,20].

Analogously, the case for anemia also lacks uniformity although a higher concurrence amongst various publications indicates that hemoglobin (Hb) deficiency correlates with poorer prognoses [3,5,6]. Clearly, lower Hb values upon admission are linked to increased disease severity. Lanser et al. observed a more pronounced decrease in Hb in severely affected patients, with worse outcomes for those presenting with anemia prior to admission [21]. It should be noted, however, that this study did not differentiate between different types of pre-existing anemia. On the other hand, certain investigations assert that survival is not correlated to hemoglobin levels but rather to parameters typically associated with hemolysis, such as red blood cell distribution width (RDW) or lactate dehydrogenase (LDH) [3]. Some researchers postulate that ferritin may influence cytokine release by macrophages, potentially establishing a detrimental feedback loop in which altered iron metabolism impairs immune response regulation, thereby exacerbating inflammation [16,22]

Anemia is a prevalent comorbidity in patients with COVID-19, it may be a previous condition or resulting from the virus-induced hyperinflammatory state combined with its hemolytic capacity. Excluding the study of Lanser and cols. none of the reported investigations analyze Hb values before infection, neither assess red blood cell parameters to determine iron status [3,4,19,21] as we did in the present study. Existing articles exploring the role of iron in COVID-19 infection have classified patients with iron deficiency based on ferritin, transferrin, and transferrin saturation index values. These parameters require cautious interpretation in the context of patients with inflammatory states, as ferritin serves as an acute-phase reactant and transferrin acts as a negative acute-phase reactant. Consequently, the inflammatory state present in COVID-19 patients may lead to an overestimation of the patient's iron stores during the initial stages and throughout the infection. The distinctive advantage of our work lies in the characterization of iron deficiency in our patients based on erythrocyte-derived parameters (MCV and MCHC). These parameters remain unaffected by the patient's inflammatory state and offer greater temporal stability than biochemical parameters, rendering a low MCV value an accurate indicator of systemic and sustained medium-term iron deficiency.

Consistent with findings from other studies, patients with anemia demonstrated worse prognoses, higher admission rates, and elevated morbidity and mortality rates, consistent with findings from other studies. Furthermore, our research reveals better outcomes in patients with iron deficiency anemia compared to those with non-iron deficiency anemia. Patients with pre-existing iron deficiency anemia displayed fewer and shorter ward and ICU admissions relative to patients with other anemia etiologies.

In our study, peak COHb levels were significantly associated with poorer prognoses (OR 2.27, p=0.002). During hemoglobin degradation, a carbon monoxide (CO) molecule is produced, which, in instances of pronounced hemolysis, results in elevated carboxyhemoglobin (COHb) levels. This parameter has been minimally investigated in COVID-19 patients, but it seems to correlate with increased severity [23], supporting the theory of hemolysis and its connection to COVID-19 severity. Numerous studies have demonstrated that other parameters related to hemolytic processes, such as LDH, RDW, and bilirubin, are elevated in COVID-19 patients, particularly in more severe cases. Our study also identified an increase in COHb values, which would be more specifically associated with a hemolytic process.

This mechanism would explain why iron overload plays a detrimental role in the prognosis of COVID-19 patients. The differences in disease severity found in our study, expressed as frequency and duration of admission, requirement for intensive care and mortality, are in line with this hypothesis. Nonetheless, non-anemic patients have a better outcome after infection than iron deficient patients. This could be explained by the fact that the morbidity and mortality of infection is closely related to tissue hypoperfusion, secondary to desaturation, which probably plays a more important role than oxidative stress.

Given these results, it would be interesting to consider treatment aimed at controlling Fe ion metabolism in those cases in which the inflammatory alteration has already begun, in order to halt oxidative stress [6,24]. Some authors suggest that iron depletion using chelators may be beneficial for the patient [25]. However, there are no interventional clinical studies in which patients have been treated with chelation, except for very small cohorts in which the assessment of chelation was not the main objective [26]. In another case report a patient with marked IDA and severe COVID infection improved after administration of erythropoietin [1,27]. The author speculates on the therapeutic mechanisms that influenced the outcome and includes that the improvement in this case may have been due not only to an increase in Hb, but also to an iron sequestration by the bone marrow that counteracts the inflammatory effect of the virus.

Our study has limitations. Primarily, it is a retrospective study and both the analytical data and the presence of comorbidities were extracted from the data in the medical record and the admission episode. Therefore, not all analytical data was collected from all patients. Although all patients were unvaccinated, the group is not homogeneous in terms of SARS-CoV-2 variants, and viral genotype was not taken into account. The groups were also not homogeneous in terms of age, although age-adjusted regression analysis and age-homogeneous subgroup analyses compensate for this shortcoming. In terms of strengths, we can mention a larger sample size than other studies on the role of iron in anemia and COVID-19, and a better assessment of iron deficiency anemia by using data on erythrocyte parameters such as MCV instead of other parameters that may be influenced by the inflammatory process.

**Conclusion**

In summary, we have found that patients with anemia have a worse prognosis in terms of hospitalization, requirement for intensive care, and mortality than other patients with COVID-19, even after adjusting for age, sex, and the presence of other comorbidities. Interestingly, among anemic patients, those with iron deficiency anemia exhibit a more favorable outcome, with a lower proportion of hospital admissions, need for intensive care, and mortality compared to other anemic patients. This association remains significant even after adjusting for the relevant confounding factors. In line, we have found that patients with iron deficiency anemia have a lower inflammatory state, as evidenced by lower levels of C-reactive protein (CRP), ferritin, and carboxyhemoglobin (COHb). Iron metabolism appears to play a crucial role in the pathogenesis of SARS-CoV-2, potentially through hemolytic effects on hemoglobin and interference with iron regulatory mechanisms. Considering the potential role of iron depletion, it would be valuable to

conduct further studies investigating the potential benefits of incorporating iron depletion strategies as adjunctive treatments to the existing therapeutic approaches. Our findings highlight the importance of evaluating the iron status, particularly iron deficiency anemia, in patients with COVID-19, as it is associated with a more favorable prognosis.

**Ethics Approval**

The study "Role of Hematic Iron and Anemia in SARS-CoV-2 Pathogenesis" was reviewed and approved by the Research Ethics Committee of Cantabria (approval code: 2023.416). The research was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. Given the retrospective nature of the study, which involved the analysis of anonymized data previously collected during routine clinical care, obtaining informed consent from participants was not feasible. The confidentiality and privacy of all participants were safeguarded throughout the study, and no identifiable information was used or disclosed.

**Author contributions**

Guillermo Velasco de Cos: Conceptualization, formal analysis, investigation, methodology, project administration, visualization, writing original draft, and writing review & editing. Armando Raul Guerra Ruiz: Conceptualization, data curation, formal analysis, investigation, methodology, project administration, supervision, visualization, validation, and writing review & editing. Rafael José García Martinez and Sarai Torres Robledillo: Investigation. María José Muruzabal Sitges: Supervision, visualization, and writing review & editing. Bernardo Alio Lavín Gómez, Seila Hernández Vicente, and David Ruiz Ochoa: Supervision and writing review & editing. María Teresa García Unzueta: Supervision.

**Disclosures**

**Conflict of interests**

The author does not have any conflict of interest to disclose in this study.

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**Data availability**

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

**Abbreviations**

IDA: Iron deficiency anemia
COHb: Carboxyhaemoglobin
LDH: Lactate dehydrogenase
Hb: Hemoglobin molecule
ROS: Reactive oxygen species
ICU: Intensive care unit
MCV: Mean corpuscular volume
MCHC: Mean corpuscular haemoglobin concentration
COPD: Chronic obstructive pulmonary disease
CRP: C-reactive protein
RDW: Red blood cell distribution width
CO: Carbon monoxide

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Research Article

# Role of Inflammatory and oxidative stress biomarkers with albuminuria: A cross sectional analysis in type 2 Diabetes Mellitus patients

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**Keywords**

Diabetic nephropathy, Type 2 diabetes mellitus, Inflammation, Oxidative stress, Albuminuria, Biomarkers

**Abstract**

**Background**: Diabetic nephropathy (DN) is a prevalent and severe complication of type 2 diabetes mellitus (T2DM), contributing to kidney disease and cardiovascular risks. Oxidative stress and inflammation play crucial roles in DN pathogenesis. This study investigates the association of inflammatory cytokines, oxidative stress markers, and cortisol with albuminuria in T2DM patients.

**Methods**: A cross-sectional study was conducted on 150 T2DM patients categorized into normoalbuminuria, microalbuminuria, and macroalbuminuria groups. Blood samples were analyzed for total antioxidant capacity (T-AOC), pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-8), high-sensitivity C-reactive protein (hsCRP), and cortisol levels. Statistical analyses included ANOVA and logistic regression.

**Results**: T2DM patients with albuminuria exhibited significantly lower T-AOC ($P=0.027$) and elevated TNF-α ($P=0.006$), IL-1 ($P<0.001$), IL-6 ($P<0.001$), IL-8 ($P<0.001$), hsCRP ($P<0.001$), and cortisol ($P<0.001$). High tertiles of TNF-α, IL-6, and hsCRP were strongly associated with increased albuminuria risk, particularly in overweight and hypertensive patients.

**Conclusion**: The findings highlight the interplay of oxidative stress, inflammation, and metabolic dysregulation in DN progression. Elevated inflammatory markers and cortisol levels correlate with albuminuria severity, emphasizing their potential as biomarkers for early DN detection. Targeting inflammatory pathways may offer therapeutic strategies to mitigate DN progression in T2DM patients.

## Introduction

Diabetic nephropathy (DN) is a common and serious complication in patients with type 2 diabetes mellitus (T2DM) and is recognized as the foremost cause of kidney disease and a major cardiovascular risk factor in the T2DM population [1]. Approximately 40% of individuals with T2DM eventually progress to DN [2]. DN is characterized by a range of abnormalities, including metabolic and hemodynamic dysregulation, aggravation of oxidative stress and the renin-angiotensin-aldosterone system, and the development of fibrosis. These factors collectively contribute to increased intraglomerular and systemic pressure, leading to symptoms indicative of renal failure, such as albuminuria, glomerular hypertrophy, and diminished glomerular filtration rate (GFR) [3]. The involvement of these diverse factors highlights the intricate and multifactorial process. DN risk can be effectively assessed by measuring albuminuria in random urine samples [4]. Thus, albuminuria is a crucial parameter for identifying diabetic nephropathy at its incipient stage and is a known risk factor for the development of overt renal failure.

Oxidative stress and the activation of inflammatory pathways are widely acknowledged as critical mediators in the initiation and development of DN. Disruption of the oxidative balance, whether through heightened oxidant production or diminished antioxidant activity, precipitates oxidative stress, resulting in renal tissue damage and injury. Oxidative damage within glomerular capillaries compromises the integrity of the glomerular filtration barrier by disrupting all of its layers. This process begins with the disruption of the functional relationship between glomerular endothelial cells and their glycocalyx layer, extending to podocytes. Consequently, this leads to significant extracellular matrix accumulation, predominantly characterized by increased production and secretion of collagen, particularly type IV collagen [5]. Additionally, research indicates that the progressive increase in free radical formation, coupled with a deficiency in antioxidants in T2DM, significantly contributes to the onset of DN [6]. These findings highlight the necessity to evaluate antioxidant levels to better understand and manage this condition. Thus, the prominent role of oxidative stress in DN underscores the intricate relationship between oxidative stress and microvascular damage, highlighting the pivotal influence of oxidant species in DN progression.

Furthermore, the involvement of immune and inflammatory pathways in the onset and progression of renal damage in T2DM has gained substantial recognition [7]. Activation of the innate immune response engages multiple cellular elements, such as macrophages and neutrophils, and triggers various acute phase responses. Furthermore, Macrophages, monocytes, and endothelial cells release pro-inflammatory cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-alpha (TNF-α), which triggers the liver to produce acute-phase proteins such as complement, C-reactive protein (CRP), and fibrinogen [8]. CRP enhances the immune response by binding to immune-complex parasites and bacteria, thereby facilitating their recognition and destruction. Additionally, it activates the classical complement pathway and induces monocytes to secrete additional tissue factors and pro-inflammatory cytokines, thereby perpetuating the inflammatory process [9].

In individuals with T2DM complicated by nephropathy, IL-6 levels are significantly elevated. Likewise, mRNA expression of IL-6 is markedly increased in renal cells, including kidney-infiltrating cells, in those with DN, compared to individuals with T2DM. Importantly, the expression levels of IL-6 mRNA are directly correlated with the extent of mesangial expansion, a defining histological feature of DN [10.11]. Furthermore, elevated IL-18 levels in DN were found to regulate the synthesis of various pro-inflammatory cytokines such as IL-1, interferon gamma (INF-γ), TNF-α, and transforming growth factor beta (TGF-β) and enhance the expression of chemokine receptors in mesangial cells, further amplifying inflammatory responses within the renal environment [12].

Cortisol, a glucocorticoid synthesized by the adrenal cortex, plays a pivotal role in the regulation of glucose, protein, and lipids metabolism [13]. Dysregulation leading to hypercortisolism has been implicated in various pathological conditions, including T2DM and cardiovascular disorders (CVDs) [14]. The incidence of hypercortisolism was also reported to be prevalent in patients with T2DM, and it was positively correlated with glycated hemoglobin (HbA1c) and albuminuria, independent of antidiabetic medications [15,16]. However, the association between serum cortisol level and cardiometabolic factors remains controversial. Some investigations, particularly those focusing on individuals with lower BMI rather than those who are overweight or obese, have yielded contradictory findings [17].

The rationale for this study is based on the hypothesis that inflammation, potentially instigated by factors beyond hyperglycemia, may significantly contribute to the pathogenesis of microvascular dysfunction, such as DN. However, the exact biological mechanisms underlying this relationship remain to be elucidated. Given this context, it is essential to investigate the total antioxidant capacity and circulating levels of various pro-inflammatory cytokines (IL-1, IL-6, IL-8, and TNF-α), acute phase protein (hsCRP), and serum cortisol in patients with T2DM. This study aims to elucidate their potential association with albuminuria, thereby advancing our understanding of the inflammatory contributions to DN.

## Materials and methods

### Study population

This cross-sectional study was conducted on patients aged greater than 18 years diagnosed with T2DM. The participants were systematically recruited from the outpatient department. The study included 150 participants, categorized into three groups: 50 individuals with T2DM with normoalbuminuria, 50 individuals with T2DM with microalbuminuria, and 50 individuals with T2DM with macroalbuminuria. Albuminuria was assessed using a single random urine sample, and the albumin-to-creatinine

ratio (ACR) was calculated to categorize participants as having normoalbuminuria (UACR < 30 mg/g), microalbuminuria (UACR between 30–300 mg/g), or macroalbuminuria (UACR > 300 mg/g). The American Diabetes Association (ADA) criteria, as described below, were used for the selection of T2DM cases: fasting plasma glucose (FPG) levels of ≥ 126 mg/dL on more than two occasions, 2-hour plasma glucose level of ≥ 200 mg/dL measured during an oral glucose tolerance test (OGTT) on one occasion, or random plasma glucose concentration of ≥ 200 mg/dl accompanied with classic symptoms of hyperglycemia or during a hyperglycemic crisis. A diagnosis of diabetes was confirmed if any of these criteria were met. The diabetes status of each participant was also confirmed through their medical records, indicating the use of oral hypoglycemic agents and/or insulin therapy. We meticulously documented the participants' medical histories, anthropometric measurements, and medicine usage. Hypertension was defined based on JNC 8 guidelines. Participants were classified as hypertensive if they had a systolic blood pressure (SBP) ≥ 140 mmHg and/or a diastolic blood pressure (DBP) ≥ 90 mmHg, as measured during the study visit, aligning with the JNC 8 recommendations for initiating treatment. Additionally, individuals were classified as hypertensive if they were currently using antihypertensive medications, regardless of their blood pressure readings at the time of the study, ensuring the identification of those with well-controlled hypertension due to medication use. By incorporating these criteria, we aimed to capture both diagnosed and treated hypertension, as well as undiagnosed or uncontrolled cases, for a comprehensive assessment of hypertension status in our study population.

**Blood investigations**

Fasting blood samples from the antecubital vein were collected by venipuncture after at least 8 hours of fasting from each patient during their examination visit. The samples were centrifuged at 2000 × g for 10 min. The plasma or serum obtained was aliquoted into polypropylene tubes for further analysis. Blood samples were analyzed for total antioxidant capacity (T-AOC) by standard ELISA method, pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-8), high-sensitivity C-reactive protein (hsCRP), and cortisol levels. Blood samples for cortisol measurement were collected in the morning, specifically between 8:00 AM and 10:00 AM, to account for the diurnal variation in cortisol levels. This timing aligns with the typical peak cortisol levels observed in the morning, ensuring consistency and reliability in our measurements. Additionally, the time of blood collection was standardized across all participants to minimize variability and ensure comparability of results.

**Ethical Consideration**

The study adhered to the ethical principles outlined in the Declaration of Helsinki (1964) and its subsequent revisions concerning biomedical research involving human subjects. This study was approved by the Institutional Human Research Ethics Committee. Prior to participation, all participants provided informed consent after being thoroughly briefed on the study protocol and objectives.

**Statistical analysis**

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 17. Continuous variables were presented as means and standard deviations, while categorical variables were expressed as numbers. Comparisons between groups were conducted using one-way Analysis of Variance (ANOVA) test. The odds ratio (OR) for detecting albuminuria risk was determined using conditional logistic regression. A p-value of less than 0.05, determined from two-sided tests was considered to denote statistical significance in all analyses.

**Results**

Table 1 provides a detailed overview of the analysis of the baseline clinical characteristics of the study participants and the statistical analysis. First findings indicated that age (P=0.104), height (P=0.131), weight (P=0.255), and BMI (P=0.788) were not significantly different among the three groups. However, systolic blood pressure (SBP) [P <0.001], diastolic blood pressure (DBP) [P=0.002], hip circumference (HC) [P=0.003], fasting blood glucose (FBS) [P <0.001], creatinine [P <0.001], total cholesterol (TC) [P <0.001], triglycerides (TG) [P <0.001] were significantly higher, and high-density lipoprotein (HDL) [P <0.001] levels were significantly higher in T2DM patients with microalbuminuria and macroalbuminuria than in those with normoalbuminuria.

**Table 1**: Baseline characteristic of the participants.

| Parameters | T2DM with normoalbuminuria | T2DM with microalbuminuria | T2DM with macroalbuminuria | P value |
|---|---|---|---|---|
| Age (years) | 42.85 ± 6.50 | 46.0 ±7.64 | 45.78 ± 10.07 | 0.1 |
| Height (m) | 1.56 ± 0.09 | 1.57 ± 0.05 | 1.59 ± 0.08 | 0.13 |
| Weight (kg) | 64.72 ± 12.61 | 62.70 ± 7.66 | 61.41 ± 9.24 | 0.25 |
| BMI (kg/m2) | 26.08 ± 4.70 | 25.55 ± 5.18 | 26.13 ± 3.98 | 0.78 |
| SBP (mm/Hg) | 127.0 ± 5.55 | 133.90 ± 6.52 | 136.35 ± 6.97 | < 0.001 |
| DBP (mm/Hg) | 78.0 ± 5.88 | 81.67 ± 2.94 | 82.65 ± 5.17 | 0.002 |
| WC (inches) | 33.26 ± 3.10 | 33.56 ± 3.83 | 34.35 ± 2.21 | 0.19 |
| HC (inches) | 36.86 ± 5.01 | 37.74 ± 4.54 | 36.95 ± 2.75 | 0.51 |
| FBS (mg/dl) | 140.92 ± 11.28 | 156.21 ± 36.8 | 168.21 ± 41.8 | <0.001 |
| Creatinine (mg/dl) | 0.94 ± 0.14 | 1.93 ± 0.72 | 3.9 ± 0.92 | < 0.001 |
| TC (mg/dl) | 172.98 ± 18.88 | 197.33 ± 22.42 | 203.33 ± 22.31 | <0.001 |
| TG (mg/dl) | 145.26 ± 2.28 | 173.52 ± 34.46 | 177.69 ± 32.05 | <0.001 |
| HDL (mg/dl) | 47.72 ± 4.06 | 43.04 ± 1.11 | 43.75 ± 0.96 | <0.001 |

Table 2 provides a comprehensive overview of the mean and standard deviation of total antioxidant capacity (ELISA based assay), circulating levels of inflammatory cytokines, hsCRP, and serum cortisol levels across different groups of study participants. Our analysis revealed that the total antioxidant capacity (T-AOC) was significantly lower in T2DM patients with microalbuminuria and macroalbuminuria than in those with normoalbuminuria (P=0.027). Furthermore, we observed significantly elevated levels of the pro-inflammatory cytokines α-TNF (P = 0.006), IL-1 (P < 0.001), IL-6 (P < 0.001), and IL-8 (P < 0.001) in T2DM patients with both microalbuminuria and macroalbuminuria, relative to those with normoalbuminuria. This indicates a heightened inflammatory state in patients with increased urinary albumin excretion, suggesting that inflammation plays a critical role in the progression of diabetic nephropathy.

**Table 2**: Total antioxidant capacity and circulating levels of inflammatory cytokines, hsCRP, and serum cortisol level in study participants.

| Parameters | T2DM with normoalbuminuria | T2DM with microalbuminuria | T2DM with macroalbuminuria | P value |
|---|---|---|---|---|
| T-AOC (mmol/l) | 2.10 ± 0.92 | 1.84 ± 0.69 | 1.71 ± 0.52 | 0.027 |
| α-TNF (pg/mL) | 11.97 ± 5.26 | 14.15 ± 2.42 | 15.17 ± 3.35 | 0.006 |
| hsCRP (mg/l) | 1.99 ± 0.78 | 3.37 ± 1.18 | 3.47 ± 0.70 | < 0.001 |
| IL-1 (pg/ml) | 4.06 ± 0.84 | 5.94 ± 1.99 | 6.49 ± 2.18 | < 0.001 |
| IL-6 (pg/ml) | 7.17 ± 1.69 | 9.30 ± 3.40 | 11.90 ± 4.31 | < 0.001 |
| IL- 8 (pg/ml) | 10.29 ± 3.66 | 12.73 ± 4.58 | 14.27 ± 4.35 | < 0.001 |
| Cortisol (mmol/l) | 357.64 ± 61.09 | 448.55 ± 135.46 | 465.55 ± 140.39 | < 0.001 |

Similarly, high-sensitivity C-reactive protein (hsCRP) (P < 0.001) and cortisol (P < 0.001) levels were significantly elevated in T2DM patients with both microalbuminuria and macroalbuminuria compared with those with normoalbuminuria. This finding reinforces the association between stress, inflammation, and kidney dysfunction in patients with T2DM.

An in-depth analysis of the association between α-TNF tertiles (Table 3), hsCRP tertiles (Table 4), IL-1 tertiles (Table 5), IL-6 tertiles (Table 6), IL-8 tertiles (Table 7), and cortisol tertiles (Table 8) with clinical characteristics demonstrated the stratified association of analytes with albuminuria in T2DM. All analytes were categorized into low, mid, and high tertiles. This stratification allows for a nuanced examination of how different analyte levels are associated with albuminuria and other variables, providing insights into the influence of analytes on albuminuria progression. The high tertile of α-TNF was significantly associated with the occurrence of microalbuminuria and macroalbuminuria in T2DM patients with a BMI > 25 kg/m², yielding odds ratios (OR) of 2.16 (95% CI: 0.65–7.19, p-trend = 0.01) and 2.4 (95% CI: 0.74–7.74, p-trend = 0.01), respectively. Additionally, there was a significant association with hypertension, with an OR of 4.16 (95% CI: 0.93–18.71, p = 0.0005) when compared to the low and mid tertiles.

**Table 3:** Association of α-TNF tertiles with clinical characteristics and albuminuria progression in T2DM patients.

| α-TNF (pg/mL) | Low tertile < 12.9 | Mid tertile 12.9-15.9 | High tertile > 15.9 | OR (95% CI) |
|---|---|---|---|---|
| **Age ≤ 45 years** | | | | |
| Controls | 20 | 4 | 5 | 1.0 (ref.) |
| T2DM with microalbuminuria | 5 | 5 | 8 | OR: 1.33; CI: 0.36 – 4.94 |
| T2DM with macroalbuminuria | 5 | 5 | 5 | OR: 0.517; CI: 0.13 – 2.07 |
| P value | 0.06 | | | |
| **Age > 45 years** | | | | |
| Controls | 14 | 3 | 4 | 1.0 (ref.) |
| T2DM with microalbuminuria | 12 | 11 | 9 | OR: 1.23; CI: 0.42 – 3.57 |
| T2DM with macroalbuminuria | 9 | 18 | 8 | OR: 0.83; CI: 0.22 – 3.1 |
| P value | 0.05 | | | |
| **BMI ≤ 25 kg/m²** | | | | |
| Controls | 13 | 4 | 5 | 1.0 (ref.) |
| T2DM with microalbuminuria | 8 | 11 | 6 | OR: 1.05; CI: 0.28 – 3.94 |
| T2DM with macroalbuminuria | 6 | 6 | 11 | OR: 2.1; CI: 0.63 – 7.04 |
| P value | 0.05 | | | |
| **BMI > 25 kg/m²** | | | | |
| Controls | 18 | 4 | 5 | 1.0 (ref.) |
| T2DM with microalbuminuria | 6 | 9 | 10 | OR: 2.16; CI: 0.65 – 7.19 |
| T2DM with macroalbuminuria | 8 | 7 | 12 | OR: 2.4; CI: 0.74 – 7.74 |
| P value | 0.01 | | | |
| **Hypertension** | | | | |
| **No** | | | | |
| Controls | 13 | 4 | 8 | 1.0 (ref.) |
| T2DM with microalbuminuria | 10 | 12 | 10 | OR: 0.97; CI: 0.34 – 2.83 |
| T2DM with macroalbuminuria | 10 | 14 | 12 | OR: 1.04; CI: 0.37 – 2.91 |
| P value | 0.23 | | | |
| **Hypertension** | | | | |
| **Yes** | | | | |
| Controls | 19 | 3 | 3 | 1.0 (ref.) |
| T2DM with microalbuminuria | 3 | 8 | 7 | OR: 3.24; CI: 0.74 – 14.26 |
| T2DM with macroalbuminuria | 3 | 4 | 7 | OR: 4.16; CI: 0.93 – 18.71 |
| P value | 0.0005 | | | |

**Table 4:** Association of hsCRP tertiles with clinical characteristics and albuminuria progression in T2DM patients.

| hsCRP (mg/L) | Low tertile< 2.8 | Mid tertile 2.8-3.6 | High tertile>3.6 | OR (95% CI) |
|---|---|---|---|---|
| **Age ≤ 45 years** | | | | |
| Controls | 14 | 9 | 3 | 1.0 (ref.) |
| T2DM with microalbuminuria | 6 | 6 | 5 | OR: 2.54; CI: 0.54– 12.08 |
| T2DM with macroalbuminuria | 5 | 5 | 11 | OR: 4.53; CI: 1.11 – 18.41 |
| P value | 0.04 | | | |
| **Age > 45 years** | | | | |
| Controls | 13 | 6 | 5 | 1.0 (ref.) |
| T2DM with microalbuminuria | 9 | 10 | 14 | OR: 2.03; CI: 0.645 – 6.42 |
| T2DM with macroalbuminuria | 6 | 7 | 16 | OR: 2.64; CI: 0.85 – 8.28 |
| P value | 0.04 | | | |
| **BMI ≤ 25 kg/m²** | | | | |
| Controls | 16 | 5 | 3 | 1.0 (ref.) |
| T2DM with microalbuminuria | 3 | 6 | 10 | OR: 4.21; CI: 1.014 – 17.48 |
| T2DM with macroalbuminuria | 4 | 5 | 14 | OR: 4.86; CI: 1.23 – 19.19 |
| P value | 0.0007 | | | |
| **BMI > 25 kg/m²** | | | | |
| Controls | 16 | 6 | 4 | 1.0 (ref.) |
| T2DM with microalbuminuria | 10 | 6 | 5 | OR: 1.54; CI: 0.36 – 6.49 |
| T2DM with macroalbuminuria | 5 | 6 | 16 | OR: 3.85; CI: 1.13 – 13.05 |
| P value | 0.005 | | | |
| **Hypertension** | | | | |
| **No** | | | | |
| Controls | 20 | 5 | 3 | 1.0 (ref.) |
| T2DM with microalbuminuria | 4 | 3 | 4 | OR: 3.39; CI: 0.65 – 17.69 |
| T2DM with macroalbuminuria | 4 | 3 | 8 | OR: 4.97; CI: 1.14 – 21.59 |
| P value | 0.02 | | | |
| **Hypertension** | | | | |
| **Yes** | | | | |
| Controls | 14 | 6 | 2 | 1.0 (ref.) |
| T2DM with microalbuminuria | 8 | 8 | 10 | OR: 4.23; CI: 0.83 – 21.39 |
| T2DM with macroalbuminuria | 6 | 6 | 23 | OR: 7.22; CI: 1.54 – 33.72 |
| P value | 0.0004 | | | |

**Table 5:** Association of IL-1 tertiles with clinical characteristics and albuminuria progression in T2DM patients.

| IL-1 (pg/mL) | Low tertile< 4.5 | Mid tertile 4.5-6.0 | High tertile> 6.0 | OR (95% CI) |
|---|---|---|---|---|
| **Age ≤ 45 years** | | | | |
| Controls | 20 | 8 | 2 | 1.0 (ref.) |
| T2DM with microalbuminuria | 5 | 5 | 6 | OR: 5.62; CI: 1.01 – 31.14 |
| T2DM with macroalbuminuria | 5 | 8 | 8 | OR: 5.71; CI: 1.1 – 29.65 |
| P value | 0.01 | | | |
| **Age > 45 years** | | | | |
| Controls | 11 | 7 | 2 | 1.0 (ref.) |
| T2DM with microalbuminuria | 12 | 8 | 14 | OR: 2.6; CI: 0.71 – 9.5 |
| T2DM with macroalbuminuria | 5 | 7 | 17 | OR: 3.51; CI: 1.04 – 11.86 |
| P value | 0.01 | | | |
| **BMI ≤ 25 kg/m²** | | | | |
| Controls | 14 | 6 | 4 | 1.0 (ref.) |
| T2DM with microalbuminuria | 8 | 5 | 10 | |
| T2DM with macroalbuminuria | 4 | 6 | 12 | |
| P value | 0.04 | | | |
| **BMI > 25 kg/m²** | | | | |
| Controls | 13 | 8 | 5 | 1.0 (ref.) |
| T2DM with microalbuminuria | 5 | 8 | 14 | OR: 2.69; CI: 0.85 – 8.55 |
| T2DM with macroalbuminuria | 5 | 8 | 15 | OR: 2.78; CI: 0.88 – 8.74 |
| P value | 0.02 | | | |
| **Hypertension** | | | | |
| **No** | | | | |
| Controls | 18 | 8 | 2 | 1.0 (ref.) |
| T2DM with microalbuminuria | 4 | 2 | 9 | OR: 8.4; CI: 1.6 – 43.9 |
| T2DM with macroalbuminuria | 4 | 2 | 10 | OR: 8.75; CI: 1.7 – 45.0 |
| P value | 0.0008 | | | |
| **Hypertension** | | | | |
| **Yes** | | | | |
| Controls | 9 | 9 | 4 | 1.0 (ref.) |
| T2DM with microalbuminuria | 9 | 9 | 9 | OR: 1.83; CI: 0.49 – 6.76 |
| T2DM with macroalbuminuria | 5 | 10 | 19 | OR: 3.07; CI: 0.92 – 10.24 |
| P value | 0.04 | | | |

**Table 6:** Association of IL-6 tertiles with clinical characteristics and albuminuria progression in T2DM patients.

| IL-6 (pg/mL) | Low tertile < 7.0 | Mid tertile 7.0-12.0 | High tertile > 12.0 | OR (95% CI) |
|---|---|---|---|---|
| **Age ≤ 45 years** | | | | |
| Controls | 12 | 16 | 2 | 1.0 (ref.) |
| T2DM with microalbuminuria | 5 | 7 | 5 | OR: 4.41; CI: 0.77 – 25.24 |
| T2DM with macroalbuminuria | 4 | 7 | 10 | OR: 7.14; CI: 1.41 – 35.91 |
| P value | 0.02 | | | |
| **Age > 45 years** | | | | |
| Controls | 7 | 10 | 3 | 1.0 (ref.) |
| T2DM with microalbuminuria | 9 | 15 | 9 | OR: 2.0; CI: 0.48 – 8.22 |
| T2DM with macroalbuminuria | 4 | 10 | 15 | OR: 3.7; CI: 0.97 – 14.74 |
| P value | 0.07 | | | |
| **BMI ≤ 25 kg/m²** | | | | |
| Controls | 9 | 10 | 3 | 1.0 (ref.) |
| T2DM with microalbuminuria | 5 | 13 | 5 | |
| T2DM with macroalbuminuria | 3 | 13 | 8 | |
| P value | 0.19 | | | |
| **BMI > 25 kg/m²** | | | | |
| Controls | 9 | 15 | 4 | 1.0 (ref.) |
| T2DM with microalbuminuria | 5 | 17 | 5 | OR: 1.29; CI: 0.31 – 5.34 |
| T2DM with macroalbuminuria | 3 | 11 | 12 | OR: 3.23; CI: 0.92 – 11.29 |
| P value | 0.03 | | | |
| **Hypertension** | | | | |
| **No** | | | | |
| Controls | 10 | 14 | 2 | 1.0 (ref.) |
| T2DM with microalbuminuria | 3 | 15 | 3 | OR: 1.85; CI: 0.28 – 12.16 |
| T2DM with macroalbuminuria | 3 | 7 | 7 | OR: 5.35; CI: 0.99 – 28.9 |
| P value | 0.02 | | | |
| **Hypertension** | | | | |
| **Yes** | | | | |
| Controls | 7 | 15 | 2 | 1.0 (ref.) |
| T2DM with microalbuminuria | 7 | 17 | 5 | OR: 2.06; CI: 0.36 – 11.63 |
| T2DM with macroalbuminuria | 3 | 16 | 14 | OR: 5.09; CI: 1.05 – 24.52 |
| P value | 0.02 | | | |

**Table 7:** Association of IL-8 tertiles with clinical characteristics and albuminuria progression in T2DM patients.

| IL-8 (pg/mL) | Low tertile < 11.0 | Mid tertile 11.0-14.0 | High tertile > 14.0 | OR (95% CI) |
|---|---|---|---|---|
| Controls | 17 | 5 | 5 | 1.0 (ref.) |
| T2DM with microalbuminuria | 5 | 5 | 7 | OR: 2.22; CI: 0.6 – 8.14 |
| T2DM with macroalbuminuria | 5 | 5 | 11 | OR: 2.82; CI: 0.85 – 9.4 |
| P value | 0.04 | | | |
| **Age > 45 years** | | | | |
| Controls | 14 | 4 | 5 | 1.0 (ref.) |
| T2DM with microalbuminuria | 10 | 10 | 13 | OR: 2.09; CI: 0.66 – 6.56 |
| T2DM with macroalbuminuria | 4 | 11 | 14 | OR: 2.06; CI: 0.64 – 6.62 |
| P value | 0.01 | | | |
| **BMI ≤ 25 kg/m²** | | | | |
| Controls | 16 | 4 | 4 | 1.0 (ref.) |
| T2DM with microalbuminuria | 6 | 5 | 8 | OR: 2.52; CI: 0.65 – 9.67 |
| T2DM with macroalbuminuria | 5 | 8 | 7 | OR: 2.1; CI: 0.53 – 8.21 |
| P value | 0.04 | | | |
| **BMI > 25 kg/m²** | | | | |
| Controls | 13 | 8 | 5 | 1.0 (ref.) |
| T2DM with microalbuminuria | 5 | 15 | 11 | OR: 1.84; CI: 0.56 – 5.99 |
| T2DM with macroalbuminuria | 6 | 10 | 14 | OR: 2.42; CI: 0.76 – 7.65 |
| P value | 0.02 | | | |
| **Hypertension** | | | | |
| **No** | | | | |
| Controls | 16 | 14 | 4 | 1.0 (ref.) |
| T2DM with microalbuminuria | 6 | 8 | 5 | OR: 2.23; CI: 0.53 – 9.34 |
| T2DM with macroalbuminuria | 3 | 8 | 10 | OR: 4.04; CI: 1.12 – 14.46 |
| P value | 0.02 | | | |
| **Hypertension** | | | | |
| **Yes** | | | | |
| Controls | 14 | 6 | 4 | 1.0 (ref.) |
| T2DM with microalbuminuria | 10 | 9 | 12 | OR: 1.16; CI: 0.41 – 3.28 |
| T2DM with macroalbuminuria | 6 | 8 | 15 | OR: 1.55; CI: 0.56 – 4.27 |
| P value | 0.04 | | | |

**Table 8:** Association of cortisol tertiles with clinical characteristics and albuminuria progression in T2DM patients.

| Cortisol (nmol/L) | Low tertile < 350.0 | Mid tertile 350.0-450.0 | High tertile > 450.0 | OR (95% CI) |
|---|---|---|---|---|
| **Age ≤ 45 years** | | | | |
| Controls | 11 | 14 | 5 | 1.0 (ref.) |
| T2DM with microalbuminuria | 5 | 6 | 7 | OR: 2.33; CI: 0.64 – 8.45 |
| T2DM with macroalbuminuria | 5 | 6 | 12 | OR: 3.13; CI: 0.96 – 10.14 |
| P value | 0.1 | | | |
| **Age > 45 years** | | | | |
| Controls | 7 | 8 | 5 | 1.0 (ref.) |
| T2DM with microalbuminuria | 8 | 16 | 8 | OR: 1.00; CI: 0.28 – 3.48 |
| T2DM with macroalbuminuria | 6 | 8 | 13 | OR: 1.92; CI: 0.59 – 6.28 |
| P value | 0.12 | | | |
| **BMI ≤ 25 kg/m²** | | | | |
| Controls | 10 | 9 | 3 | 1.0 (ref.) |
| T2DM with microalbuminuria | 4 | 10 | 8 | OR: 2.66; CI: 0.62 – 11.39 |
| T2DM with macroalbuminuria | 2 | 11 | 10 | OR: 3.18; CI: 0.77 – 13.14 |
| P value | 0.03 | | | |
| **BMI > 25 kg/m²** | | | | |
| Controls | 12 | 13 | 4 | 1.0 (ref.) |
| T2DM with microalbuminuria | 6 | 14 | 8 | OR: 1.57; CI: 0.41 – 5.9 |
| T2DM with macroalbuminuria | 3 | 10 | 14 | OR: 1.83; CI: 0.49 – 6.76 |
| P value | 0.01 | | | |
| **Hypertension** | | | | |
| **No** | | | | |
| Controls | 11 | 14 | 3 | 1.0 (ref.) |
| T2DM with microalbuminuria | 4 | 15 | 5 | OR: 1.94; CI: 0.42 – 8.95 |
| T2DM with macroalbuminuria | 2 | 9 | 6 | OR: 3.29; CI: 0.72 – 14.93 |
| P value | 0.1 | | | |
| **Hypertension** | | | | |
| **Yes** | | | | |
| Controls | 9 | 10 | 3 | 1.0 (ref.) |
| T2DM with microalbuminuria | 4 | 15 | 7 | OR: 1.97; CI: 0.45 – 8.56 |
| T2DM with macroalbuminuria | 5 | 16 | 12 | OR: 2.66; CI: 0.67 – 10.55 |
| P value | 0.1 | | | |

In continuation of our findings, the analysis demonstrated that elevated levels of hsCRP (Table 4), IL-1 (Table 5), and IL-8 (Table 7), were significantly associated with an increased risk of both microalbuminuria and macroalbuminuria in T2DM patients, regardless of age, BMI, and blood pressure. Notably, the risk of macroalbuminuria is higher than that of microalbuminuria.

Moreover, for patients aged ≤ 45 years and with a BMI greater than 25 kg/m², the high tertile of IL-6, in contrast to the mid and low tertiles, showed a significant association with an increased risk of both microalbuminuria and macroalbuminuria, irrespective of their blood pressure (Table 6). In addition, a high tertile of cortisol was significantly associated with an increased risk of both microalbuminuria and macroalbuminuria in patients with T2DM, irrespective of their BMI (Table 8).

**Discussion**

DN is characterized by a gradual decrease in GFR, the presence of albuminuria, and an increase in arterial blood pressure [17]. It has emerged as the leading cause of chronic kidney disease (CKD) in India, with matching trends observed in Western societies. It is one of the most serious long-term consequences in patients with diabetes, with considerable morbidity and mortality rates. In India, diabetes accounts for a significant proportion of end-stage renal disease (ESRD) cases, demonstrating the urgent need to understand the mechanism of its occurrence [18].

Our analysis revealed that baseline characteristics such as age, height, weight, BMI, WC, and HC were evenly distributed among the study groups (Table1), decreasing the possibility that our findings were influenced by demographic and anthropometric variables. As a result, any relationship between inflammatory biomarkers and microalbuminuria can be more firmly assigned to the clinical processes under study rather than variations in these underlying traits. However, patients with increased urinary albumin excretion exhibited more pronounced cardiometabolic risk factors, such as SBP, DBP, TC, TG, HDL, and creatinine (Table 1), indicating a higher likelihood of cardiovascular complications and renal impairment. These observations underscore the importance of the early detection and management of albuminuria in patients with T2DM to mitigate the associated risks and improve clinical outcomes.

T2DM patients with microalbuminuria and macroalbuminuria exhibited significantly lower T-AOC than those with normoalbuminuria (P=0.027). These findings emphasize the potential significance of oxidative stress in the progression of kidney damage in patients with T2DM, which is consistent with the literature stressing oxidative stress as a major contributor to diabetic sequelae, specifically, nephropathy. Oxidative stress in type 2 diabetes is caused by persistent hyperglycemia, which accelerates the production of reactive oxygen species (ROS) via mechanisms such as glucose auto-oxidation and the generation of advanced glycation end products (AGEs) [19]. Microalbuminuria, an early hallmark of diabetic nephropathy, is caused by this, which intensifies renal inflammation and fibrosis and activates vital signaling pathways like NF-κB and upregulates pro-inflammatory genes like IL-6 and TNF-α. This genetic modification of the inflammatory and fibrotic pathways reveals the importance of oxidative stress in diabetic kidney injury [20]. Various studies have reported a direct relationship between declining kidney function and increasing T-AOC reduction in patients with DN, connecting oxidative stress biomarkers to the advancement of DN [21,22]. An additional study provided more evidence that reduced antioxidant defenses, especially in the serum, are important in hastening the start of microalbuminuria by encouraging oxidative damage in renal tissues [23].

The noteworthy increase in pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6, and IL-8, in T2DM patients exhibiting microalbuminuria and macroalbuminuria suggests that individuals with DN are experiencing an elevated inflammatory state. The role of chronic inflammation in DN pathogenesis is well documented, with cytokines such as TNF-α, IL-1, IL-6, and IL-8 contributing to endothelial dysfunction, glomerular inflammation, and renal fibrosis [12]. The significant association between high TNF-α levels and an increased risk of microalbuminuria and macroalbuminuria in T2DM patients with BMI > 25 kg/m², as well as with hypertension, reveals the role of TNF-α in promoting inflammation and vascular dysfunction. Elevated TNF-α, for instance, has been shown to induce podocyte apoptosis, disrupt the glomerular filtration barrier, and promote albuminuria by increasing glomerular permeability [24]. IL-6 is another critical player, as it has been implicated in promoting mesangial cell proliferation and matrix expansion, both of which contribute to glomerulosclerosis, a hallmark of DN [25]. The significant association between high IL-6 levels and increased risk of both microalbuminuria and macroalbuminuria, particularly in younger T2DM patients (≤ 45 years) with a BMI >25 kg/m², irrespective of blood pressure, underlines the role of IL-6 in mediating inflammatory responses that contribute to early kidney damage. These findings suggest that elevated IL-6 levels may serve as a biomarker for early renal impairment in younger overweight individuals with T2DM, independent of hypertension status. This is consistent with previous evidence linking IL-6 with obesity-related inflammation and the progression of kidney disease in diabetes [26]. Similarly, IL-1 and IL-8 have been associated with the recruitment of immune cells, further amplifying inflammation and oxidative stress, leading to dysfunction of proximal tubules and podocyte damage in patients with T2DM [27].

These elevated cytokine levels suggest a sustained inflammatory response that correlates with albuminuria severity, indicating a progressive decline in renal health. This finding aligns with previous research showing that inflammation is a central mechanism in the progression from microalbuminuria to overt nephropathy in T2DM. Targeting these inflammatory pathways may offer a therapeutic strategy for preventing or delaying the progression of kidney damage in patients with diabetes. Anti-inflammatory agents, particularly those that inhibit IL-6 and TNF-α, have shown promise in preclinical models and warrant

further investigation in the context of DN [28].

Elevated cortisol levels in DN (Table 2) may reflect the chronic stress state and altered hypothalamic-pituitary-adrenal (HPA) axis regulation commonly observed in patients with T2DM. Chronic hyperglycemia and insulin resistance exacerbate HPA axis activity, leading to sustained increases in cortisol secretion. Hypercortisolemia not only contributes to metabolic dysregulation, but also exacerbates inflammation, oxidative stress, and endothelial dysfunction, all of which are key factors in the progression of DN. Recent evidence also suggests that cortisol may directly influence kidney function by promoting glomerular hyperfiltration, increasing renal sodium retention, and stimulating the production of angiotensin II, thereby accelerating the development of nephropathy in T2DM patients [29].

Similarly, hsCRP, a marker of systemic inflammation, is consistently elevated in patients with diabetic nephropathy, reflecting ongoing inflammatory processes that contribute to the progression of renal impairment. hsCRP is produced by the liver in response to pro-inflammatory cytokines such as IL-6, and its elevated levels in T2DM patients with nephropathy suggest a state of chronic low-grade inflammation. Inflammation is known to play a critical role in endothelial dysfunction, promoting atherosclerosis and further compromising renal blood flow. Moreover, high hsCRP levels have been associated with increased albuminuria, suggesting a direct link between systemic inflammation and glomerular injury in diabetic nephropathy [30]. The combined elevation of cortisol and hsCRP levels in DN may emphasize the interplay between stress, inflammation, and metabolic dysregulation in the pathogenesis of renal complications in T2DM. Therapeutic strategies aimed at reducing cortisol levels, possibly through stress management techniques or pharmacological interventions, alongside anti-inflammatory approaches targeting hsCRP and related cytokines, could potentially mitigate DN progression. Further research is warranted to explore the efficacy of such interventions in clinical settings.

This cross-sectional study has several limitations that should be considered when interpreting the results. First, the design of the study limits the ability to establish causal relationships between elevated inflammatory biomarkers, oxidative stress markers, and the progression of kidney disease in T2DM patients. Longitudinal studies are needed to clarify the temporal sequence of these associations. Second, although the study was adjusted for key confounders such as BMI, age, and blood pressure, unmeasured factors such as dietary habits, physical activity, and genetic predispositions may have influenced the observed relationships. Additionally, reliance on single-point measurements of biomarkers and albuminuria might not fully capture the dynamic changes in these parameters over time, leading to potential misclassification. The generalizability of the findings is also a concern, as the study sample may not represent the broader population of T2DM patients and ethnic, geographical, or socioeconomic differences could impact the biomarkers and outcomes studied. Lastly, potential measurement biases in the assessment of biomarkers and albuminuria due to variability in laboratory methods or sample handling may affect the accuracy and reliability of the results. These limitations highlight the need for further research to validate and extend these findings.

## Conclusion

The study findings indicate that pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-8), hsCRP, and cortisol, coupled with a reduction in total antioxidant capacity (T-AOC), were significantly associated with albuminuria in patients with T2DM, highlighting their potential roles in the progression of DN. This study revealed the intricate interplay between metabolic dysregulation, chronic inflammation, and oxidative damage in the pathogenesis of DN.

## Competing interests

The authors declare that they have no competing interests to disclose.

## Declaration of conflict of interest

The authors affirm that this study is free from any conflicts of interest.

## Ethical Approval

This investigation was conducted in accordance with the ethical guidelines of the Declaration of Helsinki on biomedical research on humans and was approved by the Institutional Human Research Ethical Committee.

## Credit Authors statement

**Deepak Parchwani:** Conceptualization, Methodology, Review, Editing.

**UdayVachhani**: Conceptualization, Project administration, Formal Analysis, Investigation.

**Sagar Dholariya**: Original Draft, Data curation, editing.

**Ashishkumar Agravatt**: Analysis, Validation, review and editing.

**Ragini Singh**: Original draft, software, data curation, and validation.

**Amit Sonagra**: Software, review and editing, supervision.

The corresponding author affirms that all listed authors have reviewed and approved their respective contributions and agree with the final version of the manuscript for submission.

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Research Article

# Risk assessment analysis of type II Diabetes Mellitus and dyslipidemia with Coronary Artery Stenosis scoring: intervention strategies

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**Keywords**

Coronary artery disease, Diabetes Mellitus, Gensini scoring system, HbA1c, Lipid profile

**Abstract**

**Background**: Diabetes is an established risk factor for coronary artery disease (CAD), with substantial evidence linking it to a higher prevalence of multivessel disease and worse cardiovascular outcomes. This study aimed to explore the relationship between lipid profile and diabetes with severity of CAD by evaluating the degree of stenosis as assessed by Gensini Score (GS).

**Methods**: A total of 300 participants were included: 100 CAD patients with diabetes, 100 CAD patients without diabetes, and 100 healthy controls. Serum lipoproteins were quantified using standard colorimetric methods, while HbA1c levels were determined through HPLC. GS was assessed using angiographic data obtained from the catheterization laboratory.

**Results**: Significant differences in lipid profile, and HbA1c were observed between diabetic CAD patients, non-diabetic CAD patients, and healthy controls. The GS in patients with CAD was markedly elevated in the diabetic CAD cohort (32.97±22.47) in comparison to the non-diabetic CAD cohort (28.70±20.60). A positive correlation was found between HbA1c ($r^2$=0.061), random blood sugar ($r^2$=0.23), and TG ($r^2$=0.00) with the GS. Conversely, HDL levels ($r^2$= -0.074) exhibited a significant negative correlation with GS.

**Conclusion**: Our results suggest that lipoproteins and diabetic indicators have a considerable impact on the severity of CAD. This highlights the need for a more personalized approach in managing diabetes and CAD, incorporating regular monitoring of glucose and lipid levels to evaluate cardiovascular risk.

## Introduction

Cardiovascular diseases remain the primary cause of mortality worldwide, responsible for an estimated 17.5 million deaths annually [1]. Diabetes is a known independent risk factor for coronary artery disease (CAD), with evidence demonstrating a higher prevalence of adverse cardiovascular events in patients with diabetes [3-6]. An earlier study reported that diabetic patients with intermediate coronary artery stenosis had the worse outcome as compared with non-diabetic ones [4]. Diabetes with cardiac complications significantly increased the risk of death, a finding that emphasizes the importance of mitigating the risk of diabetes-related complications in CAD patients [5]. In an earlier investigation by Morgan KP et al., authors documented a higher prevalence of multivessel disease, along with extensive distal involvement, and minimal collateral formation in CAD patients with type 2 diabetes mellitus (T2DM), ultimately leading to poorer outcomes [7].

Moreover, dysregulated lipid metabolism constitutes a crucial determinant in CAD and is considered a hallmark of dysfunctional angiogenesis. Numerous clinical observations have revealed reduced collateral circulation in diabetic CAD patients [8,9]. However, the impact of dyslipidemia and hyperglycemia on the relationship between impaired endothelial function and coronary artery stenosis severity in T2DM patients remains unclear. In this study, we investigated the relationship between lipoproteins, diabetic biomarkers (HbA1c and blood glucose), and the severity of coronary artery stenosis, and highlighted their potential clinical implications in T2DM patients with CAD.

## Methodology

### Participant enrollment

This tertiary care-hospital based study was conducted in the Department of Biochemistry, G.B. Pant Institute of Postgraduate Medical Education & Research, New Delhi, India. After obtaining ethical approval, we enrolled 300 participants, including 100 diabetic CAD patients (Group I), 100 non-diabetic CAD patients (Group II), and 100 age- and sex-matched healthy controls (Group III). Written informed consent was taken from all participants.The study included patients over the age of 18, who had been diagnosed with CAD through resting electrocardiography and invasive coronary angiography, showing more than 50% stenosis in at least one coronary artery. Exclusion criteria were patients below the age of 18 years, patients with renal and hepatic impairment, patients who had undergone previous procedures such as coronary artery bypass grafting, percutaneous transluminal coronary angioplasty, and stenting.

### Specimen collection and laboratory protocol

Venous blood was withdrawn from each participant. 3 mL blood was collected in EDTA vial for HbA1c, 2 mL blood in a plain vial for lipid analysis and 2 mL blood in sodium citrate vial for blood glucose analysis. HbA1c measurement was performed using HPLC. Patients exhibiting HBA1c levels >6.5% were classified as diabetics and those with <6.5% were classified as non-diabetics [10]. Serum lipoproteins and blood glucose were measured on Cobas c501 (Roche) fully automated analyser. Serum total cholesterol (TC) was measured using Cholesterol Oxidase-Peroxidase method, triglycerides (TG) using Glycerol Phosphate Oxidase-Peroxidase end-point enzymatic colorimetric method, high density lipoprotein (HDL), and low density lipoprotein (LDL) using direct enzymatic colorimetric methods. Very low-density lipoprotein (VLDL) level was calculated by dividing triglyceride level by five. For measurement of blood glucose, hexokinase method was used. Coronary angiography was performed on all recruited patients (group I and group II) using Judkin's approach [11] to examine angiographic patterns. The left coronary artery was divided into the left anterior descending, circumflex, and obtuse marginal branches, while the right coronary artery considered as a single vessel. Based on the highest level of obstruction in each projection, the arteries were classified as normal, 50%, 75%, 90%, or 100% occluded, determined through visual assessment [12]. The patients were categorized into three subgroups based on angiography results: single-vessel, double-vessel, and triple-vessel disease, (disease defined as obstruction of 50% or more) [13]. The severity of coronary artery stenosis was assessed using the Gensini score, calculated based on luminal narrowing: 1 point for 25%, 2 points for 50%, 4 points for 75%, 8 points for 90%, 16 points for 99%, and 32 points for total occlusion [14].

### Statistical analysis

Data was analyzed using the Statistical Package for the Social Sciences, version 22. For group comparisons, independent t-test and ANOVA was used for comparing two independent variables, and more than two variables, respectively. The Chi-square test assessed differences within the groups, while Pearson's correlation was employed to examine relationships between variables. All statistical analyses were performed with $p < 0.05$.

## Results

### Demographic characteristics of the study population

The mean age of group I (82 male and 11 female), group II (89 male and 18 female) and group III (54 male and 46 female) was 54.86 ± 9.80, 53.15 ± 10.30 and 43.62 ± 12.03 years respectively. In group I, 39 smokers, 42 tobacco chewers and 19 are alcoholic; in group II, 61 smokers, 39 tobacco chewers and 25 are alcoholic. However, in Group III, none of the participants are smokers, tobacco chewers, or alcoholics. 34 individuals of group I and 15 individuals of group II were diagnosed with dyslipidaemia (Table 1).

**Table 1:** Demographic characteristics of the study population.

| Parameter | Group I (n=100) | Group II (n=100) | Group III (n=100) |
|---|---|---|---|
| Age (Mean ± SD) | 54.86 ± 9.80 | 53.15 ± 10.30 | 43.62 ± 12.03 |
| Gender (n) | | | |
| Male | 82 | 89 | 54 |
| Females | 11 | 18 | 46 |
| Smoker (n) | 39 | 61 | 0 |
| Tobacco Chewer (n) | 42 | 39 | 0 |
| Alcoholic (n) | 19 | 25 | 0 |
| Dyslipidemia (n) | 34 | 15 | 0 |

Age is represented as Mean ± SD; Qualitative variables as frequencies (n)

**Comparison of biochemical parameters and Gensini score between groups I, II and III**

Table 2 illustrates comparison of biochemical parameters (diabetic parameters HbA1c, random blood sugar and lipid profile) between group I, II and III. In group I, II and III, serum random blood sugar (RBS) was 210.15±101.13, 120.61±42.52 and 129.16±9.13 respectively; and serum HbA1c was 8.84±2.02, 5.66±0.38 and 4.31±0.75 respectively. Significant difference was observed between group I, II and III, for both serum RBS and HbA1c levels. Among groups I, II and III, significant difference was observed for TC, HDL, LDL and VLDL (p=<0.001), however, for serum TG, the difference observed was non-significant (p=0.241). The Gensini scores for Group I and Group II were 32.97±22.47 and 28.70±20.60, respectively, demonstrating a significant difference ($p < 0.001$).

**Table 2:** Comparison of biochemical parameters and Gensini score between various groups.

| Parameter | Group I (N=100) | Group II (N=100) | Group III (N=100) | P Value |
|---|---|---|---|---|
| RBS (mg/dL) | 210.15 ±101.13 | 120.61 ± 42.52 | 129.16 ± 9.13 | 0.001 |
| HbA1c (%) | 8.84 ± 2.02 | 5.66 ± 0.38 | 4.31 ± 0.75 | 0.001 |
| TG (mg/dL) | 149.05 ± 49.47 | 138.14 ± 56.34 | 139.16 ± 44.70 | 0.241 |
| TC (mg/dL) | 176.32 ± 130.55 | 128.47 ± 81.96 | 101.96 ± 36.95 | 0.001 |
| HDL (mg/dL) | 39.30 ± 21.30 | 34.25 ± 11.64 | 53.57 ± 15.46 | 0.001 |
| LDL (mg/dL) | 96.23 ± 22.25 | 80.37 ± 43.74 | 79.61 ± 36.00 | 0.001 |
| VLDL (mg/dL) | 34.95 ± 26.12 | 25.70 ± 16.38 | 26.74 ± 7.24 | 0.001 |
| Gensini Score | 32.97 ± 22.47 | 28.70 ± 20.60 | - - | 0.001 |

HDL: high density cholesterol level; LDL: low-density lipoprotein; VLDL: Very low-density lipoprotein; RBS: Random Blood Sugar; TC: Total cholesterol; TG: Triglyceride

**Logistic regression analysis between biochemical parameters and Gensini score**

Table 3 and Figure 1 and 2 illustrate the logistic regression analysis between biochemical parameters and Gensini score. RBS (R2=0.023, p=0.009) and HbA1c (R2=0.161, P=<0.001) showed significant positive association with Gensini score. TG ($R^2$=0.034, p<0.001) and VLDL ($R^2$=0.009, p<0.001) showed a significant positive correlation with the Gensini score, while HDL exhibited a negative correlation ($R^2$=-0.007, p<0.001). Serum TC and LDL showed no significant association with the Gensini score.

**Table 3:** Logistic regression analysis between Gensini score and biochemical parameters.

| Parameter | $R^2$ | P Value |
|---|---|---|
| RBS | 0.023 | 0.009 |
| HbA1c | 0.161 | 0.001 |
| TC | 0.00 | 0.832 |
| TG | 0.034 | 0.001 |
| HDL | 0.074 | 0.001 |
| LDL | 0.007 | 0.145 |
| VLDL | 0.009 | 0.09 |

HDL: high density cholesterol level; LDL: low-density lipoprotein; VLDL: Very low-density lipoprotein; RBS: Random Blood Sugar; TC: Total cholesterol; TG: Triglyceride; $R^2$=coefficient of determination

**Figure 1:** Logistic regression analysis between Gensini score and Random Blood Sugar and HbA1c.

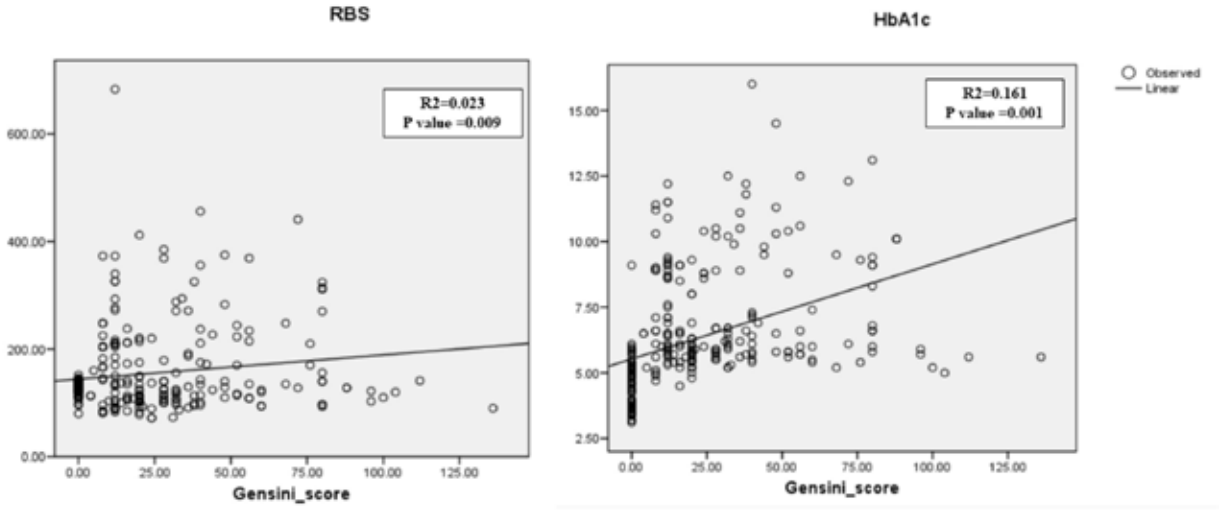
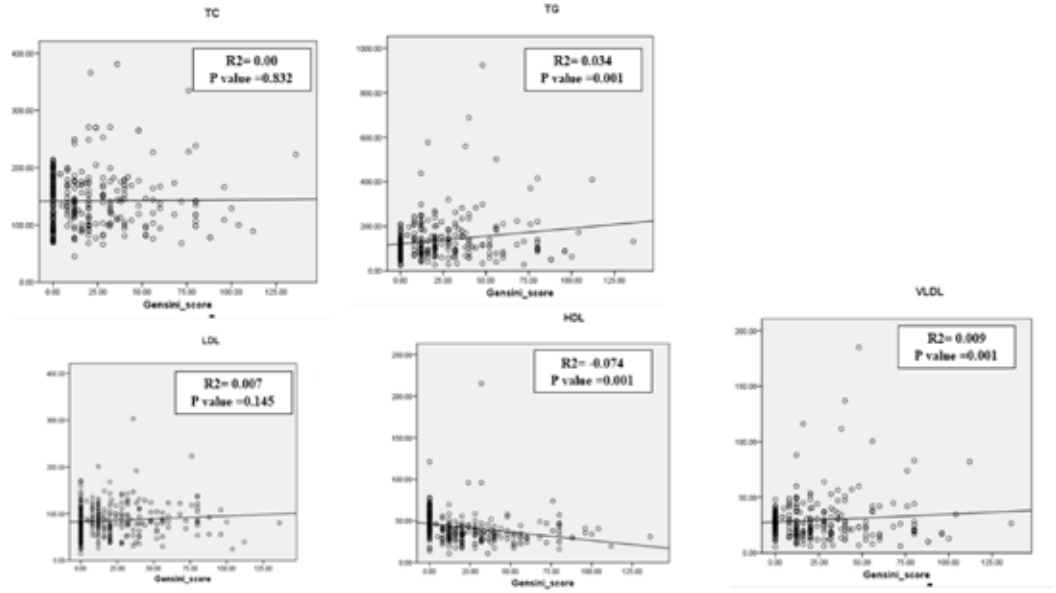



**Figure 2:** Logistic regression analysis between Gensini score and lipid profile parameters.




TC: Total cholesterol; TG: Triglyceride; HDL: high density cholesterol level; LDL: low-density lipoprotein; VLDL: Very low-density lipoprotein

## Discussion

In this cross-sectional study, we observed significant differences in diabetes-specific parameters and lipid profile between diabetic, nondiabetic CAD patients and healthy control individuals. These findings are in concordance with the observations of previous investigations. A study conducted by Ghazanfari Z. et al. identified a robust correlation between HbA1c and fasting blood sugar, particularly in individuals with diabetes [15]. Similarly, an earlier study revealed that HbA1c levels of >9.0 % are associated with higher mortality in clinically type II diabetic patients [16]. Furthermore, existing literature indicates that oxidative stress and endothelial dysfunction, commonly observed in diabetic individuals, may directly facilitate the pathogenesis of atherosclerosis and the progression of CAD [17-19]. These results imply that these parameters could assist healthcare providers in setting target plasma glucose levels with specific HbA1c values. Additionally, recent investigations have shown that while stable average glucose remains pivotal in preventing cardiovascular events, HbA1c fluctuations are an independent risk determinant for cardiovascular disease, even in patients meeting glycemic targets [20]. Yang et al. identified HbA1c variability as a predictor of in-stent restenosis in T2DM patients post-stent implantation [21]. These findings imply that HbA1c may also serve as a potential marker of glycemic control, aiding in the prevention of cardiovascular complications in CAD patients. In addition, in our study, significant differences were observed in lipid profile between diabetic, nondiabetic CAD patients and healthy controls. In agreement, Wang X et al. suggested that raised LDL and TG and decreased HDL are common in men and a significant contributor in late onset of coronary artery disease [22]. A study by Jia A et al. reported that elevated levels of serum VLDL can act as a biomarker for diabetic CAD patients [23]. The coronary artery distribution angiography investigation conducted by Hegde SS et al. posited that individuals with diabetes exhibit a significantly elevated susceptibility to multi-vessel coronary artery disease [24]. Taken together, these results highlight the clinical value of comprehensive lipid assessment and suggest that addressing dyslipidemia could potentially improve outcomes in CAD patients.

Logistic regression analysis was conducted to examine the relationship between the Gensini score (GS) and various biochemical parameters. The results revealed a positive association between RBS, HbA1c, and TG with the Gensini score, while HDL demonstrated a significant negative association. These observations are in line with the findings of earlier studies [25-27]. In a study conducted among 141 CAD patients, authors reported that GS was positively correlated with total cholesterol, LDL cholesterol and TG and a negative correlation between GS and HDL cholesterol was identified [25]. In an earlier study conducted among acute coronary syndrome patients, authors uncovered a significant association between lipid parameters (total cholesterol, non-HDL cholesterol, and the Apo B/A ratio) and the degree of CAD, encompassing vessel involvement, specific arterial regions affected, and the distribution of disease across segments [29]. Another study compared the lipid ratios in STEMI patients and suggested that lipid ratios have significant correlation with coronary artery diseases with chest pain [29]. Overall, our findings substantiate the notion that fluctuation in HbA1c and lipid profile, may be related with the severity of CAD in individuals with T2DM, and is deserving of further attention in the context of glycemic control and managing lipid profile. However, the limited sample size may constrain the external validity of the findings, hindering their extrapolation to a more diverse population. Moreover, longitudinal studies with extended follow-up could yield stronger evidence regarding the prognostic significance of lipids and diabetic markers in CAD. By addressing these constraints, a more nuanced comprehension of the role of lipids and diabetic markers in CAD can be attained, ultimately enhancing risk stratification, and facilitating the development of tailored therapeutic strategies.

## Conclusion

In conclusion, this study underscores the critical role of Type II Diabetes Mellitus and dyslipidemia in the progression of Coronary Artery Disease. Elevated random blood sugar, HbA1c, and triglycerides levels, along with reduced HDL, were closely linked to CAD severity. Looking ahead, a more personalized approach in the management of T2DM and CAD is essential, integrating regular monitoring of glucose and lipid profiles to assess cardiovascular risk. Future research should explore novel biomarkers, including VLDL and lipid ratios, to improve risk stratification. Additionally, early intervention strategies focusing on stricter glycemic and lipid control, along with lifestyle modifications, may prevent or delay the onset of severe CAD in diabetic patients, ultimately improving long-term cardiovascular health outcomes.

## Abbreviations

| | |
|---|---|
| CAD | Coronary Artery Disease |
| GS | Gensini Score |
| HDL | High Density Lipoprotein |
| LDL | Low Density Lipoprotein |
| RBS | Random Blood Sugar |
| TC | Total cholesterol |
| TG | Triglyceride |
| T2DM | Type II Diabetes Mellitus |
| VLDL | Very Low Density Lipoprotein |

**Declarations**

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None to declare.

**Conflict of interest**
The authors declare that they have no conflicts of interest.

**Ethics approval and consent to participate**
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments. The study was approved by the Institutional Ethics Committee, Maulana Azad Medical College and associated hospitals, New Delhi, India (F1/IEC/MAMC/85/03/21/no.422; Dt-30.08.2021). Written informed consent was obtained from all participants included in the study.

**Author contributions**
P.K.D. designed and supervised the study, provided facilities for testing, contributed to data interpretation and preparation, revision and finalization of the manuscript; D.S. conducted experiments and contributed to data collection, performed data analysis; D.S. and S.S. drafted the manuscript; V.M. provided the facility for the enrolment of patients; V.M. and R.M. critically reviewed the manuscript and contributed in analysis and finalisation of the manuscript; S.K. contributed in data analysis. All authors have reviewed the entire content of this manuscript and approved it for submission.

**Data sharing statement**
Data is available from the corresponding author on reasonable request.

**Clinical trial number**
Not applicable.

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Research Article

# Exploring Ethical Perspectives in Laboratory Medicine: A Survey of Laboratory Professionals at the Annual Conference in Nepal

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**Keywords**

Ethics, Laboratory Medicine, Laboratory Practice, Laboratory Ethics.

**Abstract**

This study aimed to explore the ethical perspectives of laboratory professionals in Nepal regarding laboratory medicine practices. A survey was conducted among participants at the annual conference of laboratory professionals in Nepal in April 2024, with a total of 32 complete responses collected. The survey consisted of questions categorized into three phases of laboratory practice: pre-examination (4 questions), examination (4 questions), and post-examination (5 questions). Each section addressed specific ethical considerations and practices within these phases, including issues related to sample collection, testing accuracy, result interpretation, and reporting. The responses were analyzed to identify trends, challenges, and areas where ethical standards in laboratory medicine could be improved. The findings highlight the need for greater awareness and training in ethical practices, with particular emphasis on improving consistency and transparency across all phases of laboratory work.

**Background**

Laboratory medicine plays a critical role in diagnosing, monitoring, and guiding the treatment of patients, making it essential to uphold high ethical standards throughout its processes. Ethical challenges in laboratory medicine can arise at various stages, including the pre-examination, examination, and post-examination phases each of which requires adherence to professional and ethical guidelines to ensure patient safety, privacy, and the reliability of results.

In Nepal, the healthcare system, including laboratory services, faces unique challenges such as limited resources, lack of standardized protocols, and variable training opportunities for laboratory professionals [1]. These challenges can exacerbate ethical concerns and impact the quality of laboratory services. Despite the importance of ethical decision-making in laboratory medicine, there is limited research exploring the specific ethical perspectives and practices of laboratory professionals in Nepal.

The standardization and harmonization of methods, reference intervals, test names and practices in laboratory medicine, have been central efforts of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), as well as national societies affiliated with IFCC. Given that Nepalese Association for Clinical Chemistry (NACC) is the national society, we believe this platform of annual congress is ideal for conducting the survey, as it includes members from across the country and from various types of laboratories, ensuring that the survey results will be broadly applicable. By conducting a survey at this event, this study aims to identify common ethical concerns among laboratory professionals in Nepal, assess the awareness and understanding of ethical principles across different laboratory phases, and identify areas for improvement in ethical training and practice.

**Methods**

This cross-sectional survey was conducted during the NACC Annual Congress in April 2024, targeting registered laboratory professionals attending the event, which was held in Kathmandu. [2]. A structured, self-administered questionnaire, which included informed consent and additional measures, was printed and distributed to the attendees. Participation in the survey was voluntary. Survey of this nature was exempted from ethics approval at the institution where this survey was performed. This study ensured strict anonymity and confidentiality of the collected data. The questionnaire consisted of 15 questions categorized into three phases of laboratory practice: pre-examination (4 questions), examination (4 questions), post-examination (5 questions) and others (2 questions). These questions were developed based on a review of relevant literature and consultation with expertise in the field. The questionnaire was then reviewed and approved by an independent expert in laboratory medicine. For face validity, the expert assessed the clarity and appropriateness of the questions. For content validity, the expert evaluated whether the questionnaire adequately covered key ethical aspects in laboratory medicine. While formal statistical validation was not performed, expert feedback was incorporated to refine the questionnaire. The questions were primarily multiple-choice, with respondents allowed to select more than one option. A total of 32 complete responses were received. Data were summarized using descriptive statistics, and all analyses were performed using Microsoft® Excel® 2019.

**Results**

There were total 32 responses which included 18 participants from various medical colleges and 14 from private laboratories. The participants' experience in laboratory medicine varied, with the distribution of years of experience as follows:

- 1–5 years: 19 participants
- 6–10 years: 4 participants
- 11–15 years: 6 participants
- 16–20 years: 3 participants

The responses to the questions were analyzed and are presented below. The total of 32 responses may not be reflected in some questions due to participants skipping certain questions. On assessing ethical practices in the pre-examination phase, the majority (72%) reported encountering ethical dilemmas occasionally, while 19% frequently faced such issues (Figure 1). Common ethical challenges included sample mislabeling, inadequate sample collection and improper sample transportation and storage (Figure 2).

**Figure 1:** Frequency of encounter with ethical issues in preanalytical phase.

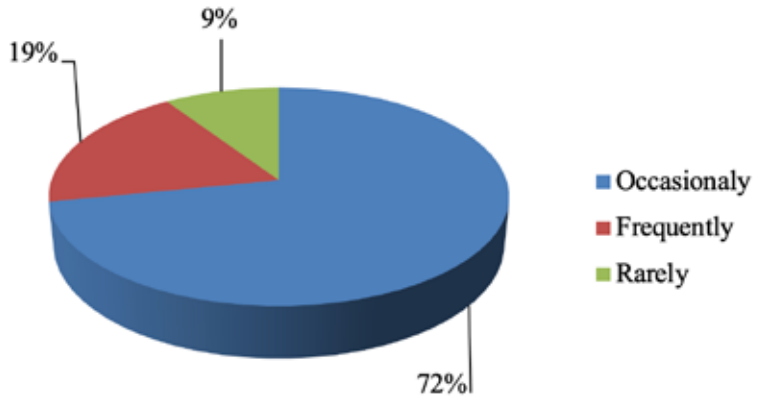
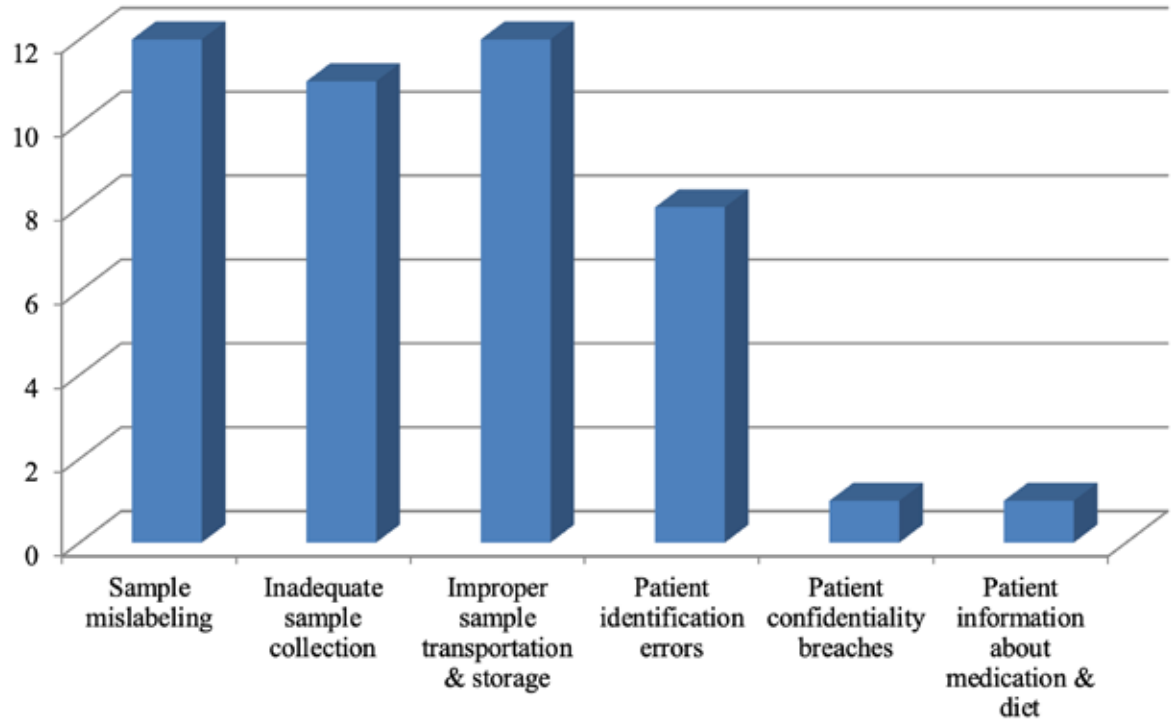



**Figure 2:** Types (X-axis) and Number of Responses (Y-axis) of ethical issues encountered in the preanalytical phase.




A significant number of respondents confirmed that all patients were treated equally during sample collection without preference, and they affirmed that tests performed were necessary and beneficial based on the best medical evidence (Table 1).

**Table 1:** Response to other questions in pre-examination phase.

| Question: Pre-examination Phase | Choice | Response (number) | Response(Percentage) |
|---|---|---|---|
| Is equal treatment given to all patients during the sample collection process without preference or expedited handling? | Yes | 26 | 86.60% |
| | No | 4 | 13.30% |
| Do you consider that all test performed are necessary and beneficial to the patient based on the best medical evidence? | Yes | 27 | 90.00% |
| | No | 3 | 10.00% |

Our study found that 100% of laboratory professionals reported refusing to analyze specimens when there were pre-examination issues and confirmed that their laboratories ensured equal treatment of all patient samples (Table 2). All respondents confirmed they ensure timely access to results, especially in emergencies, regardless of payment status. While 75% were aware of bio-banking rules and ethical considerations, only 60% had formal policies on residual sample use, revealing a gap in standardized ethics. Additionally, only 73.33% obtained patient consent for further testing of leftover samples. Lastly, 66.67% offered patients the option to consent to family members accessing their medical records (Table 3).

**Table 2:** Response to questions in examination phase.

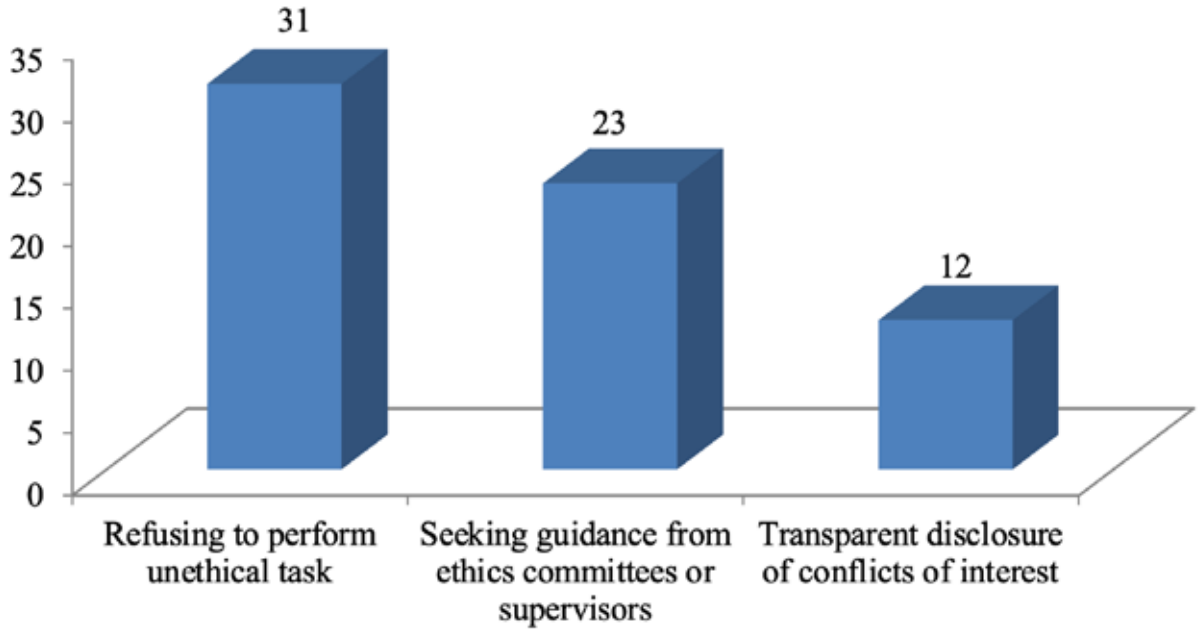
| Questions: Examination Phase | Choice | Response (number) | Response (Percentage) |
|---|---|---|---|
| Do you refuse to analyze when there is evidence of poor sample integrity, incorrect or poor labeling and other deficiencies that may compromise the test result? | Yes | 32 | 100% |
| Are rigorous quality assurance programs, including quality control and proficiency testing and laboratory accreditation, established in your laboratory? | Yes | 31 | 96.60% |
| | No | 1 | 3.30% |
| Does your laboratory ensure equal treatment of all patient samples without discrimination based on gender, age or ancestry? | Yes | 32 | 100% |
| Are special care and measures taken to maintain confidentiality in point-of-care testing settings? | Yes | 31 | 96.60% |
| | No | 1 | 3.30% |

In response to the question, "Do you think sufficient training and education regarding ethics in laboratory medicine is provided to us in Nepal?" our study reveals a significant gap in laboratory ethics training, with 70% of professionals reporting insufficient education in this field. While 23.33% felt adequately trained, the findings underscore the need for greater integration of ethics into laboratory curricula. In response to the question, "How do you handle conflicts of interest or pressures that may compromise professional integrity in laboratory medicine?" 42.86% of participants reported refusing unethical tasks, demonstrating a commitment to integrity. Additionally, 25% seek guidance from ethics committees or supervisors, and 12.50% disclose conflicts of interest (Figure 3). Fewer respondents used a combination of these approaches, highlighting the importance of mentorship and transparency in addressing ethical issues.

**Table 3:** Response to questions in post- examination phase.

| Questions: Post Examination Phase | Choice | Response (number) | Response (Percentage) |
|---|---|---|---|
| Do you ensure timely access to results, especially in emergency situations, regardless of payment status? | Yes | 32 | 100% |
| Are you aware of the rules and practices regarding bio-banking of leftover specimens and associated ethical considerations? | Yes | 24 | 75.00% |
| | No | 8 | 25.00% |
| Do you have a policy on the use of residual samples, considering ethical issues and patient consent? | Yes | 18 | 60.00% |
| | No | 12 | 40.00% |
| Do you obtain patient consent for any further testing of residual samples beyond the requested laboratory tests? | Yes | 22 | 73.30% |
| | No | 8 | 26.60% |
| Are patients given the option to provide consent for others (such as family members) to access their medical records? | Yes | 20 | 66.60% |
| | No | 10 | 33.30% |

**Figure 3:** Response of participants to ethical dilemma (X-Axis) and Number of participants (Y-axis).



**Discussion**

Our study highlights key ethical challenges and practices in the pre-examination, examination, and post examination phases of laboratory medicine in Nepal. The pre-examination phase is marked by ethical concerns such as proper patient identification, informed consent, and confidentiality. Most participants encountered ethical dilemmas occasionally, with issues such as sample mislabeling, inadequate collection, and improper storage being the most common. A majority ensured equal treatment of patients and affirmed that tests performed were beneficial based on medical evidence, indicating a strong commitment to ethical practices.

In the examination phase, confidentiality and sample integrity emerged as significant concerns. These may happen particularly in smaller laboratories and point-of-care settings. A robust quality assurance program, including proficiency testing and laboratory accreditation is essential to ensure the accuracy and reliability of results. Our findings suggest that laboratory professionals maintain high ethical standards in this phase, with 100% confirming that sample integrity is a priority and that all patients are treated equally, regardless of gender, age, or ancestry. Moreover, special care is taken to preserve confidentiality, highlighting the importance of maintaining trust in laboratory services.

The post examination phase focuses on the ethical management of patient results, including specimen storage, retention, and destruction, as well as safeguarding patient confidentiality. While 100% of respondents reported ensuring timely access to results, regardless of payment status, there were gaps in formal policies regarding the use of residual specimens and obtaining patient consent for further testing. Therefore, there is a need for clearer policies and more education around bio-banking and specimen use. In resource-limited settings, the handling of leftover samples becomes a challenge, as highlighted in the published report [3]. Additionally, while the majority allowed patients to consent to family members accessing their medical records, a significant portion did not, indicating room for improvement in practices surrounding patient autonomy.

A concerning finding in our study is that 70% of laboratory professionals felt insufficiently trained in ethics, suggesting a significant gap in education and professional development. When faced with ethical dilemmas, most professionals prioritized integrity, either by refusing unethical tasks or seeking guidance from supervisors or ethics committees. These findings highlight the need for more comprehensive ethics training, clear guidelines, and institutional support systems to address ethical conflicts effectively. A recent report by the IFCC Task Force on Ethics indicates that formal teaching of ethics is absent from many clinical chemistry and laboratory medicine training programs and that there is a perceived need for training tools, with a particular desire of directors of training programs to have online tools [4]. A recent study in Iran highlights that teaching ethical attitudes to clinical laboratory professionals through lecture-based and problem-based learning methods significantly improved their ethical attitudes, while the role-playing method showed no notable effect [5]. This suggests a substantial gap in professional development and underscores the need for targeted education and training programs. The Nepalese national society of clinical laboratory medicine should establish a clearly defined and publicly accessible policy outlining the ethical standards for its members' professional conduct. This policy, published as a code of ethics, is recommended by experts in the field [6]. The IFCC code of ethics outlines the ethical principles and standards for clinical laboratorians, emphasizing their duty to patients, colleagues, and society, with a focus on maintaining high-quality practices, confidentiality, integrity, collaboration, and social responsibility [7].

Since this study is a preliminary report, we focused on descriptive analysis to offer an initial overview of ethical perspectives in laboratory medicine. We recognize that future research with a larger dataset could build upon the findings of this study. Expanding the sample size in future studies is recommended to explore these issues more comprehensively on a national scale.

**Conclusion**

Despite the commitment of the Nepalese laboratory professionals to ethical practices, further improvements in training, policy development, and support structures are essential to ensure consistent and high-quality ethical decision-making in laboratory settings.

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**Conflict of Interest**

Authors declare no conflict of interest in the publication of this manuscript.

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Research Article

# Relationship between abnormal laboratory results and recorded clinical diagnoses in tertiary hospital settings: A retrospective observational study

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**Keywords**

Acute coronary syndrome, prostate cancer, cardiac markers, troponin, tumour markers

**Abstract**

**Background**: The relationship between laboratory results and clinical decisions, including diagnoses, is not always clear. This study aims to determine the association between abnormal laboratory test results and diagnoses recorded by the clinicians within the electronic medical records in tertiary hospital settings.

**Method**: We conducted a retrospective observational study using anonymised linked hospital data of 223,789 adult admissions between January 2020 and December 2021 in two Local Health Districts in New South Wales, Australia. Data extracted from hospital information systems included patient demographics, recorded clinical diagnoses and laboratory test results. We analysed correlations between abnormal results from common laboratory tests (nasopharyngeal/oral swab SARS-CoV-2 PCR, serum/plasma total prostate-specific antigen (PSA) antigen [PSA], free thyroxine [free T4], thyroid stimulating hormone [TSH], cardiac troponin T, and cortisol) and their respective recorded diagnoses.

**Results**: We observed the following Spearman correlation coefficients between abnormal laboratory test results and their corresponding recorded clinical diagnoses: positive SARS-CoV-2 PCR and COVID-19 ($\rho$=1.00), total PSA >10 µg/L and prostate cancer ($\rho$=0.66), free T4 >25.0 pmol/L and hyperthyroidism ($\rho$=0.58), TSH >5.00 mIU/L and hypothyroidism ($\rho$=0.56), TSH <0.30 mIU/L and hyperthyroidism ($\rho$=0.55), cardiac troponin T >20 ng/L and acute coronary syndrome ($\rho$=0.51), free T4 <8.0 pmol/L and hypothyroidism ($\rho$=0.42), and cortisol <80 nmol/L and adrenal insufficiency ($\rho$=0.33).

**Conclusions**: This study demonstrates that abnormal laboratory results play an important but varied role in clinical diagnoses. The weaker associations highlight that laboratory tests may be utilised differently in different clinical pathways, underscoring the complex relationship between laboratory

results and clinical diagnoses, and the importance of considering abnormal test results in the appropriate clinical context.

## Introduction

The ultimate goal of laboratory medicine is to improve health outcomes [1]. Studies focusing on health outcomes should be prioritised to provide evidence-based insights to inform appropriate test utilisation and clinical decision-making [2]. Historically, it has been claimed that 'laboratory data influences 70% of clinical decisions' but the evidence for this claim remains debated and was initially anecdotal [3]. Well-designed, and appropriately powered, studies are needed to provide an evidence base of the value added by laboratory medicine in improving health outcomes [3]. Outcome studies should be distinguished from those centred on clinical validation and predictive or prognostic evaluations [1].

The utility of laboratory results is a key element of value proposition for a laboratory investigation [4]. Studies are needed to determine how a laboratory result leads to better patient outcomes, including their role in informing clinical diagnosis. Evidence from such studies could then inform healthcare funding decisions. As laboratory medicine is one of the largest producers of structured healthcare data [5], there is ample opportunity for large-data analysis when pathology test results are linked to electronic medical records (EMRs). An EMR contains clinical data that can help address complex research questions, such as those concerning laboratory result utility [6]. Correlation (regression) analyses are commonly used in laboratory medicine to derive adjustment factors or risk scores, as these relationships can predict certain outcomes and help refine diagnostic and therapeutic approaches. Laboratory-based prediction models for a specific clinical outcome have been developed, such as one for chronic kidney disease progression [7]. Adopting such an approach to analysing the relationship between test results and clinical diagnoses could, likewise, aid in the development of prognostic models.

In the current study, using a large data source, we aimed to determine the degree of association between an abnormal laboratory result and a patient's subsequently recorded clinical diagnosis by the clinician within the EMR in tertiary hospital settings.

## Method

This was a retrospective observational study of adult patients (aged ≥18 years) admitted to participating hospitals in two Local Health Districts (LHDs) in New South Wales (NSW), Australia, between 1 January 2020 and 31 December 2021. The study utilised routinely collected longitudinal data from these hospitals, all of which operate an EMR which enables clinicians to create electronic laboratory test orders and record clinical diagnoses. Ethics approval was obtained from the Human Research Ethics Committee of the South Eastern Sydney Local Health District (HREC/16/POWH/412) and the New South Wales Population and Health Services Research Ethics Committee (2022/ETH0209). This work was funded by an International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) Task Force on Outcome Studies in Laboratory Medicine (TF-OSLM) Grant and the National Health and Medical Research Council (NHMRC) Partnership Projects 1111925 and 2006755. The funders played no role in designing, executing, or interpreting this study.

Data were extracted from two hospital clinical information systems: the patient administration system (PAS), which contains data on hospital admissions, and the laboratory information system (LIS), which contains data on test utilisation. Information recorded in these databases included patient demographics, recorded clinical diagnoses (using International Classification of Diseases and Related Health Problems, Tenth Revision, Australian Modification [ICD-10-AM] codes), and laboratory test results. The relevance of diagnoses was determined by one of the authors, a qualified pathologist, based on their professional judgment. (It should be noted that the diagnoses were not made by the pathologist; rather, they were the recorded clinical diagnoses made by the attending clinicians.) After linkage, pathology data were de-duplicated to one test result per admission, either the lowest or the highest (most 'severe' or 'significant') result, according to pre-determined pathology test thresholds (Supplemental file – Table 1). These thresholds were determined based on published literature [8-16] and one of the author's judgment as a qualified pathologist. In the absence of common thresholds in clinical practice guidelines, these thresholds may vary among clinicians in their clinical decision-making.

We focused on several commonly utilised laboratory tests: SARS-CoV-2 polymerase chain reaction (PCR), serum/plasma cortisol, free thyroxine (T4) and thyroid stimulating hormone (TSH), total prostate-specific antigen (PSA) and cardiac troponin T. These tests were chosen due to their widespread usage, and they generally have a clear corresponding clinical diagnosis (COVID-19, adrenal insufficiency, hyperthyroidism/hypothyroidism, prostate cancer, and acute coronary syndrome, respectively).

A rigorous data quality assessment process was employed for each dataset to evaluate the accuracy, completeness, consistency, relevance, timeliness, uniqueness, and validity of the data sources. This process included the identification of missing data, duplicates, formatting issues, and logic compliance. The linkage of hospital inpatient and pathology data was undertaken using non-identifiable patient medical record number (common to all datasets). Records were considered 'linked' if there was an exact match on all identifiers. As a given patient could have multiple visits at the same or different sites over time, only results for laboratory tests performed between the admission and discharge dates were considered.

Baseline patient characteristics, including age at admission and sex were presented for each included test. Deciles of continuous test results (excluding SARS-CoV-2 PCR positivity) and the proportion of diagnoses recorded by the clinicians falling in each

decile were calculated to descriptively evaluate the association between abnormal test results and diagnoses recorded.

Non-parametric Spearman's rank correlation coefficients were used to evaluate the correlation between the weekly volume of abnormal test results and the number of diagnoses recorded by the clinicians within the EMRs. Correlation coefficients of up to six weeks were calculated. Sensitivity and specificity of tests were assessed using a logistic regression model estimation method. Data analyses were conducted using SAS software version 9.4 (SAS Institute Inc, Cary, NC) and a P-value of 0.05 was set to declare statistical significance.

## Results

A total of 223,789 distinct admissions were linked to the pathology data between 1 January 2020 and 31 December 2021 (Table 1). The number of admissions in which a test was performed, the number of abnormal tests (based on pre-defined thresholds) and the number of diagnoses recorded by the clinicians within the EMRs are described in Table 2.

**Table 1**: Baseline Characteristics of Patients Included for Each Laboratory Test.

| | | Age (years) | | | Sex (%) | |
|---|---|---|---|---|---|---|
| **Test** | **No. of Admissions** | **Median** | **IQR*** | | **Female** | **Male** |
| Cardiac Troponin T | 52,034 | 71 | 56 | 81 | 46 | 54 |
| SARS-CoV-2 PCR | 50,798 | 70 | 52 | 81 | 49 | 51 |
| TSH | 42,150 | 75 | 58 | 84 | 56 | 44 |
| Free T4 | 9,808 | 74 | 58 | 84 | 61 | 39 |
| Cortisol | 4,964 | 72 | 59 | 82 | 54 | 46 |
| Total PSA | 1,886 | 77 | 69 | 84 | 0 | 100 |

*IQR: Interquartile range (25th percentile – 75th percentile)

PCR: Polymerase chain reaction; T4: Thyroxine; PSA: Prostate-specific antigen; TSH: Thyroid stimulating hormone.

**Table 2**: Descriptive Statistics of Pathology Test Utilisation, Abnormal Results and Recorded Diagnoses.

| **Test (Abnormal Result Threshold)** | **No. of Tests** | **No. of Abnormal Tests** | **No. of Diagnoses Recorded** | **No. with Both Diagnosis and Abnormal Test** | **% Diagnosed Among Abnormal Tests** |
|---|---|---|---|---|---|
| Cardiac Troponin T (>20 ng/L) | 52,034 | 24,175 | 5,827 | 4,901 | 20.3% |
| SARS-CoV-2 PCR (Positive) | 50,798 | 867 | 864 | 809 | 93.3% |
| TSH (>5.00 mIU/L) | 42,150 | 3,974 | 1,118 | 770 | 19.4% |
| TSH (<0.30 mIU/L) | 42,150 | 1,819 | 624 | 486 | 26.7% |
| Free T4 (>25.0 pmol/L) | 9,808 | 588 | 601 | 286 | 48.6% |
| Free T4 (<8.0 pmol/L) | 9,808 | 258 | 1,066 | 148 | 57.4% |
| Cortisol (<80 nmol/L) | 4,964 | 345 | 191 | 99 | 28.7% |
| Total PSA (>10 µg/L) | 1,886 | 510 | 294 | 232 | 45.5% |

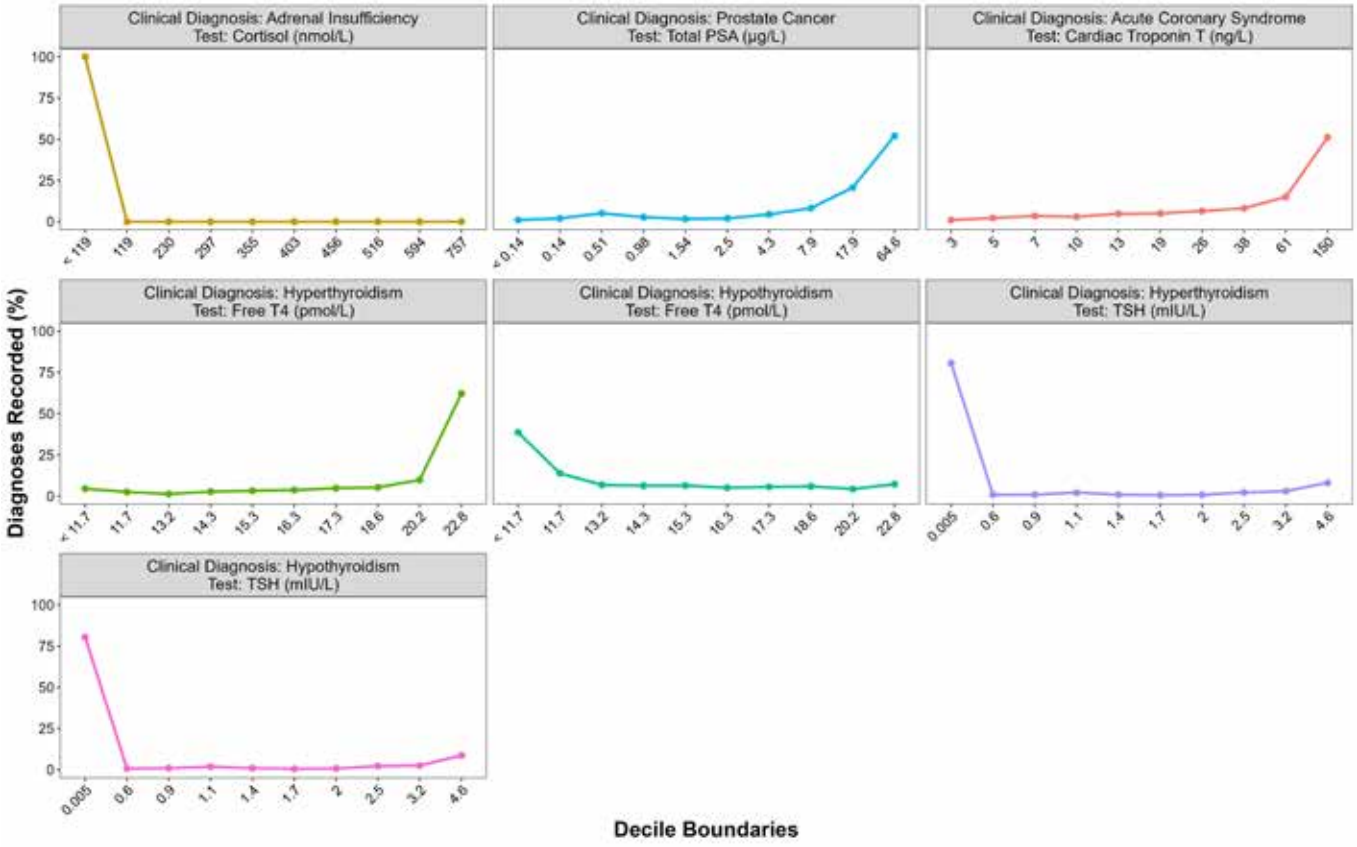
PCR: Polymerase chain reaction; T4: Thyroxine; PSA: Prostate-specific antigen; TSH: Thyroid stimulating hormone.

All tests, except for the SARS-CoV-2 PCR (nasopharyngeal/oral swab), were performed on serum or plasma.

There were 4,964 linked admissions in which a serum/plasma cortisol test result was available. Out of these, 345 (7.0%) tests were 'abnormal' (<80 nmol/L) and just over a quarter (n = 99 [28.7%]) had a recorded diagnosis of adrenal insufficiency. Similarly, 'abnormal' cardiac troponin T, TSH and free T4 were associated with less than 60% of recorded acute coronary syndrome and hyperthyroidism/hypothyroidism diagnoses. On the other hand, there was a recorded diagnosis (by the clinicians within the EMRs) of COVID-19 in 93.3% of cases of a positive SARS-CoV-2 PCR test.

Figure 1 shows the decile boundaries for the test results for each test and the proportion of associated diagnoses recorded by the clinicians at each decile. All recorded diagnoses related to adrenal insufficiency had a random serum/plasma cortisol result of less than 119 nmol/L. Not surprisingly, the majority of diagnoses associated with acute coronary syndrome were recorded in the highest decile of cardiac troponin T concentrations. Similarly, over half of the total cases of prostate cancer diagnosis were documented by the clinicians in the highest decile of total PSA levels.

**Figure 1:** Distribution of Test Results and Associated Diagnoses by Decile.




Proportion of abnormal test results and corresponding clinician-recorded diagnoses are shown across deciles of test result values.

In an assessment of the correlation between weekly number of abnormal test results at baseline and the number of recorded diagnoses (by the clinicians within the EMRs) over the following five weeks, the strongest correlations were observed at baseline (week zero) (Table 3). The correlation remained significant over the next five weeks for SARS-CoV-2 PCR positivity.

**Table 3:** Correlation Between Abnormal Test Results at Week Zero and Diagnoses Recorded Over the Following Five Weeks.

| Test (Abnormal Result Threshold) | Week | | | | | | Interpretation Based on Week 0 |
|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | |
| SARS-CoV-2 PCR (Positive) | **1** | **0.92** | **0.78** | **0.65** | **0.51** | **0.37** | Strong |
| Total PSA >10 µg/L | **0.66** | 0.14 | 0.19 | -0.02 | -0.03 | 0.16 | Moderate |
| Free T4 >25.0 pmol/L | **0.58** | 0.12 | 0.19 | 0.14 | 0.1 | 0.15 | Moderate |
| TSH >5.00 mIU/L | **0.56** | **0.34** | 0.09 | 0.17 | 0.09 | 0 | Moderate |
| TSH <0.30 mIU/L | **0.55** | **0.34** | 0.1 | 0 | 0.06 | 0.14 | Moderate |
| Cardiac Troponin T >20 ng/L | **0.51** | **0.35** | 0.15 | 0.11 | 0.05 | 0.01 | Moderate |
| Free T4 <8.0 pmol/L | **0.42** | 0.11 | 0.07 | 0.09 | 0.01 | -0.06 | Weak |
| Cortisol <80 nmol/L | **0.33** | 0.03 | 0.18 | -0.05 | -0.01 | -0.11 | Weak |

PCR: Polymerase chain reaction; T4: Thyroxine; PSA: Prostate-specific antigen; TSH: Thyroid stimulating hormone.

Spearman's rank correlation coefficient (ρ) is used to assess correlation. Statistically significant correlations ($p < 0.05$) are shown in bold. All tests, except SARS-CoV-2 PCR (nasopharyngeal/oral swab), were performed on serum or plasma.

In a clinical sensitivity-specificity analysis of admissions linked to a SARS-CoV-2 PCR test, a recorded diagnosis of COVID-19 was associated with a higher likelihood of an abnormal PCR test result (Table 4). An abnormal SARS-CoV-2 PCR test result was observed in 93.6% of admissions with a recorded COVID-19 diagnosis. The model demonstrated strong performance in predicting a COVID-19 diagnosis, with an area under the receiver operating characteristic curve [AUC] of 0.968. For several abnormal test results (TSH <0.30 mIU/L, total PSA >10 µg/L, TSH >5.00 mIU/L, cortisol <80 nmol/L, free T4 >25.0 pmol/L, cardiac troponin T >20 ng/L), the models showed high predictability (AUC, >0.7). Excellent specificity but poor sensitivity for serum/plasma TSH >5.00 mIU/L and cortisol <80 nmol/L indicate that the models are useful in predicting the absence of the associated conditions (as recorded by the clinicians) but not in identifying true cases.

**Table 4:** Diagnostic Performance of Selected Laboratory Tests.

| Test (Abnormal Result Threshold) | Sensitivity (%) | Specificity (%) | AUROC |
|---|---|---|---|
| SARS-CoV-2 PCR (Positive) | 93.6 | 99.9 | 0.968 |
| TSH <0.30 mIU/L | 77.9 | 96.8 | 0.873 |
| Total PSA >10 µg/L | 78.9 | 82.5 | 0.807 |
| TSH >5.00 mIU/L | 68.9 | 92.2 | 0.805 |
| Cortisol <80 nmol/L | 51.8 | 94.8 | 0.733 |
| Free T4 >25.0 pmol/L | 47.6 | 96.7 | 0.722 |
| Cardiac Troponin T >20 ng/L | 84.1 | 58.3 | 0.712 |
| Free T4 <8.0 pmol/L | 13.9 | 98.7 | 0.563 |

AUROC: Area under the receiver operating characteristic curve; PCR: Polymerase chain reaction; T4: Thyroxine; PSA: Prostate-specific antigen; TSH: Thyroid stimulating hormone.

All tests, except for the SARS-CoV-2 PCR (nasopharyngeal/oral swab), were performed on serum or plasma.

**Discussion**

Outcome studies of the utility of laboratory results in related clinical pathways are key to demonstrating the value of laboratory medicine [1]. One such example is a prospective cohort study involving outpatient cases attending the emergency department due to clinically suspected pulmonary embolism (PE). The prospective cohort study found that integrating pre-test clinical probability assessment with age-adjusted D-dimer cut-off, when compared with a fixed D-diner cut-off of 500 µg/L, was associated with an increased number of patients in whom PE could be excluded with a low likelihood of subsequent clinical venous thromboembolism [17]. Another study conducted at six rural health centres in Kenya demonstrated that the effective use of basic laboratory tests within primary care settings significantly improves clinical diagnosis and patient care [18].

In our study, the results of common laboratory tests were interpreted against pre-defined clinical thresholds and correlated with the diagnoses recorded by the clinicians within the EMRs utilising a retrospective observational study design, a scientifically valid approach for identifying correlations between variables. However, such designs are insufficient for establishing causal relationships due to inherent limitations, including the lack of randomisation, which is critical for isolating the effect of the exposure on the outcome [19]. Thus, we cannot infer causality, though the goal of our study is to identify statistical relationships via correlation. The presence of strong correlations between abnormal laboratory test results and diagnoses recorded by the clinicians within the EMRs supports the clinical relevance of these tests, even if causation is not implied [20].

For example, a positive COVID-19 PCR test is strongly correlated with a COVID-19 diagnosis, underscoring its diagnostic utility. These correlations support hypothesis generation for future research and can aid in developing prognostic models. Quantifying the correlation between the tests and disease is clinically insightful, whether for diagnosis or monitoring purposes. The growth and automation of clinical laboratories have enhanced the generation and availability of real-world data. Big laboratory datasets, used effectively with robust consideration of data quality and validity, can provide strong evidence in clinical and research settings. For instance, statistical correlation can help identify relationships between pathology markers and clinical decision-making and outcomes, with implications for disease diagnosis, treatment efficacy and risk assessment.

While the value of laboratory tests should ideally be judged in the context of patient history and physical examinations, our study aims to examine the overall relationship between abnormal test results and diagnoses recorded by the clinicians across a large inpatient population, not to replace comprehensive clinical evaluations. We included various uses of tests, including rule-out scenarios, and it should be emphasised that we are examining associations rather than causative links. It should also be highlighted that the recorded diagnoses are based on coded diagnoses (using ICD-10-AM codes), which can differ from biochemical diagnoses (e.g., TSH above the upper reference limit). This difference motivates us to examine whether biochemical abnormalities are associated with actual coded clinical diagnoses in the EMRs, serving as a proxy for how doctors use laboratory results. Potential pre-analytical problems include ordering the wrong tests (either wrong indication or wrong test for the clinical question). This may contribute to lower correlation for some tests, particularly if they are more complex or often misused.

Detecting the SARS-CoV-2 virus through reverse transcription PCR testing is a method for diagnosing COVID-19. However, false-negative test results may occur in a significant proportion of patients, ranging from 20% to 67%, with the quality and timing of testing being important factors [21]. In a systematic review of the effectiveness of tests to detect the presence of SARS-CoV-2 virus, pooled analysis of 16 studies (3,818 patients) estimated a clinical sensitivity of 87.8% (95% confidence interval [CI], 81.5-92.2%) for an initial reverse-transcriptase PCR test [22]. In our study, a perfect correlation ($\rho = 1.00$) between SARS-CoV-2 PCR positivity and recorded COVID-19 diagnosis was observed. Furthermore, the high sensitivity (93.7%) and specificity (99.9%) of SARS-CoV-2 PCR observed in our study is corroborated by previous reports [22].

A moderate correlation ($\rho = 0.66$) between elevated serum total PSA and prostate cancer diagnosis was observed in the present study. An elevated serum total PSA can be caused by non-malignant conditions, including benign prostate hyperplasia and prostatitis [23]. A tissue biopsy is the standard of care for the diagnosis of prostate cancer [23]. When serum total PSA concentration is above 10 µg/L, the probability of prostate cancer is high and a prostate biopsy is generally recommended [24]. There are other limitations associated with serum total PSA as a screening tool. Most males with non-elevated PSA values would not have undergone biopsy unless they had a digital rectal examination that was simultaneously abnormal. This workup bias may explain the overestimation of the sensitivity (78.9% in our study) and the underestimation of the specificity (82.5% in our study) of PSA for the detection of prostate cancer. In terms of the utility of a total PSA of >10 µg/L for the recorded diagnosis of prostate cancer, our study has lower sensitivity because we are using actual coded clinical diagnoses, not systematic biopsy of all subjects. A major possibility for the lower sensitivity is that the diagnosis was not recorded (by the clinician) or was recorded differently. Another possibility is the timing of the tests versus the diagnoses. The interval between tests and diagnoses might have been cut too finely, such that for cancer, which requires further follow-up tests (e.g., imaging, biopsy), the diagnosis might be missed because it has a longer diagnostic lag compared to something diagnosed faster (e.g., COVID-19, acute coronary syndrome).

Similarly, causes of an elevated high-sensitivity cardiac troponin are not limited to acute coronary syndrome [25]. Hence, the moderate correlation ($\rho = 0.51$) between abnormal cardiac troponin levels and a recorded diagnosis of acute coronary

syndrome observed in our study is not unexpected.

High-sensitivity cardiac troponin tests may improve the diagnosis of acute coronary syndrome but increase the detection of elevated cardiac troponin in patients without this condition [25]. Of note, the criteria for acute myocardial infarction include the detection of a rise and/or fall of cardiac troponin values with at least one value above the 99th percentile upper reference limit and at least one of five other clinical features [26]. An elevated cardiac troponin level in patients with low pre-test probability for acute myocardial infarction predominantly reflects myocardial injury rather than myocardial infarction [27]. This is reflected in the relatively low specificity (58.3%) of high-sensitivity cardiac troponin in our study. In one study, elevations in high-sensitivity cardiac troponin concentration were observed in approximately one in eight consecutive patients attending the emergency department without suspected acute myocardial infarction [27]. The study found that elevated cardiac troponin levels were associated with older age, multiple morbidities, adverse physiological indicators, and mortality [27].

Thyroid function tests should be interpreted within the overall clinical management of each patient [28]. Relying solely on biochemical markers is insufficient; instead, the individual's history and clinical presentation should also be considered [28]. Treatment decisions integrate clinical evaluations alongside careful consideration of both TSH and free T4 results [29]. In this context, the moderate correlation ($\rho$ = 0.42-0.58) between thyroid function tests (TSH/free T4) and the recorded diagnosis (by the clinicians) of hyper- and hypothyroidism observed in the present study is expected.

The diagnosis of adrenal insufficiency depends on the demonstration of inappropriately low cortisol production. Basal (early morning) serum cortisol concentration has been demonstrated as a viable first line investigation in the evaluation of patients with suspected adrenal insufficiency. Several studies have reported a threshold above which adrenal insufficiency is unlikely and an adrenocorticotropic hormone (ACTH) stimulation test is rarely indicated [30, 31]. However, the utility of baseline or random serum cortisol to diagnose adrenal insufficiency remains uncertain. A meta-analysis by Kazlauskaite et al. [32] reported that in the absence of exogenous glucocorticoids, a basal morning (0600-1000 hours) cortisol concentrations of <140 nmol/L is suggestive of adrenal insufficiency. However, Mathara Diddhenipothage and colleagues looked at random cortisol concentrations measured at any time of the day (0440-2355 hours) and did not ascertain a lower threshold below which there was adrenal insufficiency [31]. These factors may explain the high specificity (94.5%) but low sensitivity (51.8%) for a serum/plasma cortisol of <80 nmol/L in diagnosing adrenal insufficiency (as recorded by the clinicians within the EMRs) observed in our study. In patients with suspected low cortisol-binding globulin due to sepsis or cirrhosis, "subnormal" morning cortisol levels may be seen in the absence of adrenal insufficiency [33, 34].

The strength of the current study design is its large data covering a two-year period, with relatively minimal data removal. However, potential study limitations include missed recording of diagnosis, clinician discretion and subjectivity in pathology test requesting and interpretation, and the absence of a true underlying diagnostic 'reference'. We have not focused on patient admission sources (emergency versus scheduled) and pre-admission testing. While such details could provide additional context, our study's focus is on the overall association between laboratory results and clinical diagnoses, aiming for broad generalisability.

While the recorded diagnoses should ideally reflect the current admission or presentation (i.e., an acute illness resulting in a new diagnosis), it is possible that they may be influenced by pre-existing or historical diagnoses. Such misclassification represents a potential limitation and source of confounding in our analysis. This may partly explain the association of certain diagnoses with laboratory values that fall within reference intervals or pre-defined thresholds.

Regarding thyroid function tests and associated diagnoses, one limitation of our study is the inability to determine whether patients were receiving thyroid-related treatment such as thyroxine replacement or anti-thyroid therapy. Similarly, some cortisol results may reflect values obtained during stimulation or suppression testing rather than baseline levels. For example, cortisol suppression following dexamethasone or elevation following ACTH stimulation may lead to results that differ from routine, random testing. These factors could affect the observed distributions and confound the interpretation of our findings. Consequently, these results should be interpreted with caution. Future studies should aim to exclude such confounders and thereby provide more definitive insights.

Regarding the interpretation of raised biomarkers, such as cardiac troponin and PSA, and their association with myocardial infarction and prostate cancer, respectively, we acknowledge that these biomarkers can be elevated due to conditions other than the ones mentioned. However, it is important to note that our study was based on the clinical diagnoses recorded by the attending clinicians whom we assumed considered the complete clinical picture, including the potential impact of illness on biochemical tests. Our study's objective was not to determine the definitive cause of each biochemical abnormality but to explore how abnormal results align with the clinical diagnoses made in practice. The diagnostic codes used in the current study were directly entered by attending clinicians and represent the actual recorded diagnoses within the electronic medical record. These codes reflect the clinical team's evaluation of the patient's condition at the time of diagnosis (clinician-centred diagnostic codes), thus serving as an accurate representation of clinical practice. Future big-data research into the value of laboratory tests in clinical diagnoses or differential diagnoses should consider incorporating the patient's history including an understanding of the chief complaint and presenting symptoms, and the results of a physical examination (where relevant).

## Conclusions

This study examined the correlation between abnormal laboratory results and the subsequently recorded diagnoses by the clinicians within the EMRs. The documented diagnosis may be considered to represent a form of clinical decision. Among the laboratory tests examined, the correlations between abnormal results and corresponding recorded clinical diagnoses were generally of moderate strength ($\rho$ = 0.5-0.6). Our findings demonstrate that pathology results play a varied and important role in clinical diagnosis but also highlight that laboratory tests may be utilised differently in different clinical pathways. Moreover, an abnormal laboratory result may be an incidental (self-limiting) finding or is associated with other clinical conditions not examined in this study, which may weaken the strength of association with a recorded diagnosis. Finally, it should be noted that a rule-out diagnosis based on laboratory results is an equally important clinical decision as rule-in diagnosis, although the former is not usually recorded and hence, much harder to quantify.

## Declaration of conflicting interests

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

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## Ethical approval

Ethics approval was obtained from the Human Research Ethics Committee of the South Eastern Sydney Local Health District (HREC/16/POWH/412) and the New South Wales Population and Health Services Research Ethics Committee (2022/ETH0209).

## Author contributions

All authors contributed to the conception and design of the study. AG and MP obtained ethical approval while GSF and GDK conducted the data analysis. KWC and TPL drafted the initial manuscript. All authors critically reviewed, revised, and approved the final version of the manuscript for submission.

## Guarantor

KWC.

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Research Article

# Visceral Fat As The Main Obesity Index That Determines The Occurrence of Adipose Tissue Insulin Resistance

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**Keywords**
Visceral Fat, Obesity Index, Adipo-IR

**Abstract**

**Background**: Insulin resistance may occur in various organs, including adipose tissue, which causes increased lipolysis and blood free fatty acids (FFA). Insulin resistance in adipose tissue is commonly assessed using the Adipose Tissue Insulin Resistance (Adipo-IR) index, calculated using fasting insulin and FFA levels. This study aimed to evaluate which obesity index has the best predictive value to determine Adipo-IR.

**Methods**: This cross-sectional study is conducted on 80 non-diabetic adult subjects. Measurements and assessments of the relationship between obesity indices and Adipo-IR values were performed.

**Results**: Waist circumference ($r = 0.275$, $p = 0.013$), BMI ($r = 0.318$, $p = 0.004$), visceral fat ($r = 0.334$, $p = 0.002$), and body fat percentage ($r = 0.246$, $p = 0.028$) were all significantly correlated with Adipo-IR. The area under the curve (AUC) showed that visceral fat had the most significant predictive value of insulin resistance in adipose tissue compared to BMI, waist circumference, and body fat (AUC = 0.690 vs 0.663 vs 0.620 vs 0.570). Subjects with visceral fat values in the fourth quartile had a 6-fold risk of experiencing insulin resistance in adipose tissue compared to subjects in quartile 1 (OR = 6, $p = 0.014$, 95% CI 1.324-27.191).

**Conclusions**: The Adipo-IR index increases with the value of the obesity index. Visceral fat has the highest predictive value in determining the occurrence of adipose tissue insulin resistance.

## Introduction

Insulin resistance is marked by a decreased response of different tissues sensitive to insulin activity, resulting in increased insulin production to compensate for the reduced effectiveness of insulin action [1]. Insulin works by increasing the glucose entry from the blood into peripheral tissues (liver, muscle, adipose tissue) and inhibiting the process of gluconeogenesis in the liver [2]. Increased adipose tissue lipolysis due to insulin resistance results in a rise in the generation of free fatty acids (FFA), which are subsequently released into the bloodstream [1,2].

The prevalence of obesity, a worldwide issue, is rising annually. The hallmark of obesity is the increased buildup of fat in the body, which can be caused by either hyperplasia or hypertrophy of adipocyte cells [3]. White adipose tissue is a fat tissue mainly found in visceral and subcutaneous tissues, is endocrine active, and has an essential role in insulin resistance development [4]. In obesity, increased formation of FFA and dietary fat trigger lipotoxicity, which can cause worsening insulin resistance, mainly in the skeletal muscle and liver [3-5]. Adipose tissue produces a variety of adipocyte-related cytokines, such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α, which can also reduce insulin sensitivity and induce lipolysis [6].

There is a relationship between various obesity indices, both traditional waist circumference (WC), body mass index (BMI), and non-traditional ones such as visceral fat (VF) and body fat percentage (BF), with the presence of insulin resistance, especially in the liver and muscle [7-9]. The adipose tissue insulin resistance (Adipo-IR) index measures adipose tissue insulin resistance, calculated by multiplying fasting insulin by FFA levels. [10]. This study intends to establish which obesity indicator has the most predictive value to detect adipose tissue insulin resistance and to investigate the association between multiple obesity indices, particularly WC, BMI, VF, and BF, with the Adipo-IR index.

## Methods

### Study Population

This was cross-sectional study was conducted on 80 non-diabetic adult subjects who voluntarily participated by agreeing to the informed consent. Sampling and laboratory examinations were performed at Hasanuddin University Hospital from August to September 2024. The study population was clinical clerkship students, medical faculty residents, and students of the Hasanuddin University Biomedical Sciences master's program who were willing to participate. Adult participants who were at least eighteen years old met the inclusion criteria. Subjects with fasting blood glucose levels (FBG) ≥126 mg/dL and or oral glucose tolerance test (OGTT) ≥200 mg/dL, having a history of diabetes mellitus (DM), taking lipid-lowering drugs, and experiencing inflammation or infection, were excluded. This study received an ethical approval recommendation from the Hasanuddin University Research Ethics Committee with No: 649/UN4.6.4.5.31/PP36/2024.

### Obesity Index Measurement and Laboratory Procedure

Obesity indices measured include WC, BMI, VF, and BF. After measuring height and body weight (BW), BMI was calculated using the formula BMI = BW/Height$^2$. The measurement of waist circumference was taken halfway between the lower border of the 12th rib and the iliac crest. BF and VF measurements were performed using the bioelectrical impedance analysis (BIA) method. BW (coefficient variation (CV) = 0.09%), BF (CV = 1.04%), and VF (CV = 0%) measurements were performed using the Tanita BC-541 (Japan) device. The study subjects were fasted for 8-10 hours at night, and then blood samples were taken at 8 am the next day, followed by an OGTT test. FBG and OGTT examinations were carried out, according to standard protocols. A 5cc fasting blood sample was taken, then the serum was separated. Glucose examination was carried out immediately after sampling, and then the remaining fasting serum sample was stored in aliquot form at -20 °C for further examination of insulin and FFA. Glucose examination was performed using the enzymatic-colorimetry method using the Abx Pentra 400 (Horiba, United States), while insulin was examined using the electrochemiluminescence immunoassay (ECLIA) method with the Cobas e411 (Roche, Germany). Using a kit from MyBioSource (United States), the enzyme-linked immunosorbent assay (ELISA) method was used to measure the serum FFA levels. The formula = (FBG (mg/dL) x Insulin (mIU/L)/405) was used to measure the homeostatic model assessment of insulin resistance index (HOMA-IR). FFA (mmol/L) x Insulin (mIU/L) was the formula used to calculate the Adipo-IR index. Adipo-IR values ≥75th percentile are used to establish the occurrence of adipose tissue insulin resistance. Subjects with Adipo-IR values below the 75th percentile were classified as not experiencing insulin resistance in adipose tissue. The 75th percentile value of Adipo-IR in this study was 5.84.

### Statistical Analysis

The numerical data normality was evaluated by the Kolmogorov-Smirnov test. The variables of age, WC, and BF were normally distributed, while other parameters, including systolic, diastolic blood pressure, FBG, insulin, FFA, HOMA-IR, Adipo-IR index, VF, and BMI, were not normally distributed. The Spearman Correlation test was used to assess the association between the Adipo-IR index and the other parameters. Analysis with the receiver operating characteristic (ROC) curve was conducted to analyze the role of various obesity indices as adipose tissue insulin resistance predictors, followed by an assessment of the area under the curve (AUC) and determination of the best cut-off value (which had the highest combination of sensitivity and specificity) in predicting insulin resistance in adipose tissue. The Odds ratio (OR) value of the increasing risk of insulin resistance in adipose tissue along, with increasing quartiles of VF values, was performed with the Fisher-Exact or Chi-Square test.

**Results**

A total of 80 volunteers, consisting of 38 (47.5%) men and 42 (52.5%) women, were willing to join the research. The research subjects' basic characteristics are shown in Table 1. The average age of the research subjects was 31.69±4.09 years, ranging between 21 to 40 years.

**Table 1:** Basic Characteristics of Study Subjects.

| Variables | Mean±SD | Median (Min-Max) |
|---|---|---|
| Age (years) | 31.69±4.09 | 31 (21-40) |
| Systole (mmHg) | 116.20±8.52 | 120 (90-130) |
| Diastole (mmHg) | 76.11±7.48 | 80 (60-100) |
| FBG (mg/dL) | 97.23±10.83 | 95.50 (69-123) |
| Insulin (mIU/L) | 15.41±14.08 | 11.52 (4.31-70.64) |
| FFA (mmol/L) | 0.28±0.36 | 0.15 (0.01-2.15) |
| HOMA-IR (unit) | 3.82±3.97 | 2.59 (0.98-25.47) |
| Adipo-IR (unit) | 4.13±5.63 | 1.99 (0.05-24.34) |
| BMI (kg/m2) | 25.62±4.79 | 25.05 (15.30-45) |
| WC (cm) | 89.52±11.01 | 90 (61-133) |
| BF (%) | 29.25±6.50 | 29.05 (12-43.80) |
| VF (Unit) | 8.95±5.52 | 8 (1-30) |

The correlation between the Adipo-IR index and other parameters is shown in Table 2. From weakest to strongest, the Adipo-IR index correlates with BF (r = 0.246), WC (r = 0.275), BMI (r = 0.318), and VF (r = 0.334). It also exhibits a substantial association with HOMA-IR (r = 0.522).

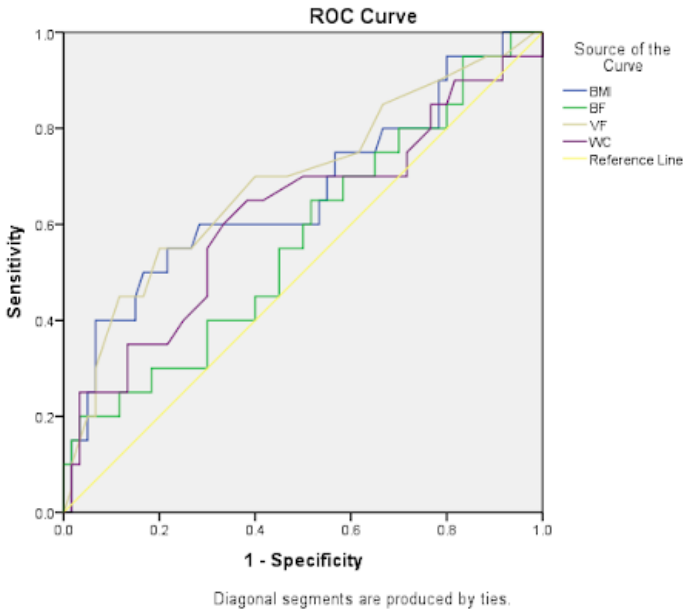
**Table 2:** Correlation Between Various Variables with Adipo-IR Index.

| Variables | Adipo-IR Index | |
|---|---|---|
| | r | p* |
| Age | 0.206 | 0.067 |
| Systole | 0.154 | 0.171 |
| Diastole | 0.013 | 0.911 |
| FBG | 0.13 | 0.251 |
| Insulin | 0.521 | <0.001 |
| FFA | 0.904 | <0.001 |
| HOMA-IR | 0.522 | <0.001 |
| BMI | 0.318 | 0.004 |
| WC | 0.275 | 0.013 |
| BF | 0.246 | 0.028 |
| VF | 0.334 | 0.002 |

*Spearman Correlation Test

The ROC curve below displays the ability of various obesity parameters to determine the occurrence of insulin resistance in adipose tissue (Figure 1).

**Figure 1**: ROC Curve of Obesity Indices as Adipose Tissue Insulin Resistance Predictors.




The AUC value shows that VF (AUC = 0.690, cut off = 8.5) and BMI (AUC = 0.663, cut off = 26.95 cm) have the strongest predictive ability in determining the occurrence of insulin resistance in adipose tissue (Table 3).

**Table 3:** AUC Values and Best Cut-off Values of Obesity Indices in Determining the Occurrence of Insulin Resistance in Adipose Tissue.

| Variables | AUC | p | 95% CI | Cut-off | Sensitivity | Specificity |
|---|---|---|---|---|---|---|
| BMI | 0.663 | 0.03 | 0.513-0.813 | 26.95 | 0.55 | 0.783 |
| WC | 0.62 | 0.108 | 0.467-0.774 | 91.5 | 0.65 | 0.617 |
| BF | 0.57 | 0.351 | 0.422-0.718 | 28.3 | 0.65 | 0.483 |
| VF | 0.69 | 0.011 | 0.547-0.833 | 8.5 | 0.7 | 0.6 |

VF, the obesity index, which has the best predictor value for assessing adipose tissue insulin resistance, will then be divided into 4 quartiles. The risk (OR) of adipose tissue insulin resistance in each quartile is assessed by comparing it with the risk in quartile 1. Those in the highest quartile had 6 times higher risk to having adipose tissue insulin resistance compared to the in the lowest quartile (Table 4).

**Table 3:** AUC Values and Best Cut-off Values of Obesity Indices in Determining the Occurrence of Insulin Resistance in Adipose Tissue.

| "VF Quartiles" | Adipo IR State | Non_Adipo IR State | p<br>OR (CI 95%) | |
|---|---|---|---|---|
| | n (%) | n (%) | | |
| 4 (>11) | 9 (47) | 10 (52.6) | 0.014[1] | 6.00 (1.324-27.191) |
| 3 (8.1-11) | 5 (26.3) | 14 (73.7) | 0.433[2] | 2.38 (0.488-11.628) |
| 2 (5.1-8) | 3 (15.8) | 16 (84.2) | 0.570[2] | 1.25 (0.222-7.051) |
| 1 (<5) | 3 (13) | 20 (87) | | Baseline |
| Total | 20 (25) | 60 (75) | | |

[1] Chi-Square Test

[2] Fisher-Exact Test

**Discussion**

In this investigation, a significant association was found between the Adipo-IR index and the obesity index, with the strongest correlation found in VF (r = 0.334) followed by BMI (r = 0.318), WC (r = 0.275), then BF (r = 0.246). Kim et al performed research on 205 adolescents and young adults (normoglycemia, pre-diabetes, and diabetes subjects) in the United States and described similar results with the findings that the Adipo-IR index correlated with visceral adipose tissue (r = 0.557) as measured by computed tomography (CT), BMI (r = 0.559) and BF (r = 0.529) [11]. Semnani-Azad et al performed a study on 468 non-diabetic adult subjects with a mean age of 50 years and revealed that the Adipo-IR index had correlation with WC (r = 0.520) and BMI (r = 0.560) [12]. Zhang et al in Beijing, China, conducted a study on 312 people with and without metabolic syndrome and found the Adipo-IR index associated with WC (r = 0.480) and BMI (r = 0.430) [13]. Several studies mentioned above showed similar results to the report of this study, although there are variations in the order of obesity indices and the strength of their correlations that can be caused by differences in race, average age, and clinical conditions of the subjects when compared to this study. In contrast, Kitaoka et al in Japan reported no correlation between the Adipo-IR index and BMI (r = -0.007, p>0.05) and WC (r = -0.062, p>0.05) in young female subjects (average age 20.7 years) [14]. This may be because the study population was women with an average BMI (20.3 kg/m2) and WC (71.2 cm) within the normal range.

In this study, it was found that compared to other obesity indices, VF had the strongest correlation with the Adipo-IR index. There are several things that can explain this. VF is ectopic fat classified as white adipose tissue, composed of larger adipocytes with fewer mitochondria than brown adipose tissue. VF is spread in the mesentery, omentum, and retroperitoneal space and is active both as an endocrine and metabolic organ that has a significant role in the occurrence of insulin resistance. Adipose tissue insulin resistance, especially in VF, causes suppression of insulin's ability to prevent lipolysis, contributing to the increased release of FFA into the blood [15-20]. In this study, it was found that VF had a stronger association with Adipo-IR than WC. This may be because WC measurements not only assess VF but also subcutaneous fat (SF). SF is reported to have a role quite different from VF, having protective properties against insulin resistance. Subjects with a low VF/SF ratio have better insulin sensitivity than subjects with a high VF/SF ratio [21]. WC measurements not only measure VF, which is associated with the insulin resistance state but also measure SF, which is protective against the occurrence of insulin resistance so that cumulatively, the correlation of WC (accumulation of VF and SF) with Adipo-IR is lower than the correlation of Adipo-IR with VF alone.

In this study, it was found that VF had the best predictive ability in assessing the occurrence of insulin resistance in adipose tissue (AUC = 0.690, cut off = 8.5) followed by BMI (AUC = 0.663, cut off = 26.95 kg/m2). Jiang et al, in Zhejiang, China, conducted a study on 499 subjects aged over 50 years and found that BMI (AUC = 0.770, cut off = 22.04 kg/m2 in men; AUC = 0.780, cut off = 21.77 kg/m2 in women) and WC (AUC = 0.790, cut off = 83.5 cm in men; AUC = 0.770, cut off = 84.75 cm in women) can be used as predictors of insulin resistance in peripheral tissue (Adipo-IR values above the 66.7th percentile are used to define insulin resistance in adipose tissue; cut off values 1.87 in men and 3.87 in women) [22]. The difference in AUC and cut-off values reported by Jiang et al with our study is due to differences in population characteristics and the Adipo-IR index percentile (cut-off) values in defining adipose tissue insulin resistance. However, the BMI cut-off value reported by this study (26.95 kg/m2) in predicting insulin resistance in adipose tissue is closer to the cut-off value used to define obesity in Southeast Asian countries, including Indonesia (range 25-30 kg/m2) [23]. This study also found that subjects with VF values in quartile 4 (>11) had a 6-fold increased risk of suffering from insulin resistance in adipose tissue compared to subjects with VF values in quartile 1 (<5). Increased VF mass can trigger the release of various pro-inflammatory adipokines, including leptin, IL-6, and TNF-α, that can trigger systemic insulin resistance in adipose tissue [24]. Increased lipid accumulation, especially in visceral tissue, will trigger lipotoxicity, metabolic disorders, and resistance in adipose tissue, thus triggering lipolysis, characterized by increased FFA release in the blood [25].

The cross-sectional study design of this research is one of its limitations, as it is unable to elucidate the causal relationship

between the variables under investigation: therefore, future longitudinal studies are needed to establish the causality. The research subjects came from a single population, so further research is needed to involve multicenter studies with different age groups and ethnicities to generalize the findings of this study. To validate the findings of this study, more research may be done utilizing the gold standard technique for evaluating VF and BF, which is magnetic resonance imaging (MRI) or CT, since the VF measurement agreement between BIA and CT methods is moderate (r = 0.387 to 0.626) [26].

## Conclusion

The Adipo-IR index increases with the value of the obesity index. Visceral fat has the highest predictive value in determining adipose tissue insulin resistance; therefore, it can be used as a routine screening protocol for assessing insulin resistance.

## Author Contributions

LBK, NAK, NN, MIB, and AA conceived the study. LBK and LPA collected samples and prepared the initial draft of this manuscript. LBK, NAK, NN, MIB, and AA conducted further reviews and provided scientific input on this manuscript.

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## Conflict Of Interest

None.

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Research Article

# Evaluation of Proflo-U® Platform for Urine Albumin Measurement in Chronic Kidney Disease Diagnosis: A Comparative Study

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**Keywords**

Chronic kidney disease (CKD), Albuminuria, Point-of-care (PoC) solutions, Proflo-U® platform, Fluorescence-based measurement, Immunoturbidity, Diagnostic accuracy

**Abstract**

**Background**: Chronic kidney disease (CKD) presents a global health challenge, requiring efficient assessment tools for effective management. Albuminuria assessment serves as a crucial indicator of CKD diagnosis, stage-classification, and progression risk. However, for the large-scale screening, the current laboratory-based methods lack portability and entail delays. Point-of-care (PoC) solutions like dipsticks offer promise, but sensitivity limitations persist.

**Objective**: To address this gap, we evaluated Proflo-U®, a novel fluorescent-based urine albumin measurement technology with the potential for PoC deployment, with the immunoturbidity-based Beckman Coulter system.

**Results**: Our study, based on a blinded comparison of 255 patient samples, revealed a high correlation (R2>0.9) between Proflo-U® and Beckman Coulter based measurement. Proflo-U® has been able to demonstrate comparable diagnostic accuracy, with strong sensitivity and specificity across different urine albumin concentration categories. Statistical analyses supported its reliability, and Receiver Operating Characteristic (ROC) analysis highlighted its clinically acceptable diagnostic accuracy.

**Conclusion**: Our findings suggest that Proflo-U® holds potential for mass screening initiatives in resource-limited settings to enable early CKD detection and management.

**Introduction**

Chronic Kidney Disease (CKD) is rapidly emerging as a significant global public health concern, placing an escalating burden on healthcare services and infrastructure worldwide [1]. Recognized by the Kidney Disease Improving Global Outcome (KDIGO) initiative, CKD is characterized by persistent abnormalities in kidney structure or function, lasting for more than three months, with profound implications for overall health. Key indicators used for CKD assessment based on function, include albuminuria levels, reflecting kidney damage, and estimated glomerular filtration rate (eGFR), indicative of kidney function [2]. These metrics serve as cornerstones for risk stratification and tailored management strategies, facilitating optimal allocation of healthcare resources and expediting referrals for patients at elevated risk of CKD progression.

Remarkably, even in the early stages of CKD, when eGFR levels may remain within normal or partially elevated ranges, the presence of mildly elevated level of albumin in urine emerges as a significant prognostic marker, impacting both diabetic and non-diabetic patient populations [3]. Alongside the recommendations set forth by organizations such as KDIGO, The National Kidney Foundation's Kidney Disease Outcomes Quality Initiative (KDOQI), American Diabetes Association, European Association for the Study of Diabetes, and the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) advocate for routine albuminuria screening, particularly in individuals with diabetes mellitus [4–6][7]. Notably, among diabetic patients with nephropathy, albuminuria consistently emerges as a robust predictor of End-Stage Renal Disease (ESRD), underscoring its clinical significance [5].

Moreover, heightened surveillance through more frequent albuminuria screening is advised for patients at increased risk of kidney disease progression, such as those with hypertension or cardiovascular disease, as well as individuals exhibiting evidence of worsening kidney damage. The primary advantage offered by urine albumin estimation as diagnostic marker is its non-invasive nature of sample while eGFR estimation either with creatinine or cystatin C is a serum biomarker and hence associated with invasive blood drawing. However, CKD that is 'not' associated with nephron or tubular damage does not show urine albumin increase at the initial stage. Moreover, the eGFR creatinine marker vary across individual based on the race, ethnicity, and muscle mass. A new biomarker i.e. Cystatin C that is associated with renal functional status, also shows elevated levels in inflammatory states and thyroid dysfunction independent of kidney disease [8]. Albuminuria is categorized into A1, A2, and A3 based on the daily excretion of albumin in urine. A1, representing levels below 30mg/24 hrs, is considered within the normal to mildly increased range. A2, ranging from 30 to 300mg/24hrs, denotes a moderately increased level, while A3, exceeding 300mg/24hrs, signifies severe elevation [9]. Interestingly, urine albumin as a biomarker is associated with different kidney disease however, the timeline of albumin increase can classify condition. For example, in case of proximal tubular nephrotoxin or acute kidney injury (AKI), there is a rapid increase in urine albumin over a short period of time that might stabilize or recover upon management. While in case of CKD, the urine albumin concentration increases gradually over a longer course of time. The latest KDIGO guideline suggests if the urine albumin level has been persistently elevated (> 30 mg/g) for greater than 3 months should be diagnosed as CKD [10,11].

While laboratory-based techniques like immunoturbidimetry, nephelometry, Flow Injection Analysis (FIA), and Sequential Injection Analysis (SIA) offer highly accurate results, their implementation is restricted to centralized laboratory settings, leading to prolonged turnaround times for patient reports [12]. To address the need for timely and accessible diagnostics, recent advancements have introduced point-of-care solutions utilizing reader devices and dipstick technology. However, conventional dipsticks exhibit limitations in sensitivity compared to quantitative methods, compromising diagnostic accuracy, particularly in detecting lower levels of albuminuria. In one large study, a protein dipstick result of trace or higher was associated with ACR values ≥30 mg/g (≥3 mg/mmol) with a sensitivity of only 69.4% and 86.8% specificity [13]. Though, easy to use and PoC deployable however, desirable diagnostic process should have high specificity and sensitivity both at A2 and A3 range while having other features likes ease of use and affordability that favors PoC deployment. Such a system will enable mass screening for early diagnosis and monitoring at resource limited setup. The Proflo-U® albumin test is expensive than the multi-parameter semi-quantitative urine dipsticks as PoC solution. However, the later has the major disadvantage of being poor sensitivity at the lower range of albumin, i.e., below 300mg/L resulting in missing of early stages of CKD patients during mass screening. Further, the readout of the test are overlapping colour indicator, that are difficult to objective estimate using visual inspection, corresponding broad range of concentration instead of a quantitative value (that necessitates the use of a relatively expensive reader). Nonetheless, if we compare the costing with the quantitative albumin estimation PoC solutions like Siemens DCA microalbumin/creatinine test and Abbott Afinion ACR test, Proflo-U® test is marginally lesser in price and comparatively has broader range of detection. Proflo-U® analytical linear range is 20-1000 mg/L while the other products have 5-200 mg/L, which is a major advantage in case of nephrological conditions. In addition, the reader device is battery operated and easy to use ad IoT enabled that offers a large range of advantages in data storage, remote maintenance, and automated operational qualifications. The innovative fluorophore nanosensor based technology has advantage over immunoturbidity be being highly thermostability, which alleviates the major limitation of cold storage and cold chain logistics in mass screening at resource limited settings [14].

To bridge this diagnostic gap, we investigated the patented

Proflo-U® platform and compared with the laboratory based immunoturbidity method for the analytical performance. Our previous published study demonstrated that Proflo-U® offers comparable albuminuria estimation to established reference standards, such as the Beckman Coulter system and Biosense assay, with a high correlation coefficient ($R2 > 0.99$, $p > 0.05$). Nevertheless, our evaluation was constrained by the utilization of spiked samples containing recombinant Human Serum Albumin (r-HSA) [14]. To ascertain the true diagnostic utility of the Proflo-U® platform, we conducted further analysis using urine samples obtained from a cohort of 255 hospitalized patients, following approval from both the research council and ethical committee at AIIMS Bhubaneswar, India. Blinded testing was performed to validate its efficacy in real-world clinical settings.

## Material and Methods

### Study Design

### Sample type

The sample type was a 24 hrs. urine sample provided to the AIIMS Bhubaneswar Biochemistry Department for urine albumin analysis from the admitted patients in the hospital.

### Sample Size

An n=238 + 20 sample size has been calculated considering the prevalence of at least 50% of the sample will have diagnostically relevant level of albumin to determine the study method at 95% Confidence of Interval with 85% sensitivity and specificity and 90% precision as parameter for calculation.

### Sample exclusion criteria

The exclusion criteria exercised for the urine samples to be tested were: 1. Urine samples with blood contamination, 2. Urine samples with dark amber color appearance and 3. Urine samples opaque and dense or preidentified UTI infection.

### Study Period

The study period was between 23rd November 2021 to 8th March 2022. The samples have been run parallelly both in the Beckman Coulter as well as Proflo-U® platform and the data has been recorded from both the system along with the patient ID.

The study design has been reviewed and passed by the AIIMS Bhubaneswar Institutional Ethics Committee Ref No. T/IM-NF/Biochem/21/74. All studies involving human subjects have been conducted in compliance with the ethical principles for medical research involving human subjects, in accordance with the Declaration of Helsinki.

### Sample analysis Method

The urine samples were coded with Hospital's Patient ID number created through the Laboratory Information Management System (LIMS). The sample freshly received by the Biochemistry department have been parallelly analyzed with Beckman Coulter Microalbumin reagent from Ireland, OSR6167 Lot no 2312 on Beckman Coulter AU5800 fully automated chemistry analyzer, S/N 2013081121, by immunoturbidity inhibition assay, according to manufacturer instruction and Proflo-U® cartridge on Proflo-U® platform according to manufacturer instruction [14]. The test has been conducted at NABL accredited AIIMS Bhubaneswar Biochemistry Laboratory. The albumin concentration obtained for each sample were noted with the patient ID both from Beckman Coulter and Proflo-U®.

### Data analysis

The data has been statistically analyzed for method correlation with R language using shiny [15]

web application framework for the Passing Bablock regression and Bland Altman (BA) plot. The Receiver Operating characteristic (ROC) in R studio package [16].

## Results and Discussion

The sample size for the study was determined according to the guidelines for Sample Size Estimation for Diagnostic Accuracy Studies, where a new diagnostic test is compared with the reference standard in a cohort provided the true disease status and prevalence are known [17]. With a fixed type I error of 0.05, a marginal error of up to 10%, and a confidence interval of 95%, the prevalence of CKD was estimated at approximately 12% (the mean reported prevalence of CKD in India ranging from 4% to 20%) [18]. Targeting a sensitivity and specificity of at least 92%, a minimum sample size of 236 was calculated [19, 20]. Permission for a sample size of 238 + 20 was obtained from the Institutional Ethics Committee. A similar sample size was utilized for the analysis of the HemoCue point-of-care system, with n=259 [21] and n=108 urinary samples were analyzed for comparison of 5 immuno-turbidimetric methods for urine albumin quantification [22].

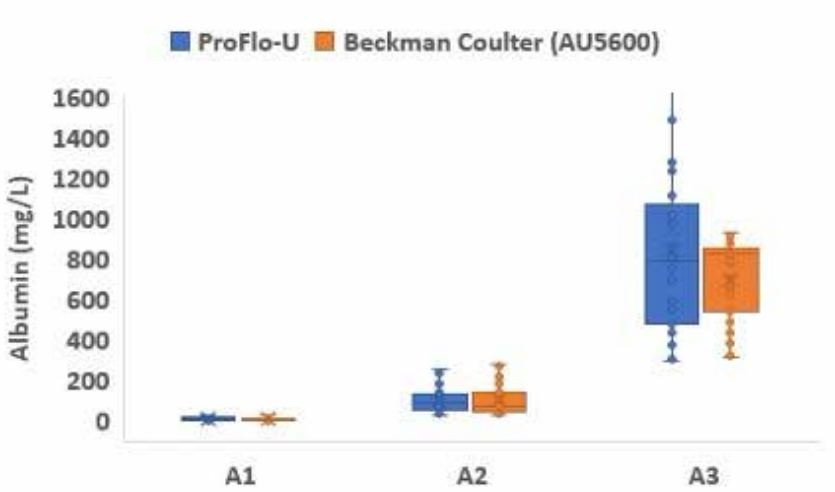
A total of 258 urine samples received at the AIIMS Bhubaneswar Biochemistry Department for urine microalbumin analysis were used for the study. Three samples lacked patient IDs and were therefore excluded, leaving n=255 urine samples for the analysis. No samples were lost, as testing was conducted promptly upon receipt by the Biochemistry Department. Results for urine albumin concentration in mg/L were tabulated (Supplementary Table 1).

The distribution of the samples between normal and elevated were almost equally represented, as 138 samples have shown below 30mg/L and 117 samples above 30mg/L concentration for the urine albumin by the Beckman Coulter System. Though, the immunoturbidity method Beckman Coulter System is not a gold standard in true sense but can be regarded as the best test under "reasonable conditions" and hence been considered as the reference method.

The urine samples have been further divided into three categories normal (A1) <30mg/L, moderately high (A2) >30mg/L but <300mg/L and severely high (A3) > 300mg/L (We have used annotation A1, A2 and A3 that generally been used for urine albumin creatinine ratio, but in this case with reference to 24 hrs

urine albumin) both for the test method Proflo-U® platform and the reference method Beckman Coulter System. The distribution of the tested samples has been represented in the Box-Whisker plot (Figure 1). In the A1 category the reference method had determined 138 samples while 132 samples by the test method, suggesting 5 samples have been wrongly categorized. In the A2 category 77 samples have been identified by the reference method while the test method had shown 84, corresponding to a difference of 6. In the category severely high category reference method has identified 41 samples while the test method could pick 39 samples indicating a non-agreement of 2.

**Figure 1**: Box-Whisker Plot of the urine albumin measured samples within the A1, A2 and A3 categories by Proflo-U® and Beckman Coulter.



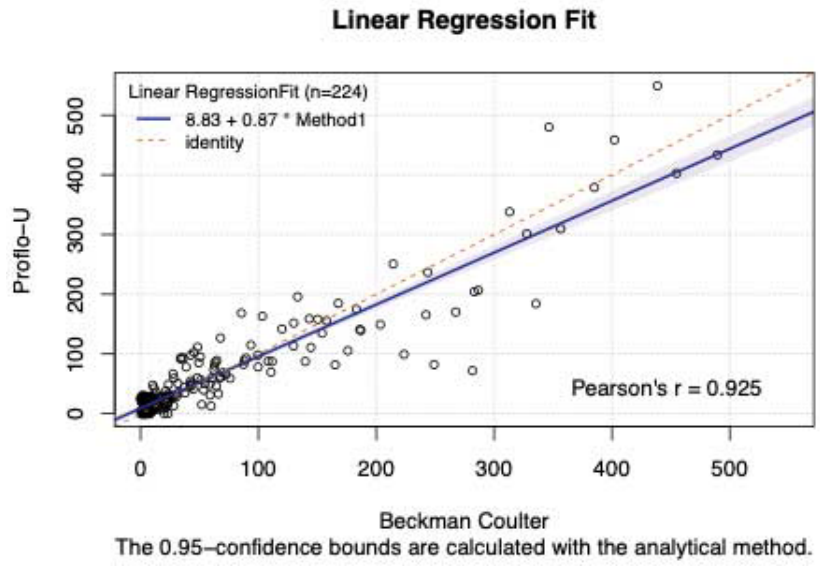

The sensitivity, specificity, positive prediction value (PPV) and negative prediction value (NPV) were calculated for each category keeping in each case the total sample size 255 (Table 1). The sensitivity with which Proflo-U® platform was able to detect the urine sample in the A1, A2 and A3 category with respect to the reference method were 94.2%, 97.37% and 95.12%, respectively. The obtained specificity with Proflo-U® platform for A1 =98.29%, A2 =94.41% and A3 =100%. In the referenced literature, the PoC urine albumin diagnostic methods compared with the laboratory based immune-nephelometry has reported comparable degree of sensitivity of 83.8% in semi-quantitative antibody-based urine albumin detection while 79.6% for the quantitative method and specificity were 93.8 and 97.1, respectively[23]. In different PoC methods for Albumin estimation the reported range for sensitivity was 86-98% and specificity were 61-94% [24]. The PPV obtained for the Proflo-U® platform for A2 was lowest with 90.5% while highest for the A3 category with 100%, similarly, the lowest obtained NPV was for the A1 category with 93.1% and highest in A3 with 99.1%. The literature reported PoC diagnostic tests has shown PPV, 95.6% for quantitative and 88.6% for the semi-quantitative method, while NPV were 85.8% for quantitative and 91% for semi-quantitative method.

**Table 1**: Presenting the distribution of sample based on urine albumin content categorized as A1 (<30mg/L), A2 (30-300mg/L) and A3 (>300mg/L) with Beckman Coulter and Proflo-U® method. The calculated sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) in percentage for the Proflo-U® with Beckman Coulter estimation as the reference method has been tabulated.

| | | Beckman Coulter | | | |
|---|---|---|---|---|---|
| | | **A1 (<30mg/L)** | **A2 (30-300mg/L)** | **A3 (>300mg/L)** | **Total** |
| **Proflo-U** | **A1 (<30mg/L)** | 130 | 2 | 0 | 132 |
| | **A2 (30-300mg/L)** | 8 | 74 | 2 | 84 |
| | **A3 (>300mg/L)** | 0 | 0 | 39 | 39 |
| | **Total** | 138 | 76 | 41 | 255 |
| 1 | **Sensitivity%** | 94.2 | 97.37 | 95.12 | |
| 2 | **Specificity%** | 98.29 | 94.41 | 100 | |
| 3 | **PPV%** | 98.5 | 90.5 | 100 | |
| 4 | **NPV%** | 93.5 | 98.8 | 99.1 | |

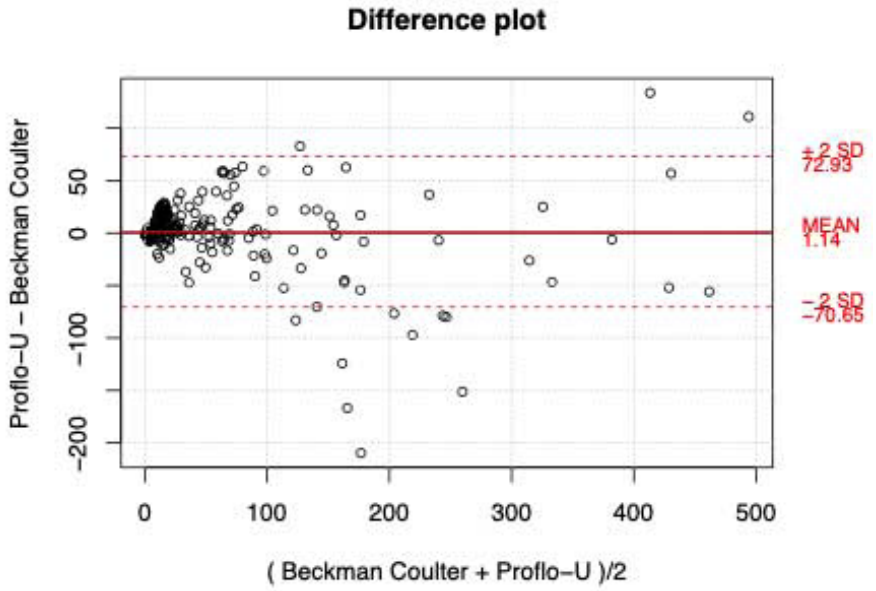
Considering the quantitative nature of both the test and reference measurement technologies, we evaluated agreement of analytical methods and possible systematic bias between them with Passing Bablok regression. Results are presented with scatter diagram and regression line where the sample size considered n=224 (samples considered with urine albumin content less than 500 mg/L, considering the reference method highest claimed analytical range 5-300 mg/L)[25] Figure 2 (The analysis for the complete cohort pool with sample size n=255 has been provided in the Supplementary Figure 1). The Pearson coefficient in both the cases are >0.9 (r=0.916 for n=255 and r=0.925 for n=224) indicating very strong relationship between the data obtained from test and reference methods.

**Figure 2:** The Passing Bablok Regression fit has been presented for the all the n =224 samples for Proflo-U® and Beckman Coulter.



The data next analyzed with the Bland Altaman plot for sample size n=255 (Supplementary Figure 2) and sample size n=224 Figure 3. The mean offset for n=224 data points was +1.14 for Proflo-U® method over Beckman Coulter where the 95% confidence Interval the Lower bound was -70.65 and upper bound is +72.93. If we consider all the data points (n=255), the data points at a range 600 above show high degree of disagreement as the range is much beyond the analytical range of the reference method and as claimed by the manufacturer might show 'Prozone or hook effect' at albumin concentration >600mg/L (Supplementary Figure 2).

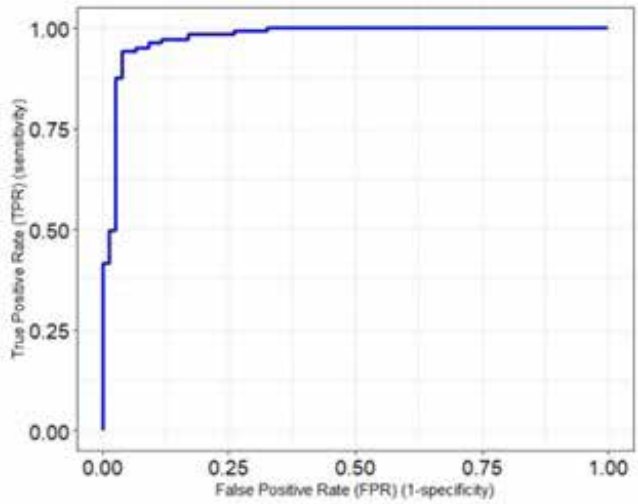
**Figure 3:** Bland-Altaman plot for method correlation where Proflo-U® serves as test method and Beckman Coulter as the reference method for sample size n =224.




To determine the diagnostic accuracy of the Proflo-U® we further analyzed the Receiver Operating characteristic (ROC) in two sets, where the set 1 for A1 and A2 category (Figure 4 A) and set 2 for A2 and A3 category (Figure 4 C). The samples were categorized based on the values obtained with the reference method Beckman Coulter as A1, A2 and A3. The data obtained by the Proflo-U® platform for the sample categorized as A1 was considered as normal and A2 as diseased for set 1. In set 1 there were total sample size was n=222 for which the ROC was analyzed. The Area Under Curve (AUC) was 0.977 with a cutoff at 30.6mg/L, where the specificity obtained 96%, sensitivity 94%, PPV= 0.977 and NPV= 0.90 Figure 4A. The response under different cut off values has been presented in Figure 4B. In set 2 the data obtained by the Proflo-U® platform for the sample categorized as A2 was considered as normal and A3 as diseased, where the sample size n=125 for which the ROC was analyzed. The Area Under Curve (AUC) was 0.998 with a cutoff at 253mg/L, where the specificity obtained 97.5%, sensitivity 100%, PPV= 0.987 and NPV= 1.00 Figure 4C. The response under different cut off values has been presented in Figure 4D.

**Figure 4:** The Receiver Operating Characteristic (ROC) curve was generated to assess the diagnostic accuracy. (A) Patient urine samples measured by the Proflo-U® platform for the prediction of A2 or urine albumin concentration range 30-300mg/L and A1 or urine albumin concentration range <30mg/L, the classification is based on the measured value obtained with the reference method Beckman Coulter. (B) Sensitivity, specificity, Positive Predictive Value and Negative Predictive Value at different cutoff for the Response Operating Curve for the data set corresponding to A1 and A2 categories. (C) Patient urine samples measured by the Proflo-U® platform for the prediction of A3 or urine albumin concentration range >300mg/L and A2 or urine albumin concentration range 30-300mg/L, the classification is based on the measured value obtained with the reference method Beckman Coulter. (D) Sensitivity, specificity, Positive Predictive Value and Negative Predictive Value at different cutoff for the Response Operating Curve for the data set corresponding to A2 and A3 categories.

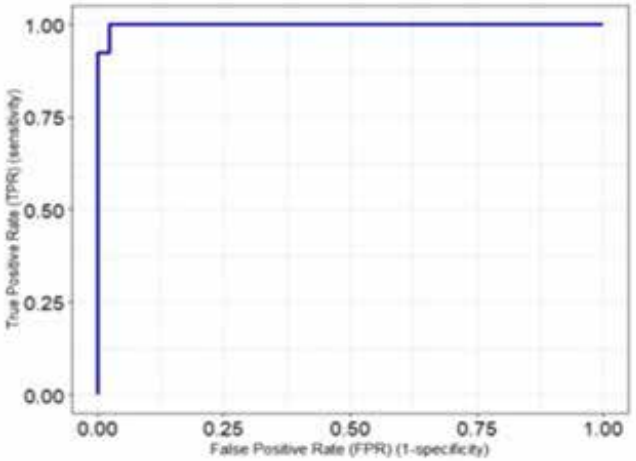
(A)




(B)

| Cut Off | Sensitivity | Specificity | PPV | NPV |
|---|---|---|---|---|
| 67.72 | 1.00 | 0.68 | 0.85 | 1.00 |
| 59.45 | 0.99 | 0.74 | 0.87 | 0.98 |
| 48.89 | 0.99 | 0.83 | 0.91 | 0.97 |
| 43.69 | 0.97 | 0.88 | 0.94 | 0.94 |
| 38.69 | 0.96 | 0.91 | 0.95 | 0.93 |
| 33.84 | 0.95 | 0.94 | 0.96 | 0.91 |
| 30.63 | 0.94 | 0.96 | 0.98 | 0.90 |
| 28.42 | 0.88 | 0.97 | 0.98 | 0.82 |
| 15.04 | 0.50 | 0.99 | 0.99 | 0.52 |
| 12.12 | 0.42 | 1.00 | 1.00 | 0.49 |

(C)




(D)

| Cut Off | Sensitivity | Specificity | PPV | NPV |
|---|---|---|---|---|
| 253.35 | 1 | 0.97 | 0.99 | 1 |
| 179.47 | 0.92 | 1 | 1 | 0.87 |

Urine albumin is an important measurement procedure used for diagnosis, risk classification, and management of CKD. The increasing prevalence of CKD can be controlled only through early diagnosis. Population level mass screening at point of care can be a major driver for this initiative. In the previous publication, Proflo-U® platform has been found suitable for the PoC deployment. This study evaluated the performance of the novel fluorescence-based point-of-care system Proflo-U® with the reference method laboratory based immunoturbidity Beckman Coulter using patient urine sample as test specimen. In the sample size 255, it has been found that the Proflo-U® platform has high correlation with the reference method and diagnostic accuracy. Suggesting, the Proflo-U® platform has the potential for application as the diagnostic method for the urine albumin measurement.

## Conclusion

The evaluation aimed to assess the Proflo-U® platform's performance in urine albumin measurement for CKD diagnosis. Utilizing patient urine samples, the Proflo-U® platform demonstrated comparable accuracy to the reference method, Beckman Coulter System, with high correlation coefficients. Despite initial limitations with synthetic samples, validation using real patient samples confirmed its efficacy in clinical settings. Sensitivity and specificity analyses showed strong performance of the Proflo-U® platform across different

albuminuria categories, aligning well with existing literature. Statistical analyses further supported its reliability, with strong agreement between methods. Receiver Operating Characteristic (ROC) analysis highlighted the Proflo-U® platform's high diagnostic accuracy, indicating its potential for early CKD detection and management, particularly in resource-limited settings. Further validation studies are recommended to ensure widespread adoption of this innovative point-of-care solution.

**Acknowledgement**

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**Contributors**

MHS has conducted the Proflo-U® platform and Beckman Coulter based experiments and collected the data. SM conducted all the Biosystem based experiments and collected the data. DB has designed the study and analyzed the data. MHS, SM and DB has prepared and reviewed the manuscript. DB was responsible for overall supervision of the project and review of the final paper.

**Supplementary Table 1:** Cohort sample n=255 with Patient ID and measured values from Proflo-U® platform (PFL) and Beckman Coulter system (BC).

| S.No. | Patient ID | PFL (in mg/L) | BC (in mg/L) |
|---|---|---|---|
| 1 | 11230570 | 1637.3 | 854.86 |
| 2 | 11230780 | 8.08 | 11.6 |
| 3 | 11230947 | 0 | 4.14 |
| 4 | 11230860 | 0 | 7.87 |
| 5 | 11240718 | 1017.2 | 853.42 |
| 6 | 11240458 | 28.02 | 24.45 |
| 7 | 11240695 | 974.27 | 837.57 |
| 8 | 11240666 | 99.37 | 223.57 |
| 9 | 11240691 | 7.23 | 4.62 |
| 10 | 11250639 | 13.08 | 28.14 |
| 11 | 11250526 | 149.15 | 203.39 |
| 12 | 11250761 | 0 | 7.52 |
| 13 | 11250387 | 15.07 | 51.97 |
| 14 | 11250648 | 0 | 20.18 |
| 15 | 11250342 | 820.8 | 720.74 |
| 16 | 11250535 | 87.39 | 111.55 |
| 17 | 11250414 | 1726.07 | 843 |
| 18 | 11250653 | 80.26 | 62.86 |
| 19 | 11250477 | 0 | 3.72 |
| 20 | 11250840 | 301.33 | 327.63 |
| 21 | 11250834 | 98.23 | 99.6 |
| 22 | 11260277 | 56.44 | 51.02 |
| 23 | 11260339 | 16.64 | 23.35 |
| 24 | 11260168 | 34.63 | 37.66 |
| 25 | 11260331 | 206.63 | 286.68 |
| 26 | 11260801 | 71.98 | 281.68 |
| 27 | 11260584 | 12.51 | 59.85 |
| 28 | 11260456 | 10.51 | 21.64 |
| 29 | 11260547 | 0 | 23.35 |
| 30 | 11260560 | 0 | 6.45 |
| 31 | 11300180 | 27.14 | 4.2 |

| | | | |
|---|---|---|---|
| 32 | 11300318 | 27.71 | 5.67 |
| 33 | 11300196 | 378.87 | 385.069 |
| 34 | 11300205 | 433.64 | 489.53 |
| 35 | 11300188 | 93.18 | 36.24 |
| 36 | 11300612 | 157.93 | 150.38 |
| 37 | 11300585 | 184.89 | 167.82 |
| 38 | 11300478 | 1483.83 | 876.22 |
| 39 | 11300431 | 195.73 | 133.21 |
| 40 | 11300420 | 78.06 | 38.58 |
| 41 | 11300435 | 40.98 | 44.75 |
| 42 | 11300462 | 309.69 | 356.51 |
| 43 | 11300580 | 28.89 | 15.76 |
| 44 | 11300532 | 9.6 | 10.75 |
| 45 | 11300537 | 47.54 | 42.91 |
| 46 | 12010251 | 26.57 | 26.05 |
| 47 | 12010240 | 3.72 | 8.82 |
| 48 | 12010193 | 338.08 | 313.13 |
| 49 | 12010296 | 25.43 | 4.44 |
| 50 | 12010196 | 89.18 | 87.94 |
| 51 | 12010244 | 26.43 | 20.38 |
| 52 | 12010140 | 82.62 | 87.47 |
| 53 | 12010159 | 48.96 | 58.73 |
| 54 | 12010173 | 16.44 | 15.1 |
| 55 | 12010606 | 1111.85 | 874.4 |
| 56 | 12010237 | 595.52 | 541.19 |
| 57 | 12010422 | 14.59 | 6.9 |
| 58 | 12010574 | 29.28 | 24.33 |
| 59 | 12010513 | 21.72 | 7.63 |
| 60 | 12010569 | 60.09 | 48 |
| 61 | 12010681 | 29.8 | 22.58 |
| 62 | 12010568 | 87.76 | 108.14 |
| 63 | 12020381 | 1.18 | 4.2 |
| 64 | 12020642 | 139.39 | 186.93 |
| 65 | 12020344 | 5.75 | 7.55 |
| 66 | 12020463 | 19.2 | 16.8 |
| 67 | 12020353 | 26.43 | 8 |
| 68 | 12020555 | 91.89 | 33.82 |
| 69 | 12020417 | 3.07 | 4.6 |
| 70 | 12020338 | 1761.82 | 860.03 |
| 71 | 12020368 | 54.81 | 42.48 |
| 72 | 12020232 | 0.75 | 3.51 |
| 73 | 12020636 | 134.68 | 154.05 |
| 74 | 12020484 | 45.4 | 42.34 |
| 75 | 12020145 | 549.31 | 438.54 |
| 76 | 12020138 | 12.73 | 8.38 |
| 77 | 12030254 | 787.36 | 842.59 |

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|---|---|---|---|
| 78 | 12030261 | 17.25 | 12.79 |
| 79 | 12030214 | 50.39 | 54.53 |
| 80 | 12030478 | 0 | 4.54 |
| 81 | 12030444 | 250.79 | 214.3 |
| 82 | 12030288 | 9.03 | 5.29 |
| 83 | 12030612 | 1.61 | 5.83 |
| 84 | 12030364 | 13.73 | 6.15 |
| 85 | 12030248 | 7.88 | 8.06 |
| 86 | 12030270 | 436.78 | 623.05 |
| 87 | 12030295 | 1135.53 | 831.37 |
| 88 | 12030288 | 1685.08 | 845.14 |
| 89 | 12030456 | 141.96 | 119.94 |
| 90 | 12030299 | 25.71 | 21.49 |
| 91 | 12030630 | 61.94 | 67.73 |
| 92 | 12030240 | 7.79 | 12 |
| 93 | 12030284 | 458.74 | 402.08 |
| 94 | 12080122 | 721.18 | 704.55 |
| 95 | 12080235 | 10.45 | 6.01 |
| 96 | 12080185 | 0 | 7.1 |
| 97 | 12080192 | 168.34 | 85.58 |
| 98 | 12080210 | 3.58 | 4.83 |
| 99 | 12080401 | 15.87 | 4.61 |
| 100 | 12080199 | 28.71 | 6 |
| 101 | 12080358 | 1768.95 | 846.11 |
| 102 | 12100550 | 7.88 | 13.64 |
| 103 | 12100548 | 10.02 | 6.68 |
| 104 | 12100429 | 27.57 | 9.41 |
| 105 | 12100559 | 89.04 | 64.71 |
| 106 | 12100445 | 479.99 | 346.42 |
| 107 | 12100493 | 44.68 | 37.37 |
| 108 | 12100619 | 158.93 | 143.07 |
| 109 | 12100568 | 5.57 | 7.24 |
| 110 | 12100593 | 26.57 | 10.56 |
| 111 | 12100526 | 758.98 | 894.36 |
| 112 | 12100636 | 1277.73 | 926.69 |
| 113 | 12100405 | 15.02 | 20.07 |
| 114 | 12140249 | 170.34 | 267.47 |
| 115 | 12140169 | 33.13 | 66.01 |
| 116 | 12140315 | 15.27 | 19.56 |
| 117 | 12140299 | 21.58 | 0.99 |
| 118 | 12140275 | 46.11 | 64.38 |
| 119 | 12140232 | 0 | 1.94 |
| 120 | 12140173 | 606.22 | 664.62 |
| 121 | 12140290 | 87.47 | 139.76 |
| 122 | 12140128 | 4.32 | 2.67 |
| 123 | 12140317 | 47.96 | 10.34 |

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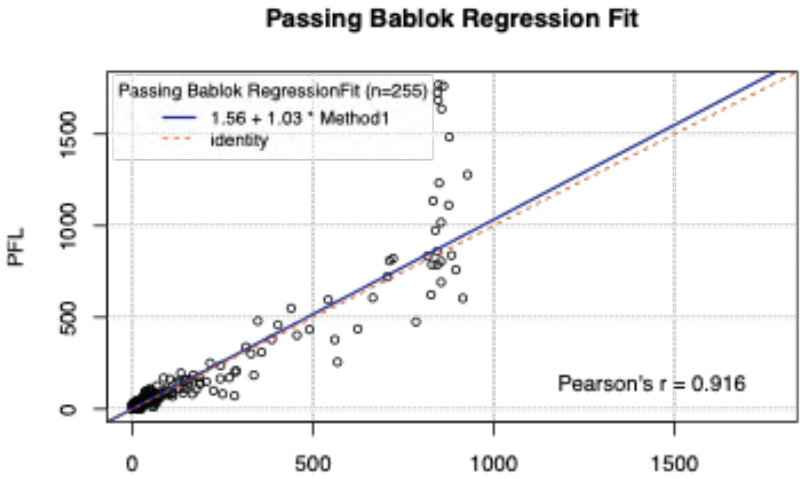
|---|---|---|---|
| 124 | 12140490 | 165.49 | 242.19 |
| 125 | 12140373 | 42.69 | 11.38 |
| 126 | 12140238 | 163.21 | 103.32 |
| 127 | 12140336 | 15.73 | 7.54 |
| 128 | 12140516 | 18.44 | 4.84 |
| 129 | 12140408 | 29.14 | 1.55 |
| 130 | 12140433 | 622.91 | 825.79 |
| 131 | 12140334 | 25.43 | 23.56 |
| 132 | 12140428 | 78.2 | 99.72 |
| 133 | 12140441 | 0 | 2.85 |
| 134 | 12150324 | 140.82 | 185.96 |
| 135 | 12150421 | 111.58 | 48.4 |
| 136 | 12150451 | 0 | 4.42 |
| 137 | 12150519 | 0 | 3.93 |
| 138 | 12150570 | 0 | 1.15 |
| 139 | 12150347 | 28.57 | 2.1 |
| 140 | 12150302 | 113.29 | 129.81 |
| 141 | 12150192 | 174.76 | 183.1 |
| 142 | 12150186 | 204.29 | 283.16 |
| 143 | 12150164 | 0 | 0.72 |
| 144 | 12150168 | 16.01 | 19.91 |
| 145 | 12150216 | 27.43 | 10.14 |
| 146 | 12150479 | 19.87 | 0.68 |
| 147 | 12150238 | 26.14 | 2.47 |
| 148 | 12150216 | 24.14 | 10.14 |
| 149 | 12150633 | 25.86 | 1.07 |
| 150 | 12150219 | 20.29 | 1.27 |
| 151 | 12160439 | 4.6 | 0.01 |
| 152 | 12160437 | 839 | 882.43 |
| 153 | 12160411 | 0.61 | 0.54 |
| 154 | 12160455 | 1232.66 | 848.04 |
| 155 | 12160521 | 25.29 | 12.81 |
| 156 | 12160388 | 13.73 | 3.05 |
| 157 | 12160544 | 93.04 | 89.43 |
| 158 | 12160480 | 0 | 1.71 |
| 159 | 12160362 | 20.29 | 12.5 |
| 160 | 12160212 | 23.72 | 16.27 |
| 161 | 12160298 | 236.81 | 243.69 |
| 162 | 12160366 | 27.71 | 7.04 |
| 163 | 12160346 | 378.01 | 559.48 |
| 164 | 12160161 | 69.5 | 110.7 |
| 165 | 12160261 | 804.91 | 853.61 |
| 166 | 12160181 | 50.53 | 31.6 |
| 167 | 12160216 | 14.87 | 3.28 |
| 168 | 12170192 | 59.09 | 75.96 |
| 169 | 12170108 | 0 | 2.24 |

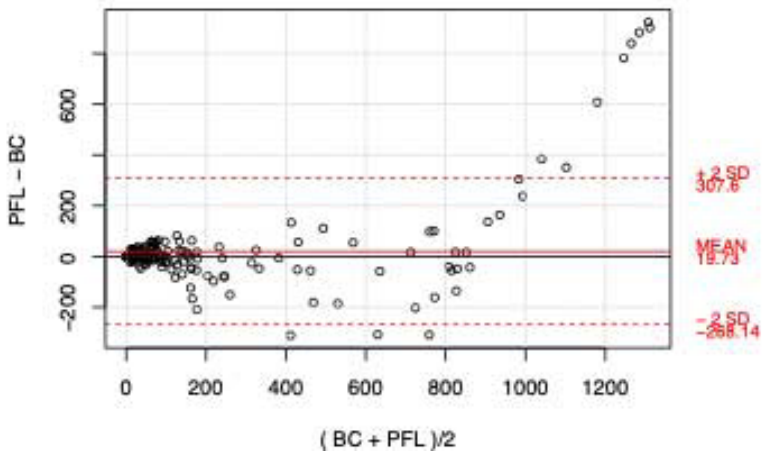
| | | | |
|---|---|---|---|
| 170 | 12170154 | 0 | 3.83 |
| 171 | 12170238 | 0.9 | 0.91 |
| 172 | 12170220 | 16.44 | 9.23 |
| 173 | 12170333 | 51.24 | 43.18 |
| 174 | 12170183 | 13.59 | 2.49 |
| 175 | 12170322 | 4 | 6.64 |
| 176 | 12170236 | 155.65 | 157.73 |
| 177 | 12170506 | 30.04 | 28.43 |
| 178 | 12170456 | 22.86 | 7.46 |
| 179 | 12170556 | 17.87 | 3.17 |
| 180 | 12170492 | 85.05 | 49.39 |
| 181 | 12170475 | 691.66 | 853.59 |
| 182 | 12170571 | 28.41 | 21.96 |
| 183 | 12170542 | 475.86 | 784.08 |
| 184 | 12170382 | 26.43 | 15.42 |
| 185 | 3030584 | 114.86 | 93.61 |
| 186 | 3030316 | 7.03 | 5.71 |
| 187 | 3030378 | 10.02 | 5.22 |
| 188 | 3030457 | 37.98 | 21.5 |
| 189 | 3030304 | 17.58 | 23.07 |
| 190 | 3030588 | 10.02 | 10.3 |
| 191 | 3040424 | 11.74 | 8.18 |
| 192 | 3040275 | 95.03 | 50.6 |
| 193 | 3040179 | 7.8 | 16.23 |
| 194 | 3040689 | 604.37 | 913.78 |
| 195 | 3040137 | 0 | 2.05 |
| 196 | 3040523 | 4.18 | 3.38 |
| 197 | 3040662 | 14.87 | 12.24 |
| 198 | 3040187 | 59.37 | 28.29 |
| 199 | 3040296 | 126.7 | 67.79 |
| 200 | 3040405 | 20.01 | 2.57 |
| 201 | 3040453 | 24.57 | 0.52 |
| 202 | 3050287 | 402.69 | 454.77 |
| 203 | 3050302 | 0 | 0.79 |
| 204 | 3050401 | 18.15 | 6.33 |
| 205 | 3050177 | 30.42 | 1.41 |
| 206 | 3050146 | 26.43 | 0.13 |
| 207 | 3050281 | 255.92 | 567.08 |
| 208 | 3050153 | 24.14 | 0.03 |
| 209 | 3050238 | 82.2 | 249.05 |
| 210 | 3050200 | 17.32 | 19.37 |
| 211 | 3050165 | 15.41 | 11.83 |
| 212 | 3070650 | 9.17 | 10.15 |
| 213 | 3070567 | 13.16 | 6.44 |
| 214 | 3070278 | 39.41 | 53.53 |
| 215 | 3070438 | 68.65 | 69.69 |

| | | | |
|---|---|---|---|
| 216 | 3070566 | 0 | 4.31 |
| 217 | 3070573 | 0 | 4.74 |
| 218 | 3070655 | 102.73 | 45.23 |
| 219 | 3070824 | 81.91 | 165.06 |
| 220 | 3070455 | 65.22 | 72.49 |
| 221 | 3070736 | 30.85 | 58.93 |
| 222 | 3070333 | 184.18 | 335.44 |
| 223 | 3070257 | 23 | 7.08 |
| 224 | 3070494 | 28.42 | 30.34 |
| 225 | 3070571 | 808.61 | 710.95 |
| 226 | 3070352 | 48.82 | 23.67 |
| 227 | 3070392 | 6.03 | 8.92 |
| 228 | 3070217 | 66.79 | 27.48 |
| 229 | 3070286 | 23 | 3.11 |
| 230 | 3070532 | 29.85 | 3.48 |
| 231 | 3080698 | 833.43 | 816.37 |
| 232 | 3080738 | 110.86 | 144.5 |
| 233 | 3080589 | 24.57 | 3.54 |
| 234 | 3080390 | 73.78 | 62.03 |
| 235 | 3080284 | 97.88 | 42.55 |
| 236 | 3080533 | 35.27 | 13.2 |
| 237 | 3080443 | 23.15 | 3.9 |
| 238 | 3080578 | 93.46 | 34.38 |
| 239 | 3080722 | 20.58 | 20.01 |
| 240 | 3080509 | 86.05 | 63.28 |
| 241 | 3080669 | 34.27 | 31.46 |
| 242 | 3080665 | 105.44 | 175.89 |
| 243 | 3080700 | 0 | 4.19 |
| 244 | 3080537 | 2.18 | 8.93 |
| 245 | 3080056 | 8.3 | 19.9 |
| 246 | 3080552 | 787.79 | 828.26 |
| 247 | 3080610 | 151.8 | 129.97 |
| 248 | 3080558 | 59.52 | 67.36 |
| 249 | 3080529 | 14.3 | 3.37 |
| 250 | 3080559 | 859.68 | 843.1 |
| 251 | 3080177 | 0 | 2.69 |
| 252 | 3080426 | 24.57 | 9.18 |
| 253 | 3080404 | 59.8 | 60.44 |
| 254 | 3080408 | 17.58 | 11.62 |
| 255 | 3080278 | 33.42 | 23.46 |

**Supplementary Figure 1:** The Passing Bablok Regression fit has been presented for the all the 255 samples for Proflo-U® and Beckman Coulter.




**Supplementary Figure 2:** The Bland-Altaman plot has been presented for the all the 255 samples for Proflo-U® and Beckman Coulter.



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Research Article

# Impact of centrifugation time reduction in GLP systems

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**Article Info**

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**Keywords**

centrifugation time, fully automated, GLP systems, turnaround time

**Abstract**

**Background**: Centrifugation of specimens is an important pre-analytical process that significantly impacts turnaround time, enabling clinicians to diagnose, treat, and monitor patients more effectively. In this study, we verified different combinations of tubes and identified optimal centrifugation settings for chemical and immunological assays.

**Methods**: We evaluated 40 leftover blood samples and collected them in 4-mL vacutainer and 2-mL vacuette lithium heparin tubes. All the tubes were centrifuged using an Abbott Automation GLP system. The first set of samples was centrifuged according to the manufacturer's guidelines. The second and third sets of samples were centrifuged at 2700×g for 7 and 5 min, respectively. All samples were analyzed through 30 chemical and 9 immunological assays on Alinity ci analyzers. The allowable total error, paired t-test, slope, intercept, and correlation coefficient (R) were used to determine the significance of differences in the first set of samples.

**Results**: Centrifugations for 7 and 5 min at 2700×g were within the acceptable range from the manufacturer's protocol after testing 39 assays in both vacutainer and vacuette lithium heparin tubes, except for LDH that was centrifuged for 5 min at 2700×g.

**Conclusions**: Shorter centrifugation times (7 min at 2700×g) can be used for different tube combinations. Centrifugation for 5 min at 2700×g affected LDH assays in vacutainer and vacuette lithium heparin tubes.

## Introduction

Clinical chemistry laboratories use fully automated GLP systems to reduce human errors and to ensure that the pre-analysis, analysis, and post-analysis processes are accurate, precise, fast, and safe. GLP systems can manage samples more efficiently by monitoring various processes, including centrifugation, decappers, aliquoters, and automatic sample transport to the analyzer via a tracking system. During post-analysis, recappers and refrigerators are used for sample management and storage.

A turnaround time (TAT) is generally employed to assess the efficiency of laboratory management, from sample receipt to reporting the results to the service recipient. Heparinized plasma is used instead of serum for performing clinical, chemical, and immunological tests to reduce the waiting time for analysis results and improve customer satisfaction. This is because lithium heparin tubes prevent blood clotting and can be immediately centrifuged. As lithium heparin inhibits the activity of thrombin III or anti-thrombin III, it helps prevent blood clotting by reducing the breakdown of red blood cells (RBCs) outside the body [1-3]. Centrifugation is an important pre-analytical process that affects the TAT and quality of assays by separating the serum and plasma from RBCs and other components, such as platelets and fibrin.

Previous studies on the effect of centrifugation on chemical test results demonstrated that the test results are not affected by a reduction in the centrifugation time [4-6]. Most of these studies have been performed using tubes from the same manufacturer but with different types of samples, such as serum or plasma. The present study aims to evaluate the effective g-force and the optimal time required for processing two types of test tubes that will be implemented in routine: 4-mL plastic vacutainer lithium heparin tubes from Becton Dickinson and 2-mL plastic vacuette lithium heparin tubes from Greiner Bio-One. The goal is to identify the minimum blood volume necessary for accurate and reliable results, enabling the method's application in patients with limited options for testing and in whom it is difficult to collect blood. The vacutainer tubes should be centrifuged for 10 min at 1000–1300×g in a swinging bucket centrifuge and for 15 min in a fixed-angle centrifuge [7], while the vacuette tubes should be centrifuged for 10–15 min at 1800–2200×g [8]. Notably, the World Health Organization (WHO) also recommends centrifuging plasma for 15 min at 2000–3000×g [9].

## Materials and Methods

### Sample collection and processing

This study was conducted at the Clinical Chemistry Laboratory of Songklanagarind Hospital, Thailand. The protocol was approved by the Ethics Committee of the Faculty of Medicine, Prince of Songkla University, Thailand (REC. 65-511-5-8).

For this study, we employed 40 leftover lithium heparin blood samples from the analysis conducted at our Clinical Chemistry Laboratory. To reduce the biological variability, we prepared the lithium heparin blood samples for blood group examination by separating them into A, B, AB, and O blood groups. Plasma having hemolysis and lipemia were excluded. We selected a set of normal and abnormal values for the test to provide a distribution of data. Approximately 20 mL of the pooled lithium heparin blood with matching blood groups were collected and mixed to obtain one sample. This sample was then divided into three 4-mL plastic vacutainer lithium heparin tubes (REF 368496) and three 2-mL plastic vacuette lithium heparin tubes (REF 454237). All the tubes were centrifuged using an Abbott Automated GLP System (Hamburg, Germany). The first set of samples (in both vacutainer and vacuette tubes) was centrifuged according to the manufacturer's guidelines (10 min at 1300×g for vacutainer tubes and 10 min at 2200×g for vacuette tubes). The second set was centrifuged for 7 min at 2700×g, and the third set was centrifuged for 5 min at 2700×g at room temperature. Subsequently, all the samples were analyzed based on 30 chemical and 9 immunological parameters, including hemolysis (HEM), icterus (ICT), and lipemia (LIP), on Alinity ci analyzers (Abbott). All analytical procedures were tested under the same conditions and completed in a time duration of 4 h.

Precision study

The total precision of all assays from internal quality control was evaluated using two levels of commercial control materials. Multichem S plus levels 1 and 3 (lot 13111220) and Multichem IA plus levels 1 and 3 (lot 37209220) were used for the immunology assays (TECHNOPATH Ballina, Co., Tipperary, Ireland). The coefficients of variation (CV) of all assays were within the acceptable criterion (<1/3rd of the total allowable error (TEa) limit), as listed in Table 1.

**Table 1:** Coefficients of variations (CV) of all assays according to internal quality measurement.

| Analyte | Abbreviation | %CV | | TEa | Resource |
|---|---|---|---|---|---|
| | | Level 1 | Level 2 | +/- | |
| Alpha-Fetoprotien, ng/mL | AFP | 3.2 | 2.71 | 20% | CLIA |
| Albumin, g/dL | ALB | 0.7 | 0.57 | 8% | CLIA |
| Alkaline phosphatase, U/L | ALP | 3.31 | 2.07 | 20% | CLIA |
| Alanine aminotransferase, U/L | ALT | 2.83 | 1.73 | 6 U/L or 15% | CLIA |
| Amylase, U/L | AMY | 2.37 | 1.65 | 20% | CLIA |
| Aspartate aminotransferase, U/L | AST | 1.35 | 0.8 | 6 U/L or 15% | CLIA |
| Direct bilirubin, mg/dL | DBIL | 5.06 | 5.29 | 20% | WLSH |
| Total bilirubin, mg/dL | TBIL | 4.82 | 5.2 | 20% | CLIA |
| Cancer antigen 125, ng/mL | CA 125 | 3.37 | 3.24 | 20% | CLIA |
| Cancer antigen 19-9, U/mL | CA 19-9 | 5.7 | 5.28 | 30% | BV |
| Calcium, mg/dL | CA | 1.29 | 1.05 | 1 mg/dL | CLIA |
| Carcinoembryonic antigen, ng/mL | CEA | 5.38 | 3.31 | 1 ng/mL or 15% | CLIA |
| Cholesterol, mg/dL | CHOL | 1.1 | 0.72 | 10% | CLIA |
| Creatine kinase, U/L | CK | 1.37 | 1 | 20% | CLIA |
| Chloride, mmol/L | CL | 0.73 | 0.74 | 5% | CLIA |
| Bicarbonate, mmol/L | CO2 | 5.89 | 5.59 | 20% | CLIA |
| Creatinine, mg/dL | CREA | 2.32 | 0.96 | 10% | CLIA |
| High-sensitivity CRP, mg/L | hs-CRP | 1.63 | 1.03 | 30% | CLIA |
| C-reactive protein, mg/L | CRP | 2 | 1.29 | 30% | CLIA |
| Low-density lipoprotein, mg/dL | LDL | 3.37 | 2.44 | 20% | CLIA |
| Ferritin, ng/mL | FER | 4.49 | 4.38 | 20% | CLIA |
| Free prostate specific antigen, ng/mL | FPSA | 2.99 | 4.31 | 30% | BV |
| Gamma-glutamyl transferase, U/L | GGT | 2.98 | 1.65 | 5 U/L or 15% | CLIA |
| Glucose, mg/dL | GLU | 0.82 | 0.75 | 8% | CLIA |
| Troponin I, ng/L | TNI | 7.08 | 4.24 | 0.9 ng/mL or 30% | CLIA |
| Iron, µmol/L | IRON | 2.23 | 1.46 | 15% | CLIA |
| Potassium, mmol/L | K | 0.63 | 0.52 | 0.3 mmol/L | CLIA |
| Lactate dehydrogenase, U/L | LDH | 4.77 | 1.84 | 15% | CLIA |
| Lipase, U/L | LP | 3 | 3.44 | 20% | BV |
| Sodium, mmol/L | NA | 0.58 | 0.68 | 4 mmol/L | CLIA |
| N-terminal pro B-type natriuretic peptide, pg/mL | NT-proBNP | 4.17 | 3.3 | 30% | CLIA |
| Phosphorus, mg/dL | PHOS | 1.87 | 1.34 | 0.3 mg/dL or 10% | CLIA |
| Total prostate specific antigen, ng/mL | TPSA | 4.99 | 4.95 | 0.2 mg/dL or 20% | CLIA |
| Total protein, g/dL | TP | 0.82 | 0.72 | 8% | CLIA |
| Triglyceride, mg/dL | TG | 1.27 | 1.39 | 15% | CLIA |
| Unsaturated iron-binding capacity, µmol/L | UIBC | 5.85 | 3.58 | 20% | AAB |
| High-density lipoprotein, mg/dL | HDL | 1.97 | 1.92 | 6 mg/dL or 20% | CLIA |
| Urea nitrogen, mg/dL | BUN | 3 | 1.69 | 2 mg/dL or 9% | CLIA |
| Uric acid, mg/dL | URIC | 0.95 | 0.79 | 10% | CLIA |

The coefficients of variation (CV) of all assays were under 1/3rd of the TEa limit derived from CLIA (Clinical Laboratory Improvement Amendments), WLSH (Wisconsin State Laboratory of Hygiene), and BV (2004 update of the Spanish Society of Clinical Chemistry and Molecular Pathology (SEQC) table of Desirable Quality Specifications based on Biological Variation).

**Statistical analysis**

Statistical analyses were performed using Excel version 2021, SPSS version 23, and EP Evaluator version 12.3.0.2 (Data Innovations, South Burlington, USA). All results are presented as mean (SD), slope, intercepts, correlation, and paired t-test. The level of statistical significance was set at $P < 0.05$ for the assay with a normal distribution and the Wilcoxon signed-rank test for a non-normal distribution by using the Kolmogorov–Smirnov test to assess the distribution. The results obtained after performing centrifugation for 7 and 5 min at 2700×g using each tube type were compared. The percentage difference (% difference) of the assay and the number of samples were calculated. For the results to be acceptable, the % difference for each test must be > 95 % within the acceptable TEa limit.

**Results**

The chemical and immunological results obtained using each tube type are summarized in Table 2. Results obtained after centrifuging each sample for 7 and 5 min at 2700×g, according to the manufacturer's guidelines, are compared.

**Table 2:** Comparison of chemical and immunological results obtained using different centrifugation settings and tube types.

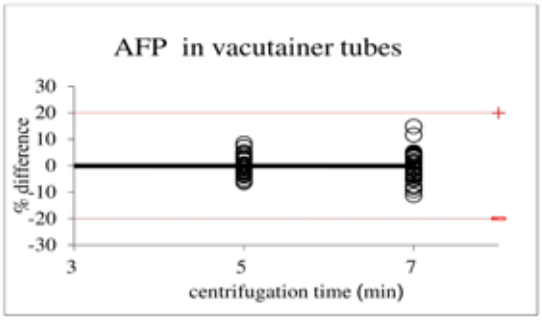
| Analyte | Vacutainer tubes | | | Vacuette tubes | | |
|---|---|---|---|---|---|---|
| | 1300×g/10 min | 2700×g/7 min | 2700×g/5 min | 2200×g/10 min | 2700×g/7 min | 2700×g/5 min |
| AFP | 265.0 (793.12) | 270.0 (830.94) | 274.6 (837.48) | 266.4 (821.86) | 265.3 (807.53) | 268.3 (815.03) |
| ALB | 4.28 (0.37) | 4.29 (0.38)[1] | 4.31 (0.39)[1] | 4.26 (0.37) | 4.30 (0.39)[1] | 4.30 (0.39)[1] |
| ALP | 80.5 (30.39) | 80.9 (30.34) | 80.8 (30.61) | 80.1 (30.90) | 80.1 (30.86) | 82.9 (30.74)[1] |
| ALT | 34.3 (24.32) | 34.1 (24.57) | 34.3 (24.48) | 34.0 (24.28) | 34.1 (24.05) | 34.0 (24.15) |
| AMY | 101.9 (40.53) | 102.0 (40.79) | 101.8 (40.58) | 101.7 (40.27) | 101.7 (40.56) | 101.7 (39.99) |
| AST | 28.6 (18.90) | 28.4 (18.84)[1] | 29.1 (19.0) | 27.8 (18.41) | 28.2 (18.55)[1] | 28.9 (18.77) |
| DBIL | 0.37 (0.41) | 0.36 (0.41)[1] | 0.37 (0.41) | 0.37 (0.41) | 0.36 (0.41) | 0.37 (0.40)[1] |
| TBIL | 0.84 (0.68) | 0.83 (0.68) | 0.85 (0.69)[1] | 0.84 (0.68) | 0.82 (0.67)[1] | 0.82 (0.68)[1] |
| CA 125 | 116.8 (330.82) | 117.9 (339.72) | 118.7 (344.53) | 116.6 (333.15) | 116.8 (329.40) | 119.3 (342.05)[1] |
| CA 19-9 | 68.3 (118.38) | 68.3 (119.03) | 68.9 (121.1) | 68.2 (118.53) | 65.4 (112.86) | 69.2 (123.88) |
| CA | 8.88 (0.45) | 8.87 (0.43) | 8.85 (0.44)[1] | 8.85 (0.45) | 8.86 (0.43) | 8.84 (0.44) |
| CEA | 21.9 (32.48) | 20.9 (31.05) | 21.3 (32.17) | 20.7 (30.39) | 20.5 (30.21) | 20.5 (30.16) |
| CHOL | 194.3 (41.40) | 194.8 (41.57) | 196.4 (42.22)[1] | 193.8 (41.09) | 195.8 (42.79)[1] | 196.3 (42.33)[1] |
| CK | 511.3 (1146.08) | 509.0 (1141.13) | 514.2 (1161.38) | 512.3 (1150.42) | 513.0 (1154.32) | 514.1 (1160.94) |
| CL | 106.0 (2.89) | 105.9 (3.00)[1] | 105.9 (3.01)[1] | 106.0 (3.02) | 106.0 (3.01) | 106.0 (2.99) |
| CO2 | 19.4 (1.76) | 19.4 (1.80) | 19.3 (1.78) | 19.1 (1.70) | 19.0 (1.60) | 19.0 (1.65) |
| CREA | 1.22 (1.14) | 1.23 (1.14)[1] | 1.23 (1.13)[1] | 1.23 (1.14) | 1.23 (1.13) | 1.23 (1.14) |
| hs-CRP | 28.5 (19.09) | 28.4 (19.15) | 28.4 (19.12) | 28.4 (19.04) | 28.4 (19.04) | 28.6 (19.12) |
| CRP | 30.8 (24.11) | 30.8 (24.15) | 30.8 (24.03) | 30.8 (24.18) | 30.7 (24.21) | 31.0 (24.11) |
| LDL | 122.4 (36.76) | 122.5 (36.56) | 123.4 (37.14)[1] | 122.0 (36.52) | 122.7 (37.06)[1] | 123.1 (36.97)[1] |
| FER | 1339.4 (1798.38) | 1342.5 (1807.55) | 1314.7 (1710.85) | 1298.6 (1699.40) | 1321.2 (1731.15) | 1324.7 (1759.94) |
| FPSA | 0.74 (0.85) | 0.75 (0.85) | 0.75 (0.85) | 0.74 (0.84) | 0.74 (0.86) | 0.75 (0.86) |
| GGT | 87.4 (62.51) | 87.0 (62.50) | 86.9 (62.1)[1] | 86.5 (63.0) | 86.4 (62.82) | 87.0 (62.57) |
| GLU | 101.5 (19.96) | 101.1 (19.86)[1] | 101.2 (20.11)[1] | 101.1 (19.96) | 100.8 (20.04) | 100.7 (20.14)[1] |
| TNI | 381.2 (858.64) | 384.7 (871.41) | 381.7 (878.91)[1] | 383.0 (883.31) | 385.4 (889.38) | 377.6 (864.60) |
| IRON | 13.5 (4.08) | 13.5 (4.09) | 13.5 (4.13) | 13.5 (4.09) | 13.5 (4.04) | 13.6 (4.10) |
| K | 4.22 (0.54) | 4.24 (0.54) | 4.22 (0.54) | 4.23 (0.53) | 4.23 (0.53) | 4.23 (0.55) |
| LDH | 427.1 (184.32) | 425.1 (179.87) | 418.4 (199.54)[1,2] | 392.8 (182.0) | 391.5 (182.48) | 417.4 (194.20)[1,2] |
| LP | 54.2 (26.17) | 54.4 (26.13) | 54.3 (26.26) | 54.1 (26.09) | 54.2 (26.22) | 54.2 (26.17) |
| NA | 139.9 (2.28) | 139.8 (2.35) | 139.7 (2.40)[1] | 139.6 (2.39) | 139.7 (2.45) | 139.6 (2.45) |
| NT-proBNP | 4487.9 (5568.05) | 4406.4 (5396.71)[1] | 4389.7 (5398.91)[1] | 4389.5 (5423.77) | 4368.9 (5362.38) | 4397.0 (5532.67) |

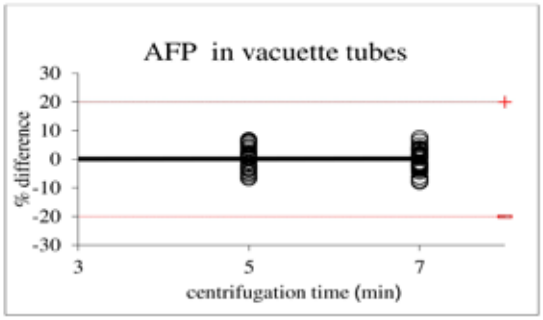
| Analyte | Vacutainer tubes | | | Vacuette tubes | | |
|---|---|---|---|---|---|---|
| | 1300×g/10 min | 2700×g/7 min | 2700×g/5 min | 2200×g/10 min | 2700×g/7 min | 2700×g/5 min |
| PHOS | 3.2 (0.40) | 3.2 (0.40) | 3.2 (0.40) | 3.2 (0.40) | 3.2 (0.40) | 3.1 (0.40)[1] |
| TPSA | 4.0 (7.37) | 4.1 (7.53) | 4.0 (7.24) | 4.0 (7.10) | 4.1 (7.55) | 4.1 (7.58) |
| TP | 7.63 (0.53) | 7.64 (0.54) | 7.70 (0.58)[1] | 7.63 (0.52) | 7.68 (0.57)[1] | 7.70 (0.57)[1] |
| TG | 134.8 (95.36) | 135.3 (97.01) | 133.3 (94.57)[1] | 131.6 (93.63) | 131.9 (94.32) | 130.1 (93.38)[1] |
| UIBC | 38.9 (9.43) | 39.0 (9.42) | 39.3 (9.63)[1] | 38.8 (9.33) | 38.9 (9.45) | 39.4 (9.69)[1] |
| HDL | 53.6 (11.79) | 53.8 (11.81) | 54.6 (12.61)[1] | 53.5 (11.89) | 54.8 (12.90)[1] | 54.8 (12.65)[1] |
| BUN | 16.8 (10.7) | 16.8 (10.78) | 16.9 (10.65)[1] | 16.6 (10.76) | 16.7 (10.65) | 16.7 (10.70)[1] |
| URIC | 5.6 (1.38) | 5.6 (1.38)[1] | 5.6 (1.38) | 5.7 (1.37) | 5.7 (1.38) | 5.6 (1.39)[1] |

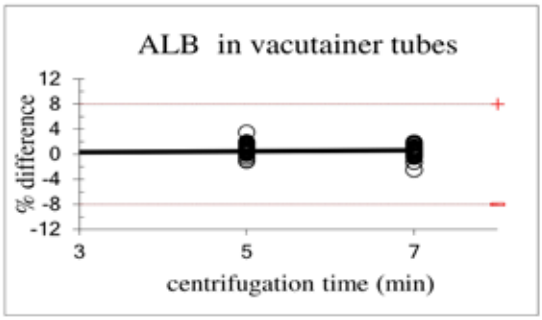
Data are presented as mean, and standard deviation (SD); [1]P<0.05 was considered statistically significant and [2] >5% of individuals exceed the allowable error limit.

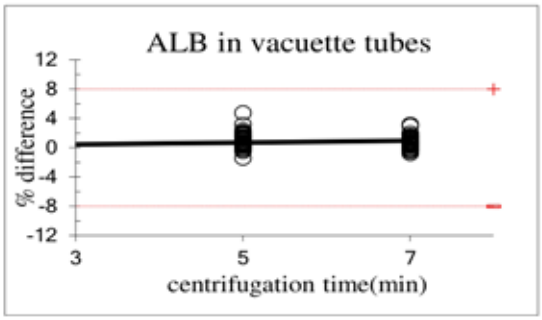
Samples in vacutainer tubes centrifuged for 7 min at 2700×g demonstrated statistical differences in the ALB, AST, DBIL, CL, CREA, GLU, LDH, NT-proBNP and URIC assay results, while samples in vacuette tubes demonstrated statistical differences in the ALB, TBIL, CHOL, LDL, TP, HDL and BUN assay results. Although the test results were different, they were within the acceptable range of TEa, as shown in Figure 1. For samples in vacutainer tubes centrifuged for 5 min at 2700xg, the ALB, TBIL, CA, CHOL, CL, CREA, LDL, GGT, GLU, TNI, NA, NT-proBNP, TP, TG, UIBC, HDL, BUN, and URIC assay results were statistically different, as were the ALB, ALP, AST, DBIL, TBIL, CA, CHOL, CL, CREA, CA125, CHOL, LDL, GGT, GLU, PHOS, TP, TG, UIBC, HDL, BUN and URIC assay results in vacuette tubes. All test results were within the acceptable range of TEa, except for LDH, which demonstrated a percentage difference of <95%, as shown in Figure 1.

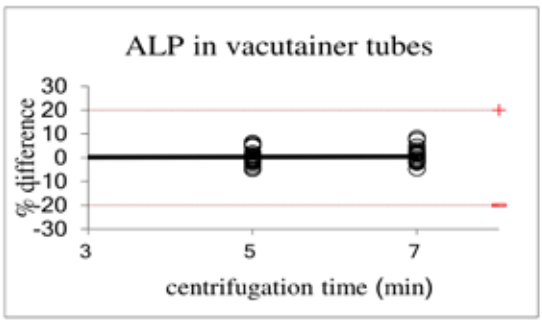
**Figure 1**: Percentage differences of assays (N=40) after centrifuging the samples in each tube for 7 and 5 min at 2700×g, according to the manufacturer's recommendations.

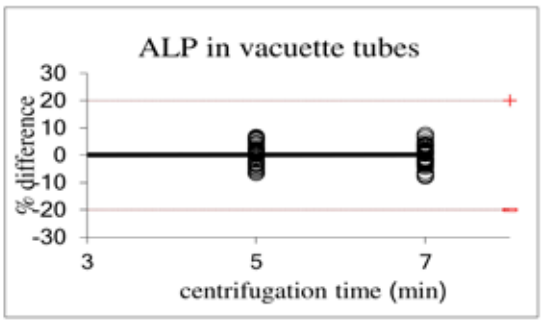


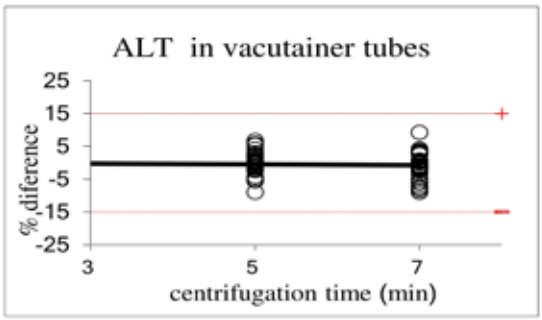



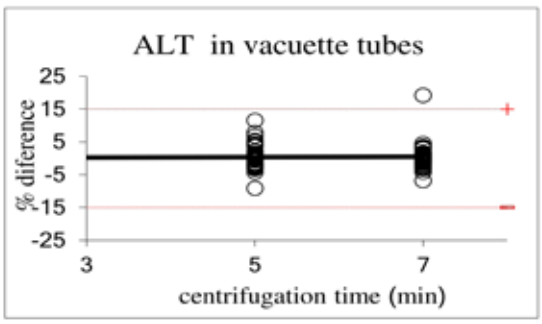



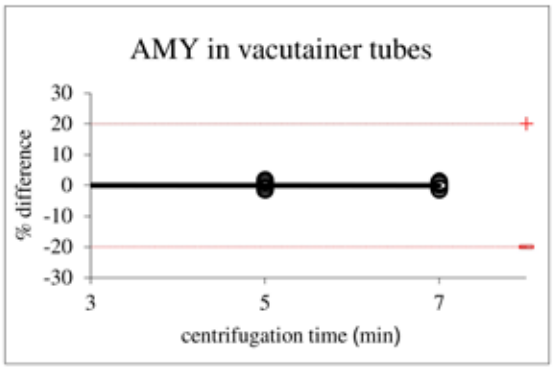



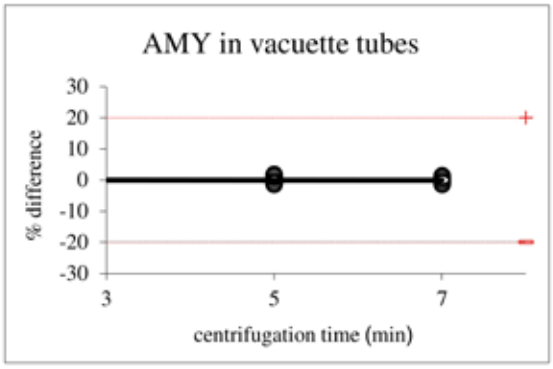



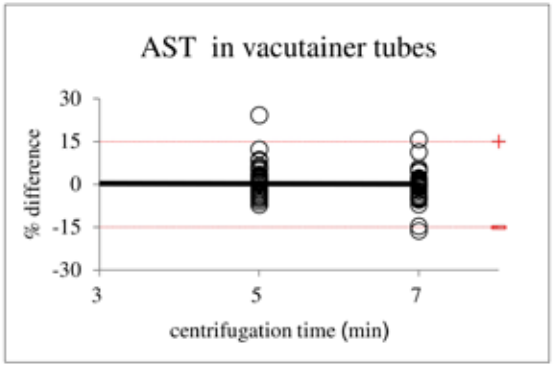



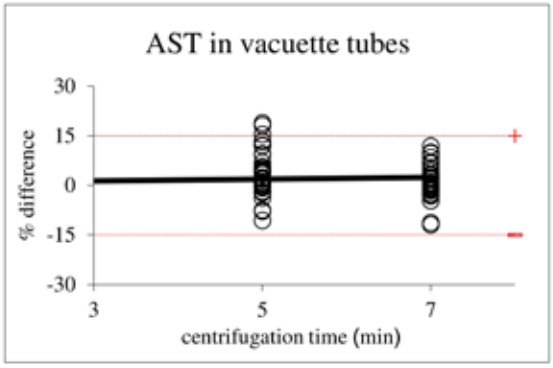



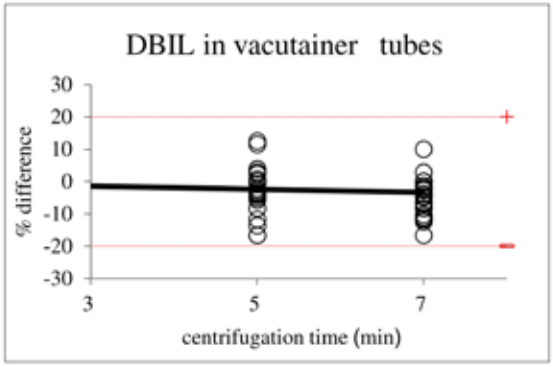



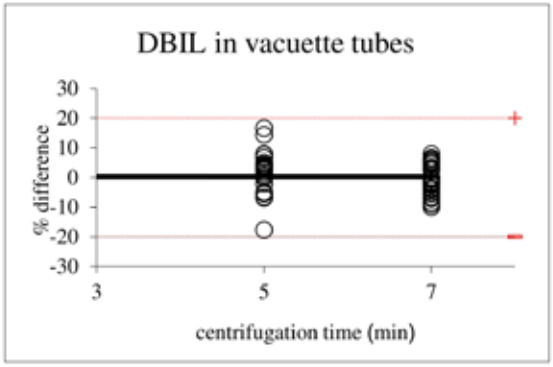

AMY in vacutainer tubes
% difference
30
20
10
0
-10
-20
-30
3
5
7
centrifugation time (min)

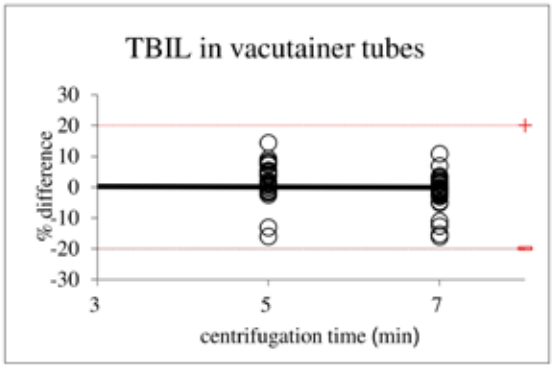

AMY in vacuette tubes
% difference
30
20
10
0
-10
-20
-30
3
5
7
centrifugation time (min)

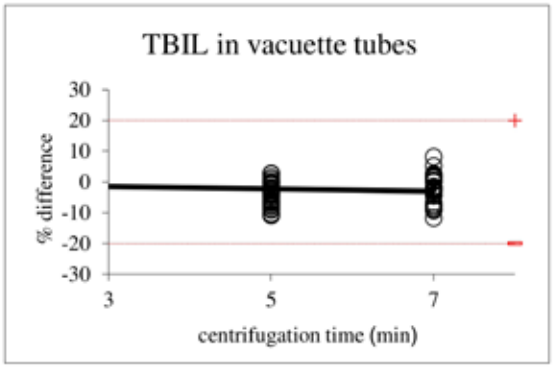

AST in vacutainer tubes
% difference
30
15
0
-15
-30
3
5
7
centrifugation time (min)

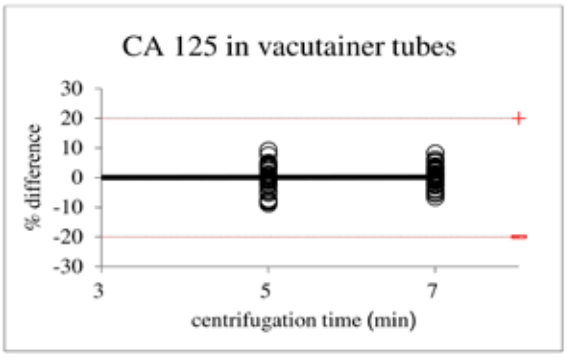

AST in vacuette tubes
% difference
30
15
0
-15
-30
3
5
7
centrifugation time (min)

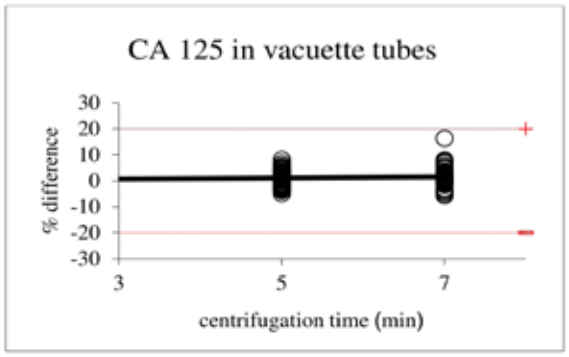

DBIL in vacutainer tubes
% difference
30
20
10
0
-10
-20
-30
3
5
7
centrifugation time (min)

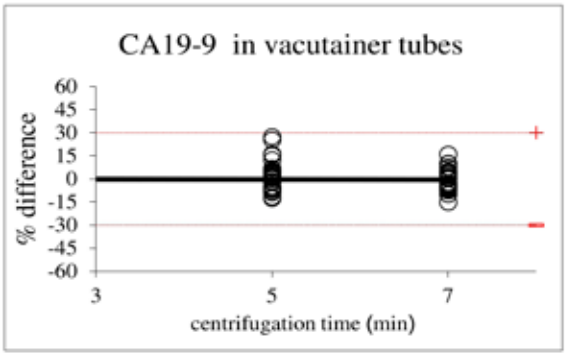

DBIL in vacuette tubes
% difference
30
20
10
0
-10
-20
-30
3
5
7
centrifugation time (min)

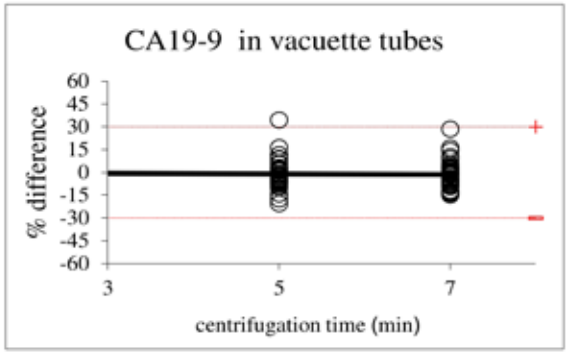

TBIL in vacutainer tubes
% difference
30
20
10
0
-10
-20
-30
3
5
7
centrifugation time (min)

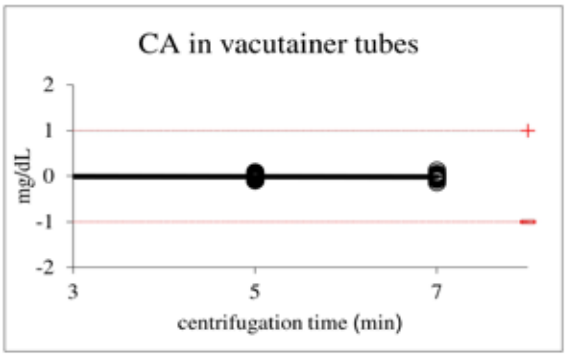

TBIL in vacuette tubes
% difference
30
20
10
0
-10
-20
-30
3
5
7
centrifugation time (min)

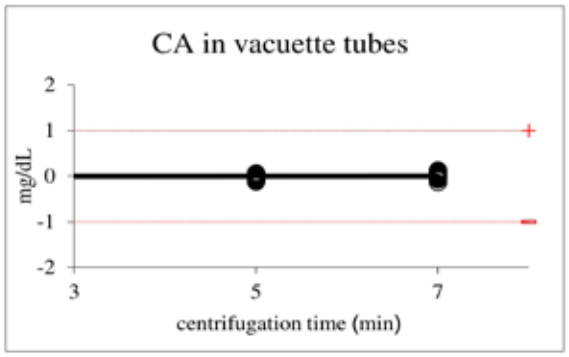

CA 125 in vacutainer tubes
% difference
30
20
10
0
-10
-20
-30
3
5
7
centrifugation time (min)

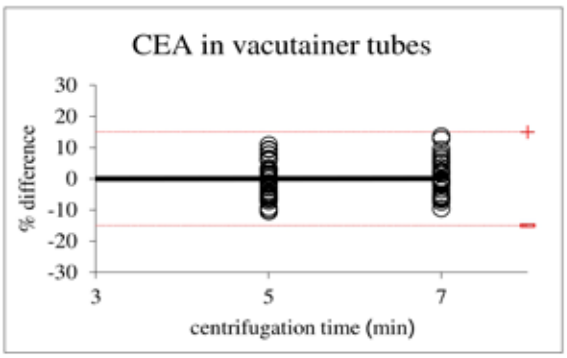

CA 125 in vacuette tubes
% difference
30
20
10
0
-10
-20
-30
3
5
7
centrifugation time (min)

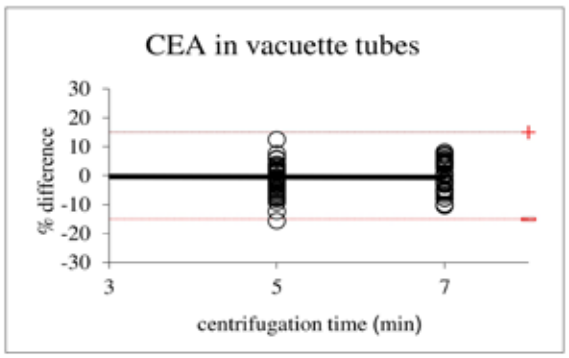

CA19-9 in vacutainer tubes
% difference
60
45
30
15
0
-15
-30
-45
-60
3
5
7
centrifugation time (min)

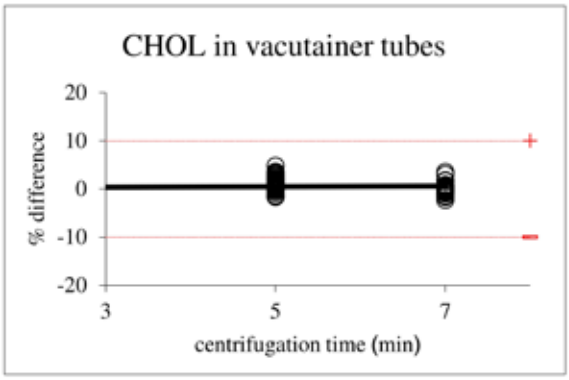

CA19-9 in vacuette tubes
% difference
60
45
30
15
0
-15
-30
-45
-60
3
5
7
centrifugation time (min)

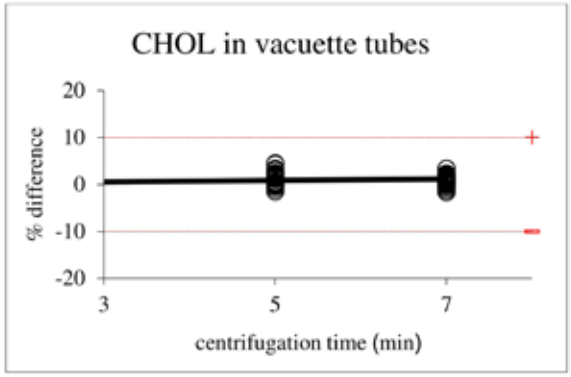

CA in vacutainer tubes
mg/dL
2
1
0
-1
-2
3
5
7
centrifugation time (min)

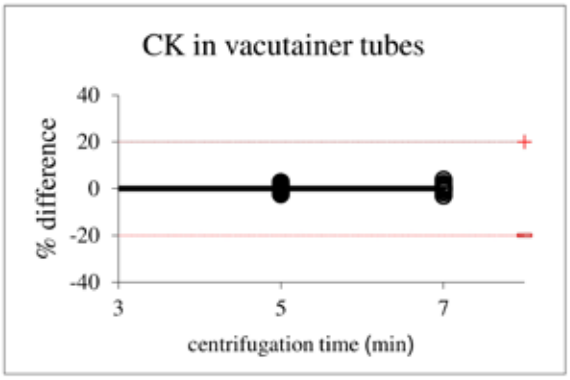

CA in vacuette tubes
mg/dL
2
1
0
-1
-2
3
5
7
centrifugation time (min)

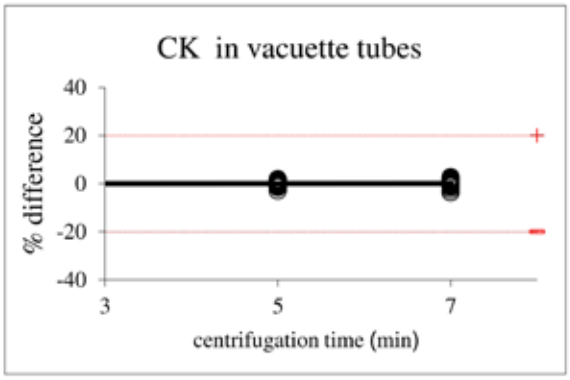

CEA in vacutainer tubes
% difference
30
20
10
0
-10
-20
-30
3
5
7
centrifugation time (min)

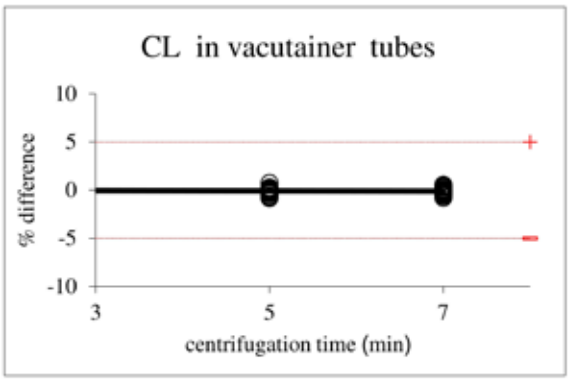

CEA in vacuette tubes
% difference
30
20
10
0
-10
-20
-30
3
5
7
centrifugation time (min)

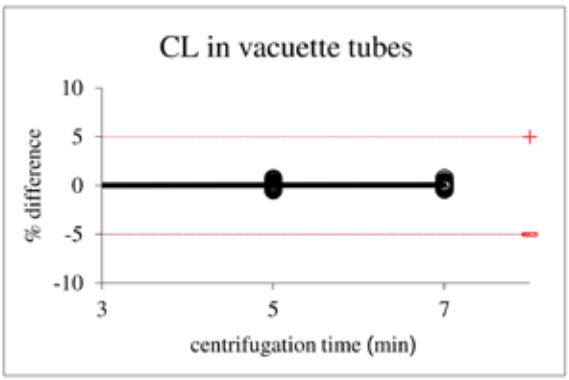

CHOL in vacutainer tubes
% difference
20
10
0
-10
-20
3
5
7
centrifugation time (min)

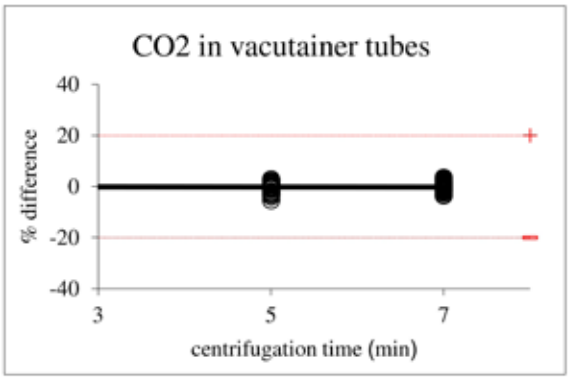

CHOL in vacuette tubes
% difference
20
10
0
-10
-20
3
5
7
centrifugation time (min)

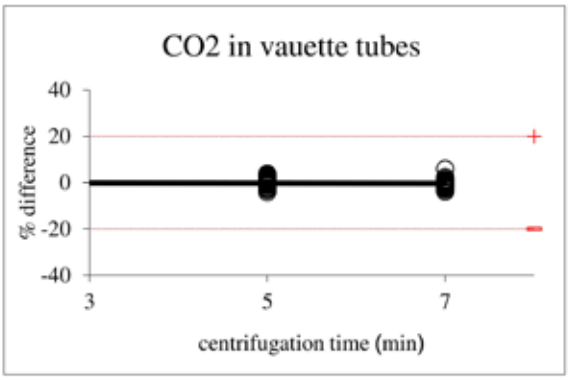

CK in vacutainer tubes
% difference
40
20
0
-20
-40
3
5
7
centrifugation time (min)

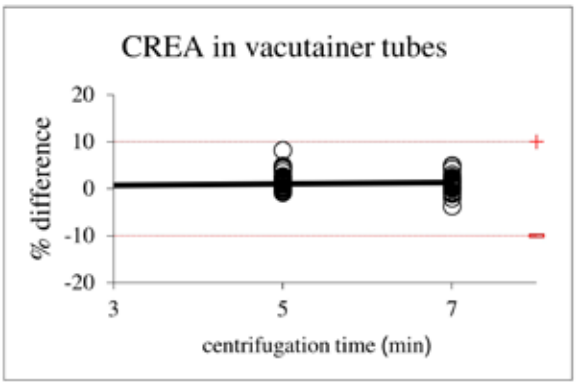

CK in vacuette tubes
% difference
40
20
0
-20
-40
3
5
7
centrifugation time (min)

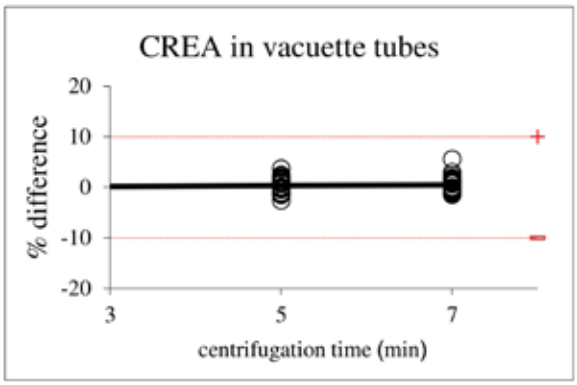

CL in vacutainer tubes
% difference
10
5
0
-5
-10
3
5
7
centrifugation time (min)

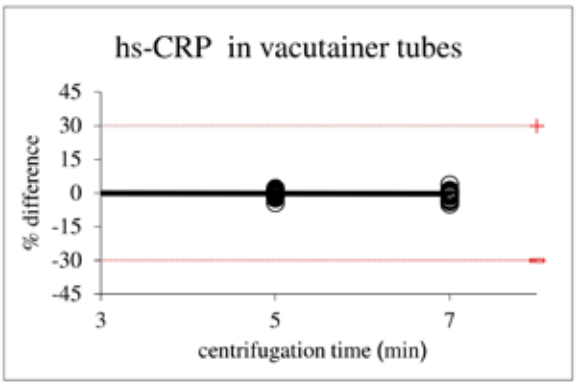

CL in vacuette tubes
% difference
10
5
0
-5
-10
3
5
7
centrifugation time (min)

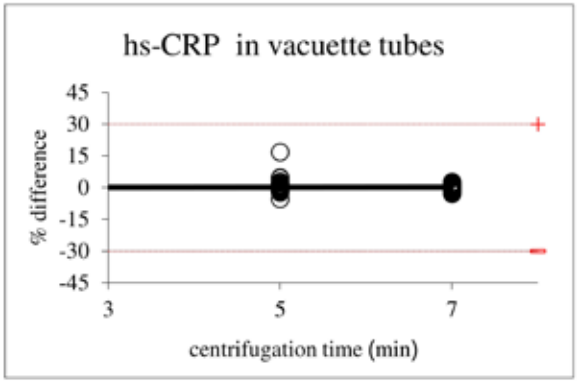

CO2 in vacutainer tubes
% difference
40
20
0
-20
-40
3
5
7
centrifugation time (min)

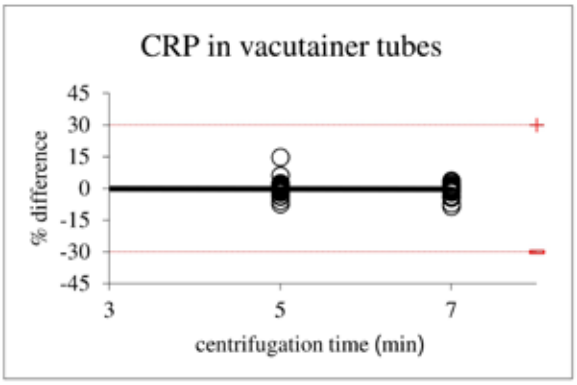

CO2 in vauette tubes
% difference
40
20
0
-20
-40
3
5
7
centrifugation time (min)

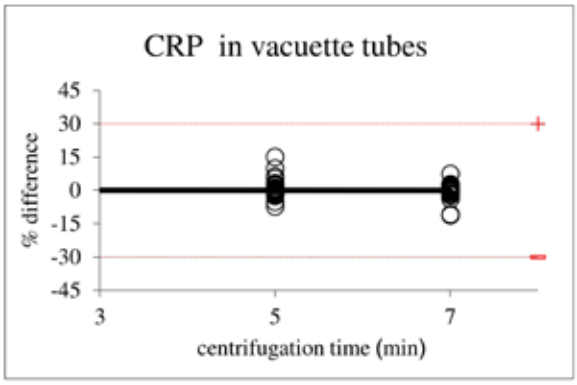

CREA in vacutainer tubes
% difference
20
10
0
-10
-20
3
5
7
centrifugation time (min)

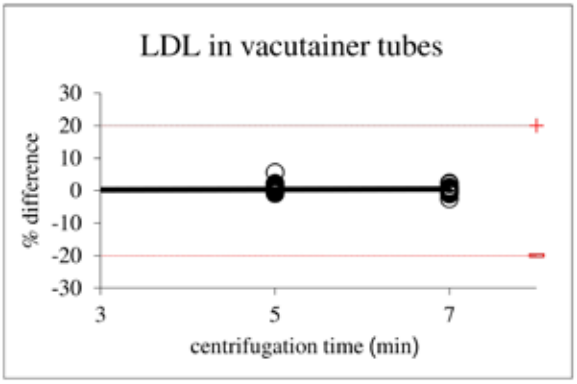

CREA in vacuette tubes
% difference
20
10
0
-10
-20
3
5
7
centrifugation time (min)

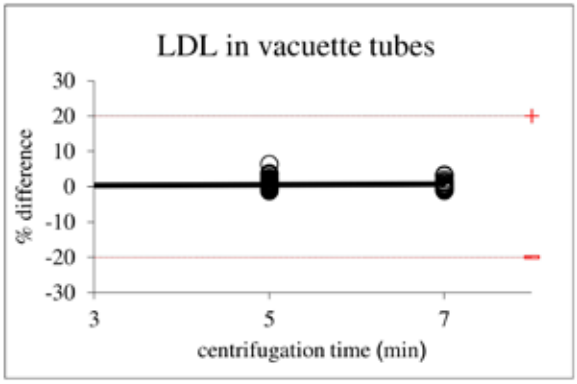

hs-CRP in vacutainer tubes
% difference
45
30
15
0
-15
-30
-45
3
5
7
centrifugation time (min)

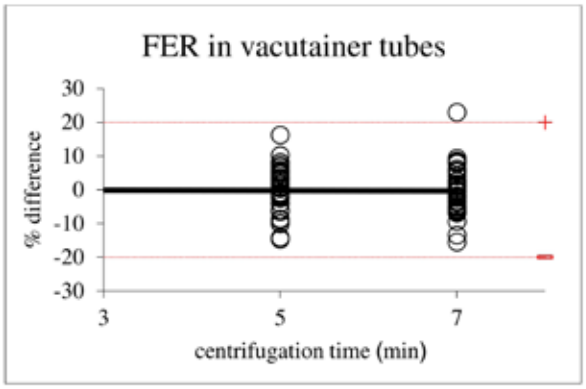

hs-CRP in vacuette tubes
% difference
45
30
15
0
-15
-30
-45
3
5
7
centrifugation time (min)

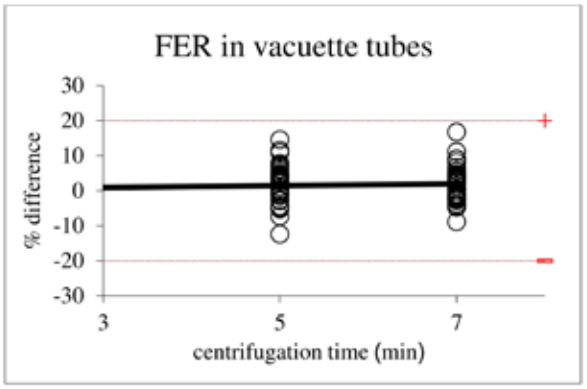

CRP in vacutainer tubes
% difference
45
30
15
0
-15
-30
-45
3
5
7
centrifugation time (min)

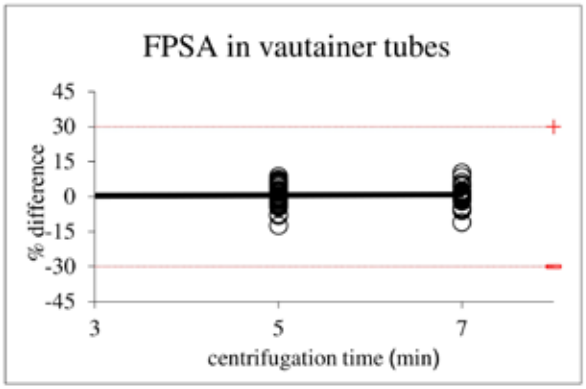

CRP in vacuette tubes
% difference
45
30
15
0
-15
-30
-45
3
5
7
centrifugation time (min)

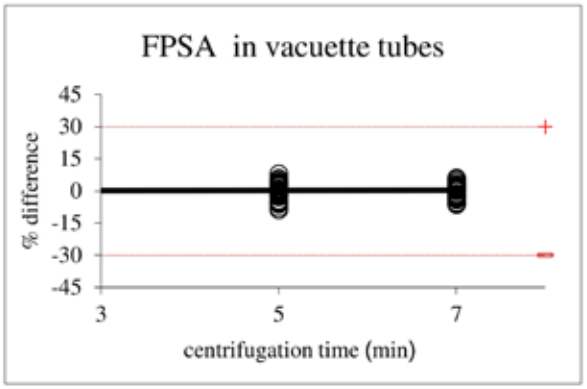

LDL in vacutainer tubes
% difference
30
20
10
0
-10
-20
-30
3
5
7
centrifugation time (min)

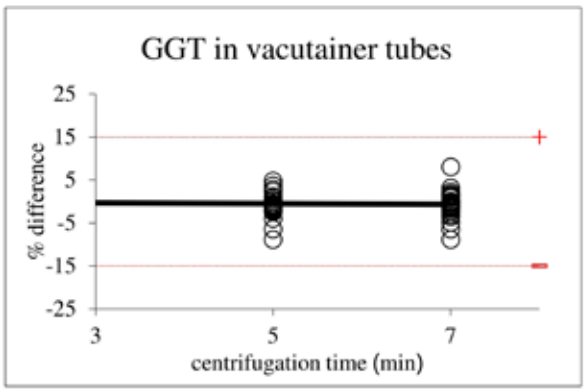

LDL in vacuette tubes
% difference
30
20
10
0
-10
-20
-30
3
5
7
centrifugation time (min)

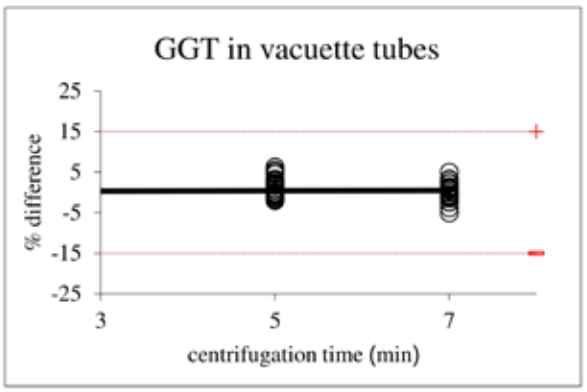

FER in vacutainer tubes
% difference
30
20
10
0
-10
-20
-30
3
5
7
centrifugation time (min)

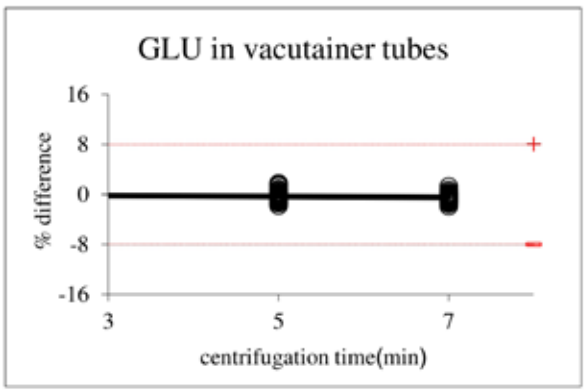

FER in vacuette tubes
% difference
30
20
10
0
-10
-20
-30
3
5
7
centrifugation time (min)

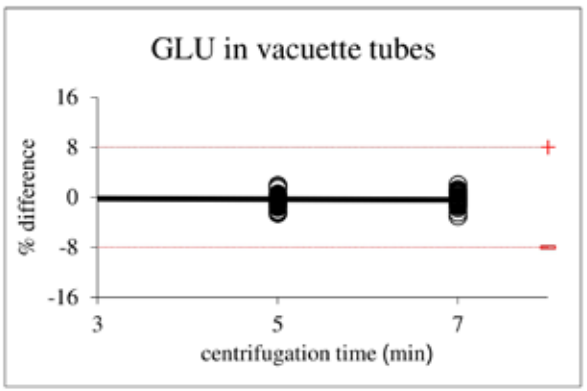

FPSA in vautainer tubes
% difference
45
30
15
0
-15
-30
-45
3
5
7
centrifugation time (min)

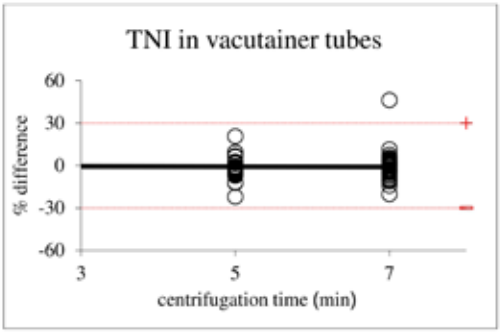

FPSA in vacuette tubes
% difference
45
30
15
0
-15
-30
-45
3
5
7
centrifugation time (min)

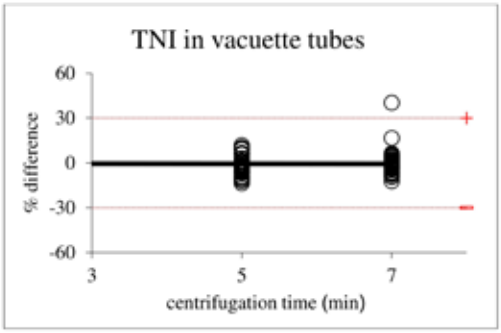

GGT in vacutainer tubes
% difference
25
15
5
-5
-15
-25
3
5
7
centrifugation time (min)

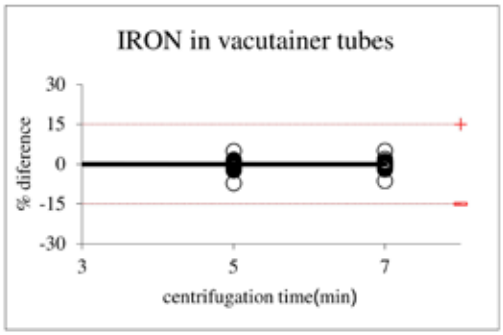

GGT in vacuette tubes
% difference
25
15
5
-5
-15
-25
3
5
7
centrifugation time (min)

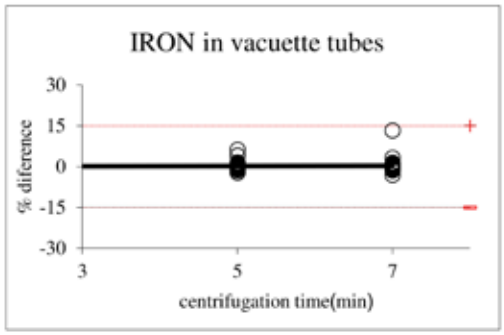

GLU in vacutainer tubes
% difference
16
8
0
-8
-16
3
5
7
centrifugation time(min)

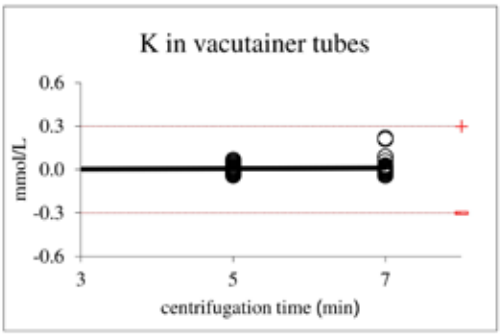

GLU in vacuette tubes
% difference
16
8
0
-8
-16
3
5
7
centrifugation time (min)

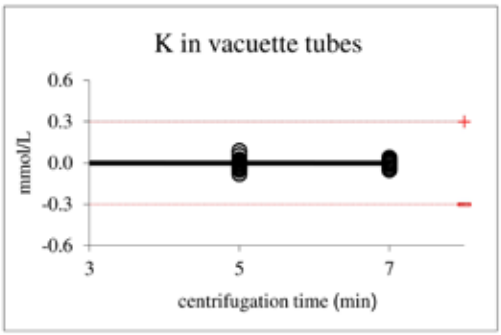

TNI in vacutainer tubes
% difference
60
30
0
-30
-60
3
5
7
centrifugation time (min)

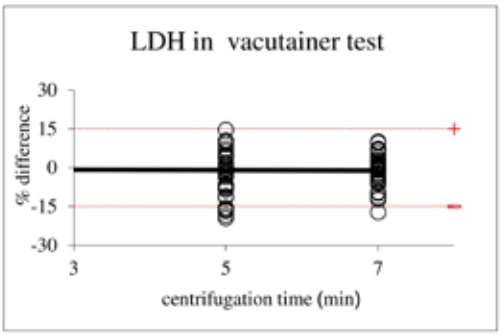

TNI in vacuette tubes
% difference
60
30
0
-30
-60
3
5
7
centrifugation time (min)

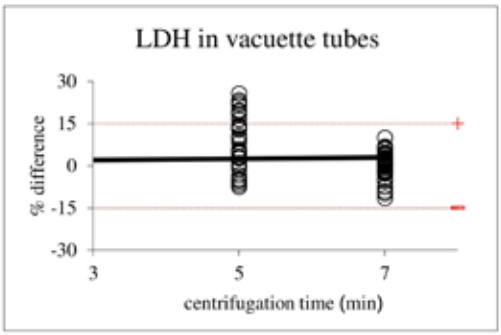

IRON in vacutainer tubes
% diference
30
15
0
-15
-30
3
5
7
centrifugation time(min)

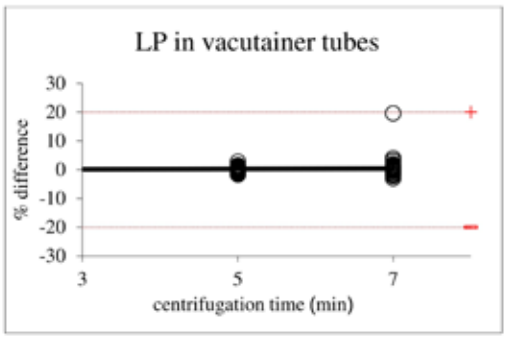

IRON in vacuette tubes
% diference
30
15
0
-15
-30
3
5
7
centrifugation time(min)

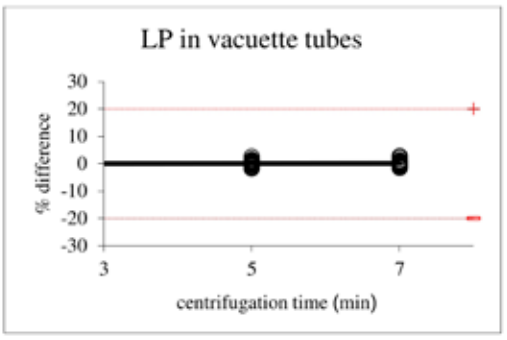

K in vacutainer tubes
mmol/L
0.6
0.3
0.0
-0.3
-0.6
3
5
7
centrifugation time (min)

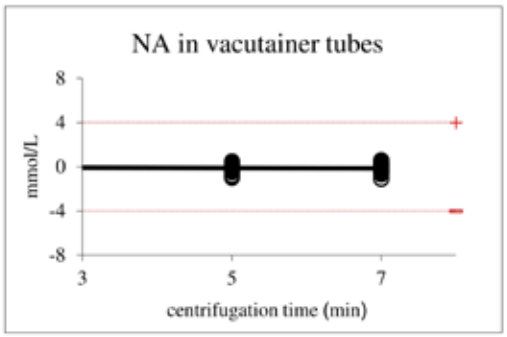

K in vacuette tubes
mmol/L
0.6
0.3
0.0
-0.3
-0.6
3
5
7
centrifugation time (min)

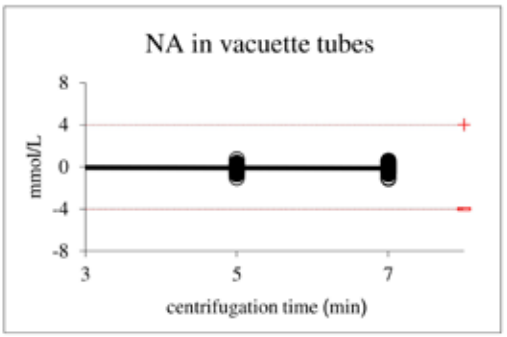

LDH in vacutainer test
% difference
30
15
0
-15
-30
3
5
7
centrifugation time (min)

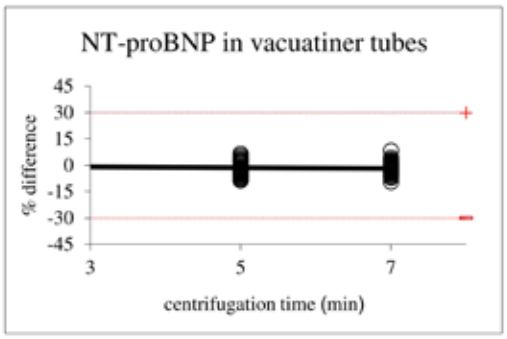

LDH in vacuette tubes
% difference
30
15
0
-15
-30
3
5
7
centrifugation time (min)

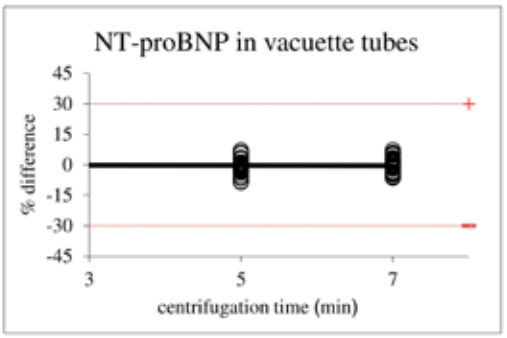

LP in vacutainer tubes
% difference
30
20
10
0
-10
-20
-30
3
5
7
centrifugation time (min)

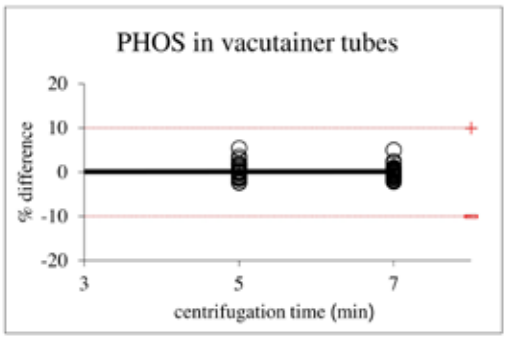

LP in vacuette tubes
% difference
30
20
10
0
-10
-20
-30
3
5
7
centrifugation time (min)

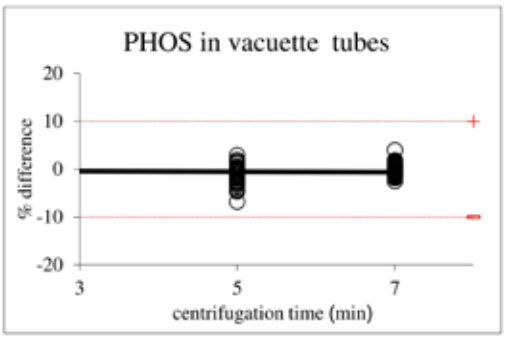

NA in vacutainer tubes
mmol/L
8
4
0
-4
-8
3
5
7
centrifugation time (min)

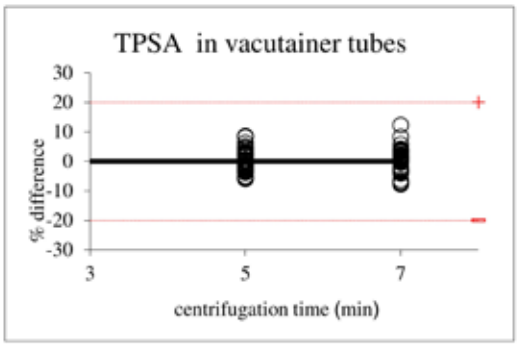

NA in vacuette tubes
mmol/L
8
4
0
-4
-8
3
5
7
centrifugation time (min)

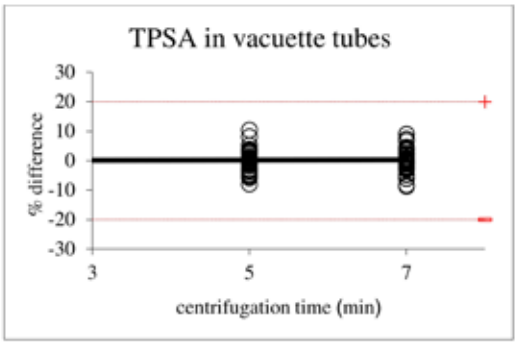

NT-proBNP in vacuatiner tubes
% difference
45
30
15
0
-15
-30
-45
3
5
7
centrifugation time (min)

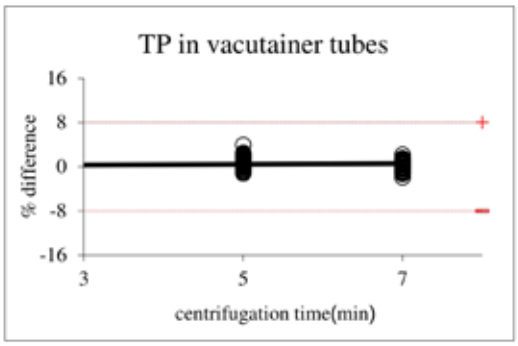

NT-proBNP in vacuette tubes
% difference
45
30
15
0
-15
-30
-45
3
5
7
centrifugation time (min)

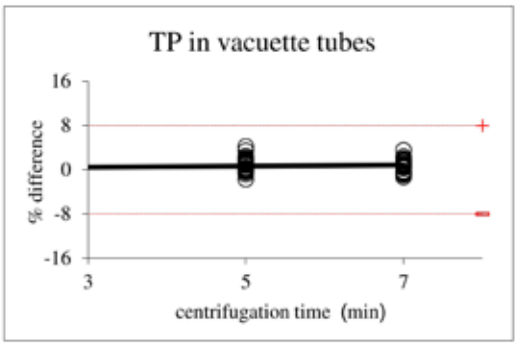

PHOS in vacutainer tubes
% difference
20
10
0
-10
-20
3
5
7
centrifugation time (min)

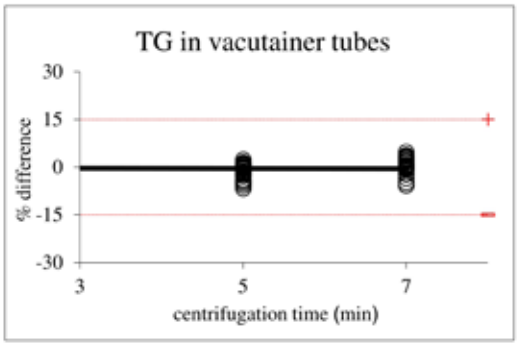

PHOS in vacuette tubes
% difference
20
10
0
-10
-20
3
5
7
centrifugation time (min)

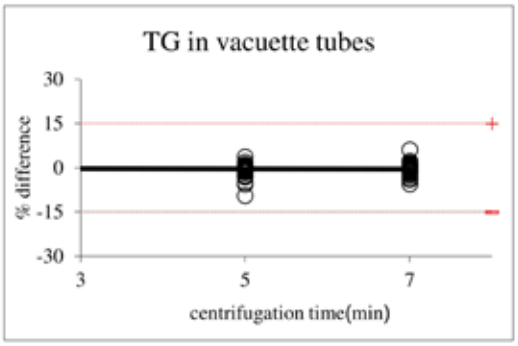

TPSA in vacutainer tubes
% difference
30
20
10
0
-10
-20
-30
3
5
7
centrifugation time (min)

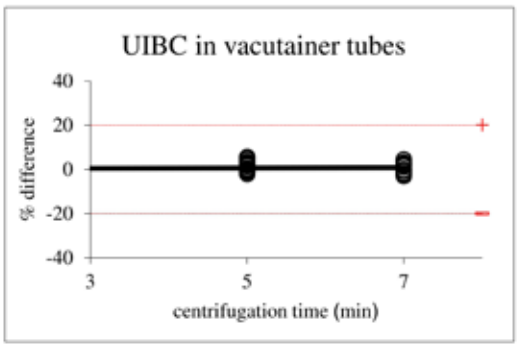

TPSA in vacuette tubes
% difference
30
20
10
0
-10
-20
-30
3
5
7
centrifugation time (min)

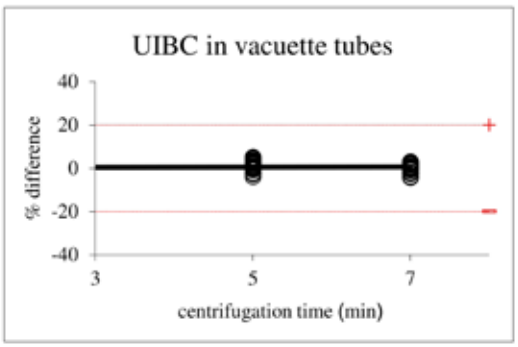

TP in vacutainer tubes
% difference
16
8
0
-8
-16
3
5
7
centrifugation time(min)

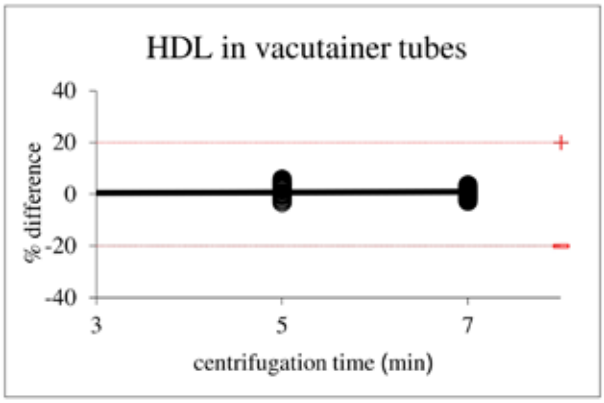

TP in vacuette tubes
% difference
16
8
0
-8
-16
3
5
7
centrifugation time (min)

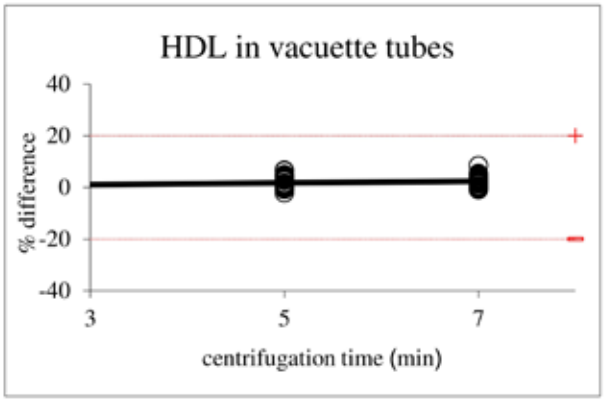

TG in vacutainer tubes
% difference
30
15
0
-15
-30
3
5
7
centrifugation time (min)

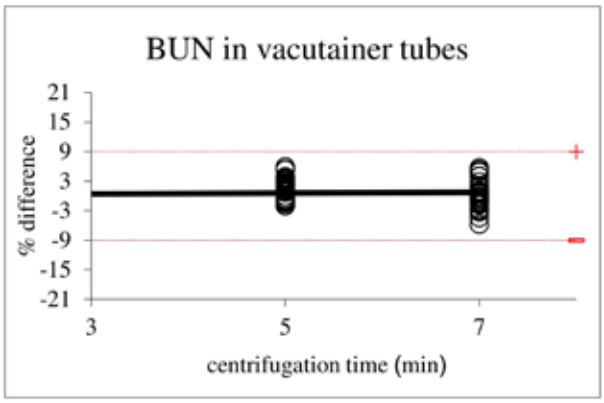

TG in vacuette tubes
% difference
30
15
0
-15
-30
3
5
7
centrifugation time(min)

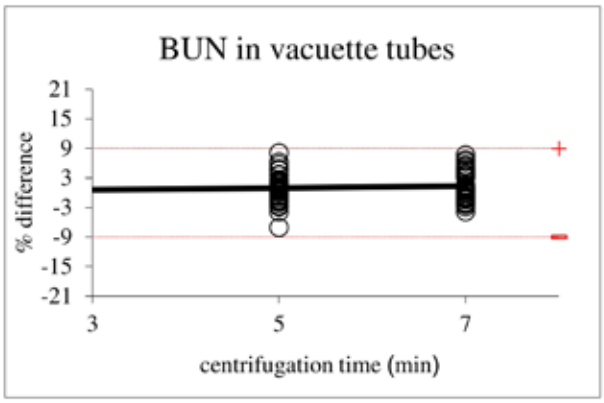

UIBC in vacutainer tubes
% difference
40
20
0
-20
-40
3
5
7
centrifugation time (min)

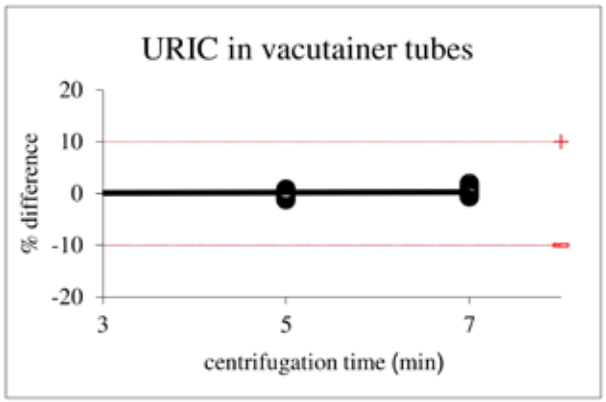

UIBC in vacuette tubes
% difference
40
20
0
-20
-40
3
5
7
centrifugation time (min)

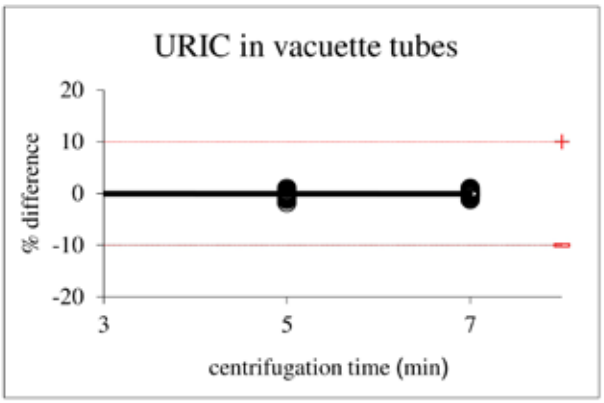



The percentage differences of each test must be >95% within acceptable TEa limits except LDH in vacutainer and vacuette tubes at 5 min.

The index results for hemolysis (HEM), icterus (ICT), and lipemia (LIP) cannot be interpreted since most of the measured values were below the lowest detection limit of the assay. The Passing–Bablok regression analysis results showed that the slope and intercept lie in the 95% confidence interval (95% CI) (indicated with parentheses. Furthermore, the correlation coefficient obtained using vacutainer and vacuette tubes for centrifugation at 7 and 5 min at 2700×g correlated well with the acceptable limit (>0.9). The corresponding results are listed in Tables 3–6.

**Table 3**: Comparison of chemical and immunological assays after centrifugation at 2700×g for 7 min and 1300×g for 10 min on vacutainer tubes.

| Tests | Slope (95% CI) | Intercept (95% CI) | Correlation coefficient (AL) | Correlation |
|---|---|---|---|---|
| AFP | 0.981 (0.950 to 0.999) | 0.0816 (-0.0135 to 0.1691) | 0.9962 | Acceptable |
| ALB | 1.022 (1.000 to 1.061) | -0.079 (-0.235 to 0.010) | 0.9948 | Acceptable |
| ALP | 1.000 (1.000 to 1.016) | 0.000 (-1.057 to 0.000) | 0.9986 | Acceptable |
| ALT | 1.011 (0.998 to 1.024) | -0.552 (-0.900 to -0.141) | 0.9994 | Acceptable |
| AMY | 1.000 (1.000 to 1.000) | 0.0 (0.0 to 0.0) | 0.9999 | Acceptable |
| AST | 1.008 (0.985 to 1.038) | -0.229 (-0.908 to 0.215) | 0.9969 | Acceptable |
| DBIL | 1.000 (0.995 to 1.000) | -0.010 (-0.010 to -0.007) | 0.9995 | Acceptable |
| TBIL | 1.000 (1.000 to 1.000) | 0.000 (0.000 to 0.000) | 1.0000 | Acceptable |
| CA 125 | 0.999 (0.988 to 1.015) | 0.3592 (-0.5550 to 1.0302) | 0.9999 | Acceptable |
| CA 19-9 | 0.979 (0.952 to 1.007) | -0.0840 (-0.4126 to 0.4198) | 0.9986 | Acceptable |
| CA | 0.952 (0.917 to 1.000) | 0.410 (-0.010 to 0.728) | 0.9933 | Acceptable |
| CEA | 0.956 (0.934 to 0.993) | 0.1833 (-0.0030 to 0.2749) | 0.9990 | Acceptable |
| CHOL | 1.007 (0.992 to 1.020) | -0.892 (-3.341 to 1.711) | 0.9988 | Acceptable |
| CK | 1.000 (0.986 to 1.000) | 0.0 (0.0 to 2.1) | 0.9999 | Acceptable |
| CL | 1.026 (1.000 to 1.064) | -2.93 (-6.90 to -0.10) | 0.9959 | Acceptable |
| CO2 | 1.020 (0.960 to 1.091) | -0.41 (-1.85 to 0.75) | 0.9837 | Acceptable |
| CREA | 1.000 (0.995 to 1.000) | 0.010 (0.010 to 0.013) | 0.9999 | Acceptable |
| hs-CRP | 1.001 (0.995 to 1.006) | -0.056 (-0.166 to 0.055) | 0.9998 | Acceptable |
| CRP | 1.004 (0.996 to 1.012) | -0.060 (-0.349 to 0.033) | 0.9998 | Acceptable |
| LDL | 0.996 (0.987 to 1.004) | 0.389 (-0.484 to 1.532) | 0.9996 | Acceptable |
| FER | 0.988 (0.966 to 1.019) | 2.9823 (-11.220) to 16.4556) | 0.9988 | Acceptable |
| FPSA | 1.005 (0.996 to 1.016) | 0.0006 (-0.0018 to 0.0045) | 0.9996 | Acceptable |
| GGT | 1.000 (0.996 to 1.000) | 0.0 (0.0 to 0.1) | 0.9997 | Acceptable |
| GLU | 0.997 (0.986 to 1.005) | -0.06 (-0.98 to 0.92) | 0.9995 | Acceptable |
| HEM | 1.250 (1.000 to 1.500) | -0.250 (-1.500 to 0.500) | 0.9624 | Not interpret |
| ICT | 1.000 (1.000 to 1.000) | 0.00 (0.00 to 0.00) | 0.9466 | Not interpret |
| TNI | 1.001 (0.981 to 1.019) | -0.253 (-1.138 to 0.440) | 0.9996 | Acceptable |
| IRON | 1.000 (0.991 to 1.007) | 0.000 (-0.082 to 0.106) | 0.9988 | Acceptable |
| K | 1.000 (0.993 to 1.036) | 0.000 (-0.143 to 0.029) | 0.9947 | Acceptable |
| LIP | 0.900 (0.769 to 1.000) | -4.000 (-5.000 to -2.269) | 0.9568 | Not interpret |
| LDH | 1.032 (0.938 to 1.146) | -16.2 (-66.1 to 14.7) | 0.9826 | Acceptable |
| LP | 1.000 (0.991 to 1.007) | 0.0009 (-0.3491 to 0.4433) | 0.9981 | Acceptable |
| NA | 1.038 (0.985 to 1.100) | -5.43 (-14.11 to 2.07) | 0.9872 | Acceptable |
| NT-proBNP | 0.985 (0.970 to 0.998) | 3.728 (-11.824 to 16.353) | 0.9996 | Acceptable |
| PHOS | 1.000 (0.968 to 1.036) | 0.000 (-0.128 to 0.098) | 0.9949 | Acceptable |
| TPSA | 1.010 (0.992 to 1.030) | -0.0034 (-0.0162 to 0.0044) | 0.9986 | Acceptable |

| | | | | |
|---|---|---|---|---|
| TP | 1.023 (0.976 to 1.063) | -0.144 (-0.459 to 0.205) | 0.9922 | Acceptable |
| TG | 1.014 (0.998 to 1.026) | -1.41 (-2.75 to 0.44) | 0.9994 | Acceptable |
| UIBC | 1.000 (1.000 to 1.034) | 0.000 (-1.303 to 0.000) | 0.9972 | Acceptable |
| HDL | 1.000 (0.978 to 1.022) | 0.150 (-0.966 to 1.419) | 0.9978 | Acceptable |
| BUN | 1.000 (0.985 to 1.008) | 0.000 (-0.126 to 0.253) | 0.9996 | Acceptable |
| URIC | 1.000 (0.991 to 1.008) | 0.020 (-0.028 to 0.063) | 0.9997 | Acceptable |

The 95% confidence interval (95% CI) for the slope and intercept is indicated with parentheses. Results cannot be interpreted because most measured values are below the lowest detection limit of the assay.

**Table 4**: Comparison of chemical and immunological assays after centrifugation at 2700×g for 5 min and 1300×g for 10 min on vacutainer tubes.

| Tests | Slope (95% CI) | Intercept (95% CI) | Correlation coefficient (AL) | Correlation |
|---|---|---|---|---|
| AFP | 1.001 (0.984 to 1.034) | -0.0022 (-0.1145 to 0.0463) | 0.9994 | Acceptable |
| ALB | 1.081 (1.038 to 1.119) | -0.301 (-0.475 to -0.125) | 0.9950 | Acceptable |
| ALP | 1.000 (1.000 to 1.027) | 0.000 (-2.446 to 0.000) | 0.9978 | Acceptable |
| ALT | 1.005 (0.995 to 1.014) | -0.165 (-0.470 to 0.168) | 0.9996 | Acceptable |
| AMY | 1.000 (1.000 to 1.000) | 0.0 (0.0 to 0.0) | 0.9998 | Acceptable |
| AST | 1.026 (1.000 to 1.057) | -0.272 (-1.062 to 0.300) | 0.9978 | Acceptable |
| DBIL | 1.000 (1.000 to 1.033) | 0.000 (-0.011 to 0.000) | 0.9993 | Acceptable |
| TBIL | 1.000 (0.993 to 1.026) | 0.010 (-0.011 to 0.015) | 0.9987 | Acceptable |
| CA 125 | 1.004 (0.980 to 1.037) | -0.0108 (-0.8573 to 0.9488) | 0.9999 | Acceptable |
| CA 19-9 | 0.975 (0.935 to 1.032) | 0.1540 (-0.8069 to 0.6894) | 0.9972 | Acceptable |
| CA | 0.992 (0.951 to 1.034) | 0.050 (-0.328 to 0.411) | 0.9928 | Acceptable |
| CEA | 0.972 (0.944 to 1.003) | 0.0987 (-0.0297 to 0.2011) | 0.9979 | Acceptable |
| CHOL | 1.026 (1.004 to 1.045) | -3.155 (-6.989 to 0.926) | 0.9981 | Acceptable |
| CK | 1.000 (0.996 to 1.005) | 0.0 (-0.4 to 0.9) | 0.9999 | Acceptable |
| CL | 1.040 (1.000 to 1.074) | -4.32 (-7.96 to -0.05) | 0.9958 | Acceptable |
| CO2 | 1.000 (0.942 to 1.074) | 0.00 (-1.43 to 1.08) | 0.9814 | Acceptable |
| CREA | 0.993 (0.979 to 1.000) | 0.018 (0.010 to 0.036) | 0.9999 | Acceptable |
| hs-CRP | 1.000 (0.994 to 1.005) | -0.005 (-0.117 to 0.111) | 0.9999 | Acceptable |
| CRP | 0.998 (0.992 to 1.006) | 0.027 (-0.128 to 0.182) | 0.9999 | Acceptable |
| LDL | 1.012 (0.998 to 1.025) | -0.844 (-2.215 to 1.018) | 0.9993 | Acceptable |
| FER | 0.993 (0.965 to 1.037) | 4.1364 (-11.1582 to 19.2708) | 0.9964 | Acceptable |
| FPSA | 1.011 (0.993 to 1.028) | 0.0001 (-0.0055 to 0.0030) | 0.9993 | Acceptable |
| GGT | 1.000 (0.992 to 1.000) | 0.0 (0.0 to 0.3) | 0.9998 | Acceptable |
| GLU | 1.005 (0.993 to 1.022) | -1.12 (-2.82 to 0.13) | 0.9991 | Acceptable |
| HEM | 1.000 (0.875 to 1.273) | 0.000 (-0.864 to 0.438) | 0.9561 | Not interpret |
| ICT | 1.000 (1.000 to 1.000) | 0.00 (0.00 to 0.00) | 0.8584 | Not interpret |
| TNI | 0.983 (0.963 to 1.004) | -0.197 (-0.793 to 0.600) | 0.9985 | Acceptable |

| | | | | |
|---|---|---|---|---|
| IRON | 1.004 (0.997 to 1.018) | -0.046 (-0.215 to 0.052) | 0.9986 | Acceptable |
| K | 1.000 (0.988 to 1.026) | 0.000 (-0.112 to 0.048) | 0.9988 | Acceptable |
| LDH | 0.980 (0.936 to 1.031) | 7.8 (-13.0 to 29.6) | 0.9892 | Acceptable |
| LIP | 0.875 (0.750 to 1.000) | -3.750 (-5.000 to -2.000) | 0.9715 | Not interpret |
| LP | 1.003 (0.995 to 1.010) | -0.0633 (-0.3844 to 0.2892) | 0.9998 | Acceptable |
| NA | 1.056 (1.000 to 1.108) | -8.04 (-15.23 to -0.20) | 0.9890 | Acceptable |
| NT-proBNP | 0.976 (0.954 to 0.990) | 0.374 (-13.074 to 11.777) | 0.9993 | Acceptable |
| PHOS | 1.000 (0.962 to 1.046) | 0.000 (-0.139 to 0.128) | 0.9923 | Acceptable |
| TPSA | 0.988 (0.975 to 1.002) | 0.0031 (-0.0025 to 0.0145) | 0.9988 | Acceptable |
| TP | 1.100 (1.061 to 1.146) | -0.689 (-1.052 to -0.392) | 0.9925 | Acceptable |
| TG | 1.003 (0.993 to 1.018) | -1.18 (-2.86 to 0.47) | 0.9994 | Acceptable |
| UIBC | 1.002 (1.000 to 1.037) | -0.058 (-1.217 to 0.000) | 0.9977 | Acceptable |
| HDL | 1.069 (1.039 to 1.106) | -2.835 (-4.786 to -1.115) | 0.9970 | Acceptable |
| BUN | 0.988 (0.972 to 1.000) | 0.394 (0.200 to 0.593) | 0.9997 | Acceptable |
| URIC | 1.004 (1.000 to 1.011) | -0.013 (-0.058 to 0.005) | 0.9998 | Acceptable |

For the slope and intercept, the 95% confidence interval (95% CI) is indicated with parentheses. Results cannot be interpreted because most measured values are below the lowest detection limit of the assay.

**Table 5**: Comparison of chemical and immunological assays after centrifugation at 2700×g for 7 min and 2200×g for 10 min on vacuette tubes.

| Tests | Slope (95% CI) | Intercept (95% CI) | Correlation coefficient (AL) | Correlation |
|---|---|---|---|---|
| AFP | 1.004 (0.987 to 1.027) | 0.0016 (-0.0745 to 0.0470) | 0.9994 | Acceptable |
| ALB | 1.074 (1.045 to 1.115) | -0.281 (-0.458 to -0.159) | 0.9965 | Acceptable |
| ALP | 1.000 (1.000 to 1.000) | 0.000 (0.000 to 0.000) | 0.9993 | Acceptable |
| ALT | 0.994 (0.984 to 1.006) | 0.194 (-0.147 to 0.571) | 0.9991 | Acceptable |
| AMY | 1.000 (1.000 to 1.000) | 0.0 (0.0 to 0.0) | 0.9999 | Acceptable |
| AST | 1.023 (1.000 to 1.067) | -0.413 (-1.283 to 0.350) | 0.9982 | Acceptable |
| DBIL | 1.000 (0.992 to 1.000) | 0.000 (0.000 to 0.002) | 0.9997 | Acceptable |
| TBIL | 1.000 (0.957 to 1.000) | -0.010 (-0.010 to 0.018) | 0.9982 | Acceptable |
| CA 125 | 1.010 (0.990 to 1.033) | -0.0478 (-0.8831 to 0.6025) | 0.9999 | Acceptable |
| CA 19-9 | 0.957 (0.923 to 1.007) | 0.4234 (-0.5283 to 1.1428) | 0.9984 | Acceptable |
| CA | 0.968 (0.912 to 1.018) | 0.300 (-0.148 to 0.780) | 0.9890 | Acceptable |
| CEA | 0.985 (0.970 to 1.004) | 0.0574 (-0.0931 to 0.1737) | 0.9996 | Acceptable |
| CHOL | 1.034 (1.016 to 1.050) | -4.828 (-7.919 to -1.361) | 0.9990 | Acceptable |

| | | | | |
|---|---|---|---|---|
| CK | 1.000 (0.994 to 1.002) | 0.0 (-0.3 to 0.9) | 1.0000 | Acceptable |
| CL | 1.000 (0.963 to 1.014) | 0.00 (-1.41 to 3.96) | 0.9950 | Acceptable |
| CO2 | 0.963 (0.897 to 1.000) | 0.57 (-0.15 to 1.86) | 0.9827 | Acceptable |
| CREA | 1.000 (0.993 to 1.000) | 0.000 (0.000 to 0.012) | 1.0000 | Acceptable |
| hs-CRP | 0.998 (0.990 to 1.004) | 0.042 (-0.053 to 0.272) | 0.9999 | Acceptable |
| CRP | 0.998 (0.993 to 1.003) | -0.033 (-0.147 to 0.105) | 0.9999 | Acceptable |
| LDL | 1.011 (0.998 to 1.026) | -0.822 (-2.342 to 0.424) | 0.9993 | Acceptable |
| FER | 1.024 (1.000 to 1.039) | -4.7694 (-12.7687 to 6.1334) | 0.9992 | Acceptable |
| FPSA | 1.005 (0.990 to 1.020) | 0.0005 (-0.0031 to 0.0031) | 0.9990 | Acceptable |
| GGT | 1.000 (0.993 to 1.000) | 0.0 (0.0 to 0.4) | 0.9999 | Acceptable |
| GLU | 1.001 (0.989 to 1.023) | -0.49 (-2.53 to 0.87) | 0.9987 | Acceptable |
| HEM | 1.051 (0.961 to 1.333) | -1.253 (-3.333 to -0.627) | 0.9561 | Not interpret |
| ICT | 1.000 (1.000 to 1.000) | 0.00 (0.00 to 0.00) | 0.9526 | Not interpret |
| TNI | 1.006 (0.993 to 1.018) | -0.692 (-1.248 to 0.012) | 0.9999 | Acceptable |
| IRON | 0.996 (0.983 to 1.000) | 0.044 (0.000 to 0.224) | 0.9990 | Acceptable |
| K | 1.000 (1.000 to 1.025) | 0.000 (-0.100 to 0.000) | 0.9993 | Acceptable |
| LDH | 1.000 (0.968 to 1.033) | 1.0 (-12.3 to 12.6) | 0.9962 | Acceptable |
| LIP | 1.000 (1.000 to 1.000) | 0.000 (0.000 to 0.000) | 0.9918 | Not interpret |
| LP | 1.007 (1.001 to 1.013) | -0.2321 (-0.5360 to 0.0858) | 0.9999 | Acceptable |
| NA | 1.021 (0.977 to 1.063) | -2.87 (-8.63 to 3.30) | 0.9920 | Acceptable |
| NT-proBNP | 0.997 (0.985 to 1.012) | 0.147 (-13.333 to 10.524) | 0.9996 | Acceptable |
| PHOS | 1.007 (0.975 to 1.039) | -0.031 (-0.137 to 0.074) | 0.9958 | Acceptable |
| TPSA | 1.007 (0.987 to 1.027) | -0.0017 (-0.0161 to 0.0094) | 0.9986 | Acceptable |
| TP | 1.096 (1.061 to 1.142) | -0.678 (-1.037 to -0.425) | 0.9940 | Acceptable |
| TG | 1.008 (1.000 to 1.018) | -0.48 (-1.52 to 0.40) | 0.9997 | Acceptable |
| UIBC | 1.000 (1.000 to 1.015) | 0.000 (-0.560 to 0.000) | 0.9981 | Acceptable |
| HDL | 1.065 (1.035 to 1.098) | -2.258 (-4.149 to -0.697) | 0.9970 | Acceptable |
| BUN | 0.983 (0.964 to 1.000) | 0.302 (0.100 to 0.611) | 0.9993 | Acceptable |
| URIC | 1.009 (1.000 to 1.016) | -0.042 (-0.079 to 0.005) | 0.9998 | Acceptable |

For the slope and intercept, the 95% confidence interval (95% CI) is indicated with parentheses. Results cannot be interpreted because most measured values are below the lowest detection limit of the assay.

**Table 6**: Comparison of chemical and immunological assays after centrifugation at 2700×g for 5 min and 2200×g for 10 min on vacuette tubes.

| Tests | Slope (95% CI) | Intercept (95% CI) | Correlation coefficient (AL) | Correlation |
|---|---|---|---|---|
| AFP | 1.003 (0.989 to 1.044) | 0.0223 (-0.1137 to 0.0723) | 0.9981 | Acceptable |
| ALB | 1.074 (1.042 to 1.114) | -0.280 (-0.459 to -0.137) | 0.9944 | Acceptable |
| ALP | 1.000 (0.966 to 1.016) | 3.000 (2.040 to 5.000) | 0.9977 | Acceptable |
| ALT | 0.996 (0.982 to 1.004) | 0.105 (-0.186 to 0.549) | 0.9995 | Acceptable |
| AMY | 1.000 (1.000 to 1.000) | 0.0 (0.0 to 0.0) | 0.9998 | Acceptable |
| AST | 1.058 (1.010 to 1.120) | -0.878 (-2.116 to 0.235) | 0.9960 | Acceptable |
| DBIL | 1.000 (1.000 to 1.034) | 0.000(-0.005 to 0.000) | 0.9993 | Acceptable |
| TBIL | 0.991 (0.966 to 1.000) | -0.016 (-0.020 to 0.000) | 0.9993 | Acceptable |
| CA 125 | 1.027 (1.016 to 1.038) | -0.2656 (-0.8344 to 0.1033) | 1.0000 | Acceptable |
| CA 19-9 | 1.005 (0.955 to 1.034) | -0.3446 (-0.9598 to 0.4621) | 0.9979 | Acceptable |
| CA | 0.989 (0.937 to 1.036) | 0.079 (-0.331 to 0.542) | 0.9916 | Acceptable |
| CEA | 0.987 (0.963 to 1.019) | 0.0572 (-0.0669 to 0.1761) | 0.9992 | Acceptable |
| CHOL | 1.033 (1.011 to 1.052) | -4.294 (-7.763 to 0.344) | 0.9983 | Acceptable |
| CK | 1.000 (0.994 to 1.002) | 0.0 (-0.3 to 0.7) | 0.9999 | Acceptable |
| CL | 0.981 (0.952 to 1.000) | 1.98 (0.00 to 4.98) | 0.9950 | Acceptable |
| CO2 | 1.000 (0.933 to 1.040) | -0.05 (-0.80 to 1.20) | 0.9816 | Acceptable |
| CREA | 1.000 (0.996 to 1.000) | 0.000 (0.000 to 0.005) | 1.0000 | Acceptable |
| hs-CRP | 1.000 (0.996 to 1.007) | 0.004 (-0.114 to 0.081) | 0.9987 | Acceptable |
| CRP | 1.000 (0.994 to 1.007) | 0.045 (-0.088 to 0.220) | 0.9992 | Acceptable |
| LDL | 1.009 (0.993 to 1.026) | -0.489 (-2.238 to 1.224) | 0.9988 | Acceptable |
| FER | 1.032 (0.985 to 1.048) | -1.1126 (-12.9257 to 12.5014) | 0.9987 | Acceptable |
| FPSA | 1.012 (0.988 to 1.032) | -0.0005 (-0.0042 to 0.0023) | 0.9993 | Acceptable |
| GGT | 1.000 (0.988 to 1.000) | 0.0 (0.0 to 1.2) | 0.9997 | Acceptable |
| GLU | 1.011 (0.993 to 1.027) | -1.44 (-2.97 to 0.23) | 0.9988 | Acceptable |
| HEM | 1.000 (0.750 to 1.214) | -1.500 (-2.964 to 0.000) | 0.9505 | Not interpret |
| ICT | 1.000 (1.000 to 1.000) | 0.00 (0.00 to 0.00) | 0.9541 | Not interpret |
| TNI | 0.993 (0.970 to 1.015) | -0.619 (-1.224 to -0.095) | 0.9991 | Acceptable |
| IRON | 1.000 (0.990 to 1.010) | 0.000 (-0.110 to 0.140) | 0.9992 | Acceptable |
| K | 1.032 (1.000 to 1.064) | -0.137 (-0.270 to 0.000) | 0.9980 | Acceptable |
| LDH | 1.012 (0.917 to 1.111) | 14.8 (-21.0 to 56.9) | 0.9838 | Acceptable |
| LIP | 1.000 (1.000 to 1.056) | 1.000 (-0.083 to 1.000) | 0.9931 | Not interpret |
| LP | 1.003 (0.994 to 1.012) | -0.1131 (-0.4541 to 0.2308) | 0.9998 | Acceptable |
| NA | 1.018 (0.967 to 1.069) | -2.47 (-9.65 to 4.62) | 0.9899 | Acceptable |
| NT-proBNP | 0.996 (0.978 to 1.011) | -2.128 (-15.960 to 16.839) | 0.9985 | Acceptable |

| | | | | |
|---|---|---|---|---|
| PHOS | 1.061 (1.000 to 1.103) | -0.235 (-0.366 to -0.030) | 0.9907 | Acceptable |
| TPSA | 1.001 (0.985 to 1.024) | -0.00152 (-0.02010 to 0.01167) | 0.9984 | Acceptable |
| TP | 1.104 (1.065 to 1.153) | -0.721 (-1.091 to -0.425) | 0.9917 | Acceptable |
| TG | 1.006 (0.997 to 1.019) | -1.20 (-2.45 to -0.20) | 0.9998 | Acceptable |
| UIBC | 1.033 (1.000 to 1.063) | -0.697 (-1.935 to 0.850) | 0.9972 | Acceptable |
| HDL | 1.066 (1.041 to 1.092) | -2.210 (-3.636 to -0.703) | 0.9971 | Acceptable |
| BUN | 1.000 (0.979 to 1.012) | 0.150 (-0.062 to 0.390) | 0.9995 | Acceptable |
| URIC | 1.008 (1.000 to 1.014) | -0.050 (-0.087 to -0.010) | 0.9998 | Acceptable |

For the slope and intercept, the 95% confidence interval (95% CI) is indicated with parentheses. Results cannot be interpreted because most measured values are below the lowest detection limit of the assay.

**Discussion**

Various blood-collection tubes are available on the market for efficient laboratory testing as well as for storing blood samples for an appropriate period. Each tube type is designed for using samples of different types and quantities with different anticoagulant techniques. Each manufacturer typically provides recommendations for the centrifugation of serum or plasma at different speeds and times, which are followed by most laboratories. According to the Clinical & Laboratory Standards Institute (CLSI) guidelines, the centrifugation time and g-force recommended by the manufacturer of blood collection tubes [10] or those by the WHO should be followed. Although nowadays laboratories tend to use fully automated systems, these systems are found to delay the pre-analysis steps. The entire process of centrifugation, starting with queuing, loading, balancing, centrifugation, slowing down to stop, and sample unloading from the centrifuge, depending on the setup and workload, can take a minimum of 15–20 min. Earlier studies have investigated the effects of changing the speed or reducing the time required to ensure a continuous analysis, while maintaining the quality of the analysis [11-14].

Centrifugation for 7 min at 2700×g did not affect the chemical and immunological assays in both vacutainer and vacuette tubes. Although statistically significant differences were observed, the percentage of differences was within the acceptable TEa limit. The number of tests performed exceeded the acceptable limit (<5%), which is consistent with the results reported by Minder et al. [4]. No significant differences were observed when whole blood samples were centrifuged as per the WHO guidelines (15 min at 2180×g, 10 min at 2180×g, and 7 min at 1870×g) to reduce the TAT significantly. Additionally, Tanitsaranon et al. [11] reported that centrifugation can be carried out at 1300×g for 10 min. Further, the results obtained when centrifugation was carried out for 7 min at 2200×g and 5 min at 2750×g using lithium heparin vacutainer tubes were found to be acceptable. These results suggest that centrifugation should be performed at higher speeds for shorter times to improve the TAT.

Chemical and immunological tests were performed by centrifugation at 2700×g for 5 min using both vacutainer and vacuette tube types. Statistically significant differences were observed, although the percentage difference was within the acceptable TEa limit. Except for the LDH test that was carried out in both vacutainer and vacuette tubes, only six samples, or 15% and 8 samples or 20% of the total number of cases, respectively, were found to have a percentage difference outside the TEa limit. This finding is in contrast to that reported by Koenders et al. [12], who did not find any differences between serum and plasma samples when the centrifugation duration was reduced from 10 min to 5 min at 1885×g. However, similar results were reported by Moller et al. [13], who found that when the serum and plasma samples were centrifuged for 10 min at 2200×g and 5 min at 3000×g, not only did the overall LDH test results exceed the total error acceptance limit; however, the were higher after centrifugation for 5 min at 3000×g, even in a vacutainer tube, owing to the higher g-force (2700×g).

In a study conducted by Cadamuro et al. [14], results of centrifugation performed on samples of serum and plasma vacutainer tubes for 10 min at 2000×g and 7 and 5 min at 3000×g demonstrated that the serum and heparin samples could be centrifuged at higher speeds (3000×g) for a shorter duration (5 min) when using plasma for blood collection. However, a separate LDH reference value may be required for this.

In this study, it was found that centrifugation performed for 7 and 5 min at 2700×g, performed as per the manufacturer's recommendation, did not affect the HEM index. As listed in Table 2, we did not find any increase in the K, PHOS, and AST levels in the plasma. A comparison of the LDH values of the vacutainer and vacuette tubes demonstrated that the vacutainer tubes had higher LDH values. Centrifugation for 5 min at 2700×g in vacuette tubes increased the LDH values, probably because of the shorter centrifugation period, distinct tube characteristics, and a difference in the lithium content from that of vacutainer tubes. Both vacutainer and vacuette tubes use spray-dried lithium heparin. According to the IFCC [15] recommendation for LDH

measurements, serum is the preferred sample for LDH to avoid platelet aggregation or platelet rupture. The increase in LDH levels may have been due to platelet lysis or optical interference caused by intact platelets [16]. This is consistent with the study by Lippi et al. [6], which investigated lithium heparin tubes being centrifuged at 1200×g for 1, 2, 5, 10, and 15 min. They found that centrifugation time was inversely related to the residual blood cell composition measured in the plasma. Plasma platelet counts were significantly increased in samples centrifuged for 10 min or less, while red and white blood cell counts were significantly increased in samples centrifuged for 2 min or less and for 1 min, respectively. While the LDH of vacutainer tubes after centrifugation for 7 and 5 min at 2700×g became lower than that after 10 min at 1300×g, it was found that increasing the g-force resulted in a decrease in LDH values, possibly due to the lower platelet count. This is consistent with the study conducted by Jesting et al. [17], who found that centrifugation for 10 min at 3000×g significantly reduced platelet counts in plasma compared with centrifugation at 2000×g. However, for routine centrifugation, plasma lithium heparin is often preferred. Research by Minder et al. [4] demonstrated a reduction in centrifugation time from 15 min to 7 min, representing a 47% decrease. Similarly, Koenders et al. [12] reported a reduction to 5 minutes, a 66% decrease compared to WHO guidelines, significantly enhancing laboratory turnaround time (TAT). Consistent with these findings, reducing centrifugation time from 10 min to 7 min resulted in a 30% decrease, leading to a substantial reduction in laboratory TAT for both routine and stat samples, by an average of 19% and 24%, respectively, depending on the workload of each cycle.

## Conclusion

This study was designed to determine the appropriate g-force and time for centrifugation using different combinations of vacutainer and vacuette tubes to reduce the centrifugation duration and improve the TAT in the laboratory. The resultant efficiency of centrifugation procedure when carried out according to the manufacturer's guidelines and the conditions designed by us, while maintaining the efficiency, effectiveness, quality, accuracy, and reliability of centrifugation, were compared. We found that centrifugation at 2700×g for 7 min could be used for both vacutainers and vacuette lithium heparin tubes. This study can significantly impact the turnaround time, enabling clinicians to make accurate and timely diagnoses, allowing for immediate adjustments to treatment plans, particularly for critically patients. This improvement increases the likelihood of effective treatment, mitigates the risks associated with prolonged test result waiting times, and enhances patient satisfaction. The scope of future research will aim at studying the impact of the varying needs and constraints under which laboratories operate, including workload, personnel, automated analyzers, the use of different brands or types of tubes, and the determination of TAT taking each of these into careful consideration.

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## 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.

## Authors's contributions

Pensiri Choosongsang was the principal investigator, conceived, designed, and performed the experiments, performed data curation, and analyzed data and for editing the manuscript. Yupawadee Yamsuwan designed and performed the experiment and editing the manuscript. Sarayut Petchaithong, Thawin Prasongsab and Naphatohn Bhornsrivathanyou collected specimens and performed the experiment. Phattanapong Choosongsang performed data curation and analyzed data, and was responsible for drafting and editing the manuscript. The final version of the article was reviewed and approved by all authors.

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Research Article

# Conceptualization of the Use of Artificial Intelligence by Clinical or Research Laboratory Professionals: Challenges for Its Implementation in Mexico

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AI Ethics, Artificial Intelligence, Clinical Laboratory, Conceptualization, Mexico, Laboratory technologies

**Abstract**

**Introduction**: Artificial intelligence (AI) is revolutionizing the healthcare sector via advanced tools to improve diagnostic accuracy, operational efficiency, and decision-making. In clinical laboratories (CLs), the integration of AI automates the processing and analysis of large volumes of data, enables the early detection of diseases, and supports personalized medicine. Determining how personnel involved in the use of AI in CLs conceptualize AI can enable the identification of challenges and difficulties impeding its implementation.

**Methods**: An observational, prospective, cross-sectional, and comparative study was performed using an online survey for CL or research professionals in public or private CL throughout Mexico. The survey had 36 questions aimed at obtaining sociodemographic information and conceptualization of different aspects of AI, namely, familiarity, use, concerns, limitations, and useful applications.

**Results**: Overall, 125 men and 237 women (aged 19–81 years) participated in this study. The survey results showed that CL or research professionals were familiar with AI in general. They preferred to use AI to reduce pre-analytical errors (67%) and save time (65%). Lack of knowledge and training (74%) and fear of being replaced (66%) were identified as major AI-related concerns; the ethical aspects of AI were also a main concern. Only 4.7% of respondents had received formal AI training, but 84.8% were willing to take AI courses.

**Conclusion**: The findings highlight opportunities and priorities to promote AI-related public and educational policies, regulate AI adoption in CLs, develop optimal training strategies, as well as foster ethics, avoiding the exclusion of particular social groups.

## Introduction

Quality management in clinical laboratories (CLs) must evolve to address not only process control but also the complexity of patients and their environments. Quality in CLs encompasses multiple aspects, such as total control, assurance, reengineering, innovation, and continuous improvement, all of which require a data-driven approach to ensure reliable results. It is critical that standardized laboratory test results are obtained accurately within defined limits, minimizing pre-analytical, analytical, and post-analytical errors and considering inter- and intra-assay variations, along with the heterogeneity of human factors that influence decision-making [1-3].

Since the 1950s, automation in clinical practice has transformed data management and administrative calculations. In the last 20 years, artificial intelligence (AI) has accelerated this evolution with advanced applications in automated image analysis, algorithms, expert systems for diagnosis, and mass processing of clinical data. AI currently enables the analysis of large volumes of data (big data), facilitating the development of predictive models and optimizing diagnostic quality in clinical practice. Its implementation has improved test accuracy, operational efficiency, and the ability to quickly predict errors or diseases [4-7].

Now the integration of AI in CLs is revolutionizing healthcare by enabling advanced analysis, diagnostic algorithms, personalized treatment, and process optimization. Globally, AI has demonstrated benefits in areas such as digital pathology, molecular test interpretation, and medical image analysis [7-9], however, in low- and middle-income countries, such as Mexico, its implementation faces significant challenges due to inequalities in access to technology, variability in CL infrastructure, and the need for specialized training. Although Mexico has witnessed isolated efforts to integrate these tools, a comprehensive assessment of their adoption, benefits, and specific obstacles nationally has not yet been conducted. To effectively integrate AI into their work, CL staff must conceptualize its application in existing processes, understand its benefits, and develop training strategies that ensure its ethical and efficient use [9-13].

As reported in various studies, AI-based technologies have significantly improved diagnostic accuracy, operational efficiency, and the personalization of healthcare. In particular, advanced machine learning (ML) algorithms have achieved performance comparable and even superior to that of human experts in detecting diseases such as skin cancer [14]. Furthermore, these tools are used to interpret molecular tests, identify abnormalities in clinical analyses, and predict complications in patients with chronic diseases. However, a range of studies have highlighted the need for further research and strict regulations to ensure their safety and utility as support systems for healthcare professionals, contributing to the improvement of clinical outcomes, reduction of waiting times, lowering of healthcare costs, and reduction of workload [14-19].

In response to the need to improve care for chronic diseases worldwide, AI is increasingly contributing to control of these and other diseases. A recent bibliometric analysis on the use of AI in chronic diseases examined 341 publications from 775 institutions in 55 countries, published in 175 journals between 2013 and 2024, indicating that 95% of these studies focused on four key areas: diagnosis, healthcare, telemedicine, and health technology [18]. Elsewhere, analysis of current trends highlighted the rise of mobile health and ML as fundamental pillars in the progress of AI clinically [18-21].

The incorporation of artificial AI into CL processes almost imperceptibly induces behavioral changes, which are closely linked to the conceptualization of these technologies and the perceived risks and benefits. In this context, the Prochaska and DiClemente model posits that behavioral change is not a linear process but rather progresses through distinct stages: precontemplation, contemplation, preparation, action, maintenance, and termination. Each stage represents a different level of readiness for change, and individuals may transition between stages at any time. This model also underscores the importance of motivation and self-efficacy in facilitating behavioral change [22].

This study was conducted to assess the conceptualization of AI by CLs and research professionals, identifying the challenges facing its implementation and the opportunities to optimize its use in Mexico. An analysis based on the findings of a nationwide survey explored the main applications of AI in CLs, the expected benefits, the barriers to its adoption, and the perceptions of AI among the specialized personnel at CLs. The findings of this work should provide a foundation for the development of public policies that regulate the implementation of AI, the establishment of training strategies for those tasked with using AI tools in CLs, and measures that encourage the responsible adoption of AI in order to improve the quality and accessibility of diagnostic services in Mexico.

## Materials and Methods

This research was carried out via a survey aimed at CLs or research professionals working in any field (administrative or analytical processes), male or female, of any age, and with at least a technical degree, from public institutions or private laboratories throughout Mexico.

The survey was designed based on a previously validated instrument [23] to which questions were added to analyze additional variables considered important in the context of the present study. The instrument consisted of a total of 36 questions. The first section of the survey prior to the questions, included a description of the objective of the research and requested informed consent, along with assurances that participation was voluntary and anonymous. The first eight questions were

related to sociodemographic variables (e.g., age, sex, years of experience in their current position, educational level) and where in the laboratory/office the respondents performed their professional activities; 25 questions were directed to the conceptualization of different aspects of AI such as familiarity, daily use of AI-based applications, AI knowledge, processes for which AI could potentially be useful, and fears and challenges regarding its implementation. Of these latter 25 questions, 18 were multiple-choice questions for which one or more answers were allowed; for the other seven questions, a 5-point Likert scale ranging from 1 ("highly disagree") to 5 ("highly agree") were made. Finally, three optional questions were included to collect additional comments on the content of the instrument.

To ensure linguistic accuracy and cultural relevance, four experts validated the translation of the survey instrument into Spanish, considering the cultural context of the Mexican study group. They assessed the appropriateness of the questions, the accuracy of additional items, and the order of presentation to ensure alignment with the research objectives. Furthermore, they evaluated the response options to maximize the likelihood of obtaining truthful responses. A pre-test (pilot test) was conducted with 10 workers from various laboratory departments to identify and mitigate potential sources of random error.

The surveys were distributed from November 2024 to January 2025 through WhatsApp or email, and the corresponding Google forms link to access the survey to representatives of Colleges of Professionals in the CL field, both public and private, and to researchers in research laboratories at universities and research centers in all eight geographical regions into which Mexico is divided. The survey was accompanied by a 35 s video featuring an AI-created character, who welcomed the participants and explained the scope and objectives of the research, in addition to thanking those responding to the survey.

### Statistical analysis

Statistical analysis was performed using NCSS software version 2020, with a methodological approach that combined descriptive statistics and nonparametric inference tests. Categorical data were summarized using absolute frequencies and percentages, while quantitative variables were analyzed with measures of central tendency and dispersion. For comparisons of qualitative variables between groups, the chi-square test ($\chi^2$) was applied as a non-parametric method, selected for its suitability to the type of data and the assumptions of the study. The interpretation of the results was based on the p-value obtained, with the threshold for statistical significance set at $\alpha<0.05$. This criterion allowed the determination of statistically relevant associations between the analyzed variables. The process included initial characterization of the sample using distribution tables; bivariate analysis with contingency tables; the application of corrections for tables with low expected frequencies; and confirmation of the validity of the assumptions on which the choice of each statistical test was based. This methodological approach guaranteed rigorous treatment of the data, which is particularly important in studies with non-normally distributed data or small samples.

## Results

The survey was initially distributed to 491 contacts via email and WhatsApp, with a request to further disseminate it using the snowball method. However, not all emails and messages were confirmed as received, making it impossible to determine the exact number of surveys distributed and, consequently, the response rate.

A total of 364 responses to the survey on AI in CLs were received from November 15, 2024, to January 15, 2025. Two responses were excluded from the analysis: one because the individual was from another country (Portugal) and the other due to inconsistencies found in the responses. As such, the analysis was carried out on 362 surveys, corresponding to 125 men and 237 women between the ages of 19 and 81 (mean±SD: 43±13.6). The subjects varied in their educational level, years of experience in their current job, and whether their professional activities were performed in the CLs or involved administrative work. They worked in either public or private laboratories, which were classified by size (i.e., number of employees) and geographical area (Table 1; Figure 1). Overall, 53% of the participants reported having 1 to 10 years of experience (47 of them with <5 years of work experience) and 47% had ≥11 years in their current position. Of the 76 managers, 68 also performed laboratory tests, while 8 only performed managerial activities. Meanwhile, 29 participants only worked in administrative areas, and the rest (n=257) performed both laboratory and administrative activities, particularly in micro- and small laboratories.

**Table 1**: Participant and laboratory characteristics.

| **PARTICIPANTS** | **n** | **%** |
|---|---|---|
| **Sex** | | |
| Female | 237 | 65.4 |
| Male | 125 | 34.5 |
| **Age (years)** | | |
| 18–26 | 45 | 12.4 |
| 27–59 | 266 | 73.4 |
| >60 | 51 | 14 |
| **Education:** | | |
| Technical baccalaureate | 42 | 11.6 |
| Bachelor's degree | 175 | 48.3 |
| Postgraduate training in a CL specialism | 43 | 11.8 |
| Postgraduate degree | 102 | 28.1 |
| **Professional activities:** | | |
| Managers | 76 | 21.0 |
| Administrative | 204 | 56.3 |
| ***Laboratory:** | | |
| Hematology | 178 | 49.1 |
| Immunology | 157 | 43.3 |
| Bacteriology and microbiology | 131 | 36.1 |
| Clinical pathology | 94 | 25.9 |
| Molecular biology | 87 | 24 |
| **Years of experience:** | | |
| 1–10 | 192 | 53.0 |
| 11–20 | 82 | 22.6 |
| 21–30 | 47 | 12.9 |
| >30 | 41 | 11.3 |
| **LABORATORIES** | | |
| **Sector**: | | |
| Public | 158 | 43.6 |
| Private | 204 | 56.3 |
| **Size (number of employees)**: | | |
| Micro- (<10) | 116 | 32.0 |
| Small (10–50) | 155 | 42.8 |
| Medium (51–100) | 54 | 14.9 |
| Large (>100) | 37 | 10.2 |
| **Type**: | | |
| Clinical laboratory | 342 | 94.4 |
| Research laboratory | 20 | 5.5 |

*Only the five most common fields are shown.

**Figure 1:** Geographical regions of Mexico and their constituent states.


1. Northwestern: Baja California, Baja California Sur, Chihuahua, Sonora, Sinaloa, and Durango; 2. Northeastern: Coahuila, Nuevo Leon, and Tamaulipas; 3. Western: Colima, Jalisco, Michoacan, and Nayarit; 4. Eastern: Hidalgo, Puebla, Tlaxcala, and Veracruz; 5. North-Central: Aguascalientes, Guanajuato, San Luis Potosi, Zacatecas, and Queretaro; 6. South-Central: State of Mexico, Morelos, and Mexico City; 7. Southwestern: Chiapas, Guerrero, and Oaxaca; and 8. Southeastern: Campeche, Yucatan, Quintana Roo, and Tabasco,

**Familiarity**

The subjects were asked about their degree of familiarity with the use of AI in general and with AI specific to CLs. Only two considered themselves experts (0.5%). Table 2 shows the results of familiarity for both AI in general and AI specific to CLs. The analysis shows that the participants were significantly less familiar with specific AI applications in CLs. In addition, participants with postgraduate training in a CL specialism or postgraduate degrees were more familiar than those with bachelor's or technical baccalaureate degrees (p=0.0007). Meanwhile, 140 of the participants (38.6%) asserted that AI was not used in their laboratory, 108 (29.8%) reported that it was, and 114 (31.4%) were not sure.

**Table 2:** Degree of familiarity with AI in general and specific to CLs.

| Degree of familiarity | AI in general n (%) | AI specific to CLs n (%) | p |
|---|---|---|---|
| Expert | 2 (0.5) | 1 (0.2) | p=0.564 |
| Very familiar | 45 (12.4) | 21 (5.8) | p=0.004 |
| Something familiar | 197 (54.4) | 128 (35.3) | p=0.001 |
| Little or not at all familiar | 118 (32.6) | 213 (58.8) | p=0.00001 |
| Total | 362 (100) | 362 (100) | |

**Daily use of AI tools**

First, the respondents were asked whether they used AI applications on a daily basis, with three possible and exclusive answers ("Yes," "No," and "I'm not sure"). They were provided with a list of common applications from which they could choose all those that they used on a daily basis. MetaAI (WhatsApp) was the most widely used (65.7%), followed by ChatGPT (45.5%) and Google Lens (33.7%) (Table 3).

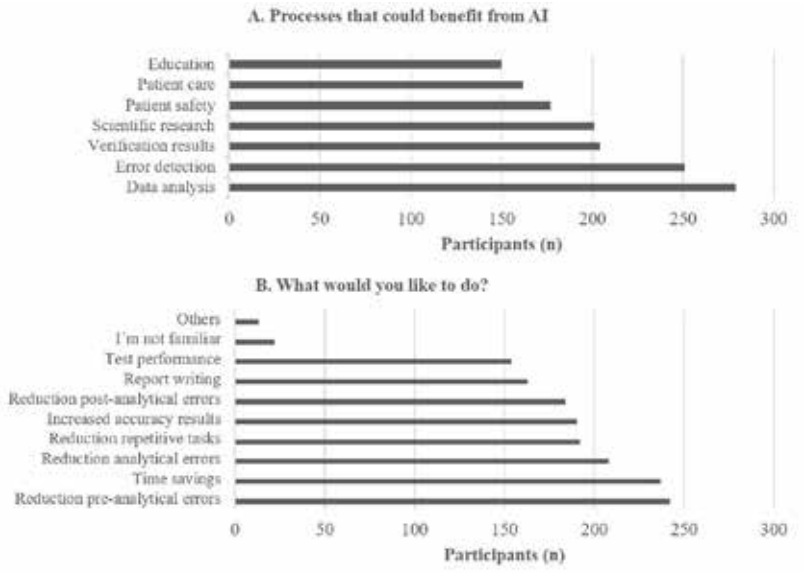
**Table 3:** Daily use of AI tools.

| Application | n | % |
|---|---|---|
| MetaAI (WhatsApp) | 238 | 65.7 |
| ChatGPT | 165 | 45.5 |
| Google Lens | 122 | 33.7 |
| Snapchat | 72 | 19.8 |
| Google Bard | 53 | 14.6 |
| Grammarly | 36 | 9.9 |
| Bing | 23 | 6.3 |
| Copy.ai | 12 | 3.3 |
| Perplexity | 10 | 2.7 |
| Scite | 7 | 1.9 |
| Quillbot | 3 | 0.8 |
| Tome | 1 | 0.2 |
| DeepSeek | 1 | 0.2 |
| Others | 43 | 12.5 |
| I don't know or use any | 49 | 13.5 |

**Benefits of AI in the laboratory**

Overall, 78% (n=283) of the subjects were sure that AI was used for laboratory processes, while 19% (n=70) reported that this may be the case and 2% (n=9) were not sure. According to the participants, the three types of tasks for which AI could be most beneficial were data analysis, error detection, and verification and presentation of results. Meanwhile, the three purposes for which the professionals would most like to use AI in CLs were reduce errors in pre-analytical stage, to save time, and to reduce errors in the preanalytical and analytical stage (Figure 2).

**Figure 2:** Tasks for which AI could be most beneficial and what would you like to do.



**Processes that can be automated**

More than 50% of the participants asserted that AI tools could enable the automation of administrative processes, quality control, repetitive tasks, and numerical data management in CLs (Table 4). Participants with a higher academic degree (Postgraduate training in a CL specialism or postgraduate degree) showed a greater tendency to assert that AI could be used to predict values based on other results ($p<0.00001$). Overall, 75.4% (n=273) of respondents supported the idea of implementing AI tools in the laboratory, and 52.2% (n=189) thought that such implementation should be carried out in the near future. Moreover, those with higher academic degrees also exhibited a greater tendency to think that AI could increase the precision and reliability of the results in CLs ($p=0.0374$ and $p=0.0005$, respectively).

**Table 4:** Processes that could benefit from AI in CLs.

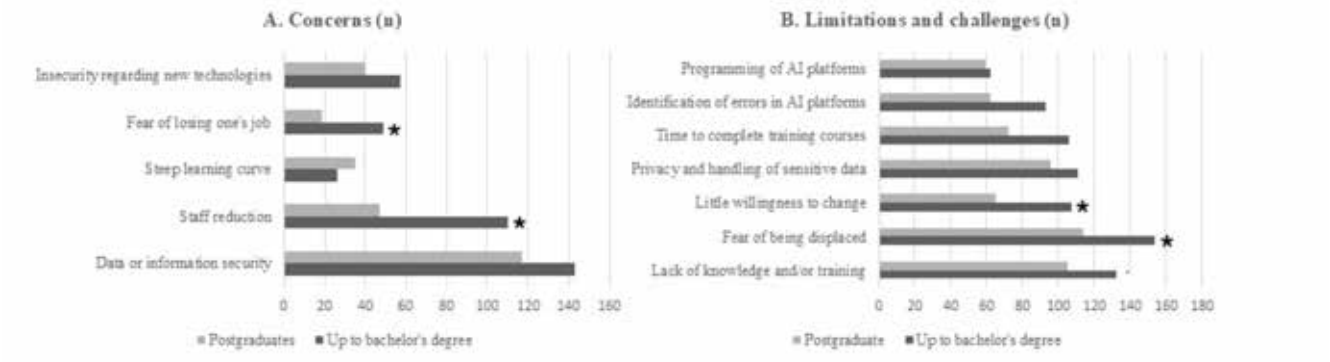
| Processes | n | % |
|---|---|---|
| Administrative processes | 282 | 77.9 |
| Quality control and/or error identification | 251 | 69.3 |
| Repetitive tasks | 210 | 58 |
| Handling numerical data | 200 | 55.2 |
| Workflow | 172 | 47.5 |
| Set custom reference ranges | 163 | 45 |
| Efficiency of diagnostic processes | 157 | 43.3 |
| Accuracy of diagnostic processes | 155 | 42.8 |
| Suggestions for additional testing | 147 | 40.6 |
| Predicting values based on other results | 133 | 36.7 |
| Interpretation of results | 132 | 36.4 |
| Interaction with patients | 132 | 36.4 |

Significantly, 52% of participants considered that AI will replace humans in performing certain tests in CLs, with this proportion not differing depending on the type or size of the laboratory. However, this rate did differ depending on the academic level of the participants, with those with higher educational levels being more likely to hold this opinion ($p=0.0005$).

**Concerns, limitations, and challenges**

Overall, 73.5% of the participants were concerned about their own lack of knowledge or training in the use of AI, 65.7% were afraid of losing their jobs to AI, and 58.2% were concerned about the lack of willingness of CL management to introduce AI tools into the workplace. Regarding ethical aspects, 71.8% were concerned about the security of the data fed into AI tools, and 50% were concerned about privacy and the handling of sensitive data. Three participants expressed concern about who would be held responsible for AI errors. Figure 3 shows the number of responses for each concern or limitation and challenges, and distinguishes the differences according to academic degree.

**Figure 3:** Concerns, Limitations and changes about AI.

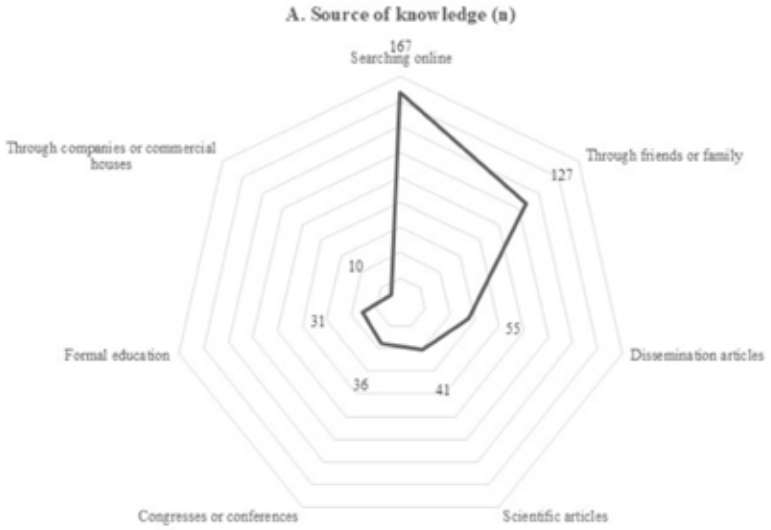



*$p<0.05$

**Formal AI-related education and sources of knowledge about AI**

Only 17 of the participants (4.7%) had received formal training in AI. Figure 4 shows data on how the participants learned about AI. Nonetheless, 84.8% reported that they were willing to be formally trained in AI and were not concerned about the steep learning curve.

**Figure 4:** Sources of AI knowledge among CL professionals.




**Discussion**

To develop public policies that effectively regulate the implementation of AI, and to design optimal training strategies and implement measures that promote its ethical and responsible adoption, there is a need to understand how CL professionals conceptualize or perceive AI. This would in turn improve the quality and accessibility of diagnostic services and benefit patients and the medical community.

AI applications, particularly those based on ML and deep learning (DL), play a crucial role in the pre-analytical, analytical, and post-analytical phases of the testing process in clinical laboratories. Significant advancements have been documented in various fields, including hematology, cytology, histopathology, biochemistry, immunology, microbiology, and urinary sediment analysis. AI applications have also demonstrated success in diagnosing malignant diseases and enabling longitudinal monitoring of biomarkers, allowing for the prediction of patient treatment outcomes [24–29]. Similarly, the utility of convolutional neural networks (CNNs), which utilize three-dimensional data, has been highlighted in the recognition of peripheral blood cell images [30].

A few recent studies have specifically attempted to analyze how CL professionals conceptualize AI, with results from Pakistan, Europe, and the USA [23, 31-33] and how health professionals and hematology students understand AI [34]. The shared objectives of these studies included exploring and analyzing perceptions of AI, conceptualization and/or attitudes toward it, degree of familiarity with AI, formal education in AI-focused courses, and willingness to adopt AI technologies in decision-making processes in diagnosis and treatment, as well as identifying opportunities, concerns, and challenges facing the implementation of AI. With the exception of the work by Jafri et al. [33] in which structured interviews with 13 participants were performed, other studies involved online surveys with between 10 and 36 questions, with our study considering the highest number of aspects.

To the best of our knowledge, this is the first study of CL professionals from public or private institutions on their conceptualization of AI in laboratories in Latin America. It was conducted without external funding and differs from other studies in that it includes professionals at research laboratories in addition to CL staff. Our study also distinguished between those working at micro-, small, medium, and large laboratories, defined according to the number of employees.

This study was based on a previously validated instrument to which questions were added to obtain a broader overview of attitudes towards AI and the status of its implementation in CLs, including sociocultural characteristics and the social context

of the Mexican population. This constitutes a major strength of our study, as well as having included different categories of operational and administrative CL-related work. The survey was self-administered, anonymous, and electronically distributed, minimizing interviewer bias (e.g., tone of voice and gestures) and encouraging honest responses, particularly because not address sensitive or personal topics. Nevertheless, a limitation was the lack of control over the response rate, which may have impacted the findings by introducing response bias, reducing statistical power, and limiting the generalizability of the results to a broader population. Despite these limitations, we believe that the variability in profiles among CL workers across the eight geographic regions of Mexico—both in public and private sectors—is not significant enough to compromise the objectives of this exploratory study. We ensured that responses were obtained from all geographic regions (Figure 1).

The limited number of participants can be explained by the large number of questions in the survey, the target group's resistance to evaluation, or concerns among the participants regarding cybersecurity, since most had to use personal electronic devices to complete the survey. Nonetheless, all geographical regions of Mexico were represented in the sample. For comparison, in other similar studies, the analysis was carried out on 13 (Pakistan), 195 (in 34 European countries), 342 (Pakistan), and 1,721 participants (USA) [23, 31-33]. Official Mexican data indicate that women make up more than 55% of the labor force in CLs; they most commonly have a background in chemistry, and 11% of those employed in this sector have a master's or doctorate degree [35]. This contrasts with 28% of participants who had a postgraduate degree in our sample. Furthermore, the majority of respondents worked in private laboratories (56%), where AI might well be more widely used.

The characteristic of being "Somewhat familiar" with AI dominated in all of the studies performed (54% to 64%), including ours. However, this contrasts with the low familiarity with AI specific to CLs, the perceived need for CL staff to receive AI-related courses and training, and concerns about regulation, transparency, and ethics.

When the respondents were asked if they used AI applications on a daily basis and, if so, which ones, the answers were inconsistent. Specifically, those who answered "No" selected several applications, which shows an imprecise understanding of which tools are actually AI-based.

Regarding the first four benefits that professionals would like to pursue if they were to apply AI (save time, perform tests, reduce errors, and reduce repetitive tasks), the comparison with other studies showed that these potential benefits were mentioned at higher rates in Mexico than elsewhere [23,31].

Studies on the perceptions of AI have reported that such perceptions are influenced by cultural, educational, structural, and economic factors [22, 23, 31-33]. In this study, more than half (58.1%) of the participants expressed their own unwillingness to change regarding the adoption of AI, an attitude that should be modified through specific training to take advantage of the precontemplation and contemplation stages they find themselves; and that, according to the Prochaska and DiClemente model, are phases that a person needs to overcome in a change process [22].

The results of this study show that in Mexico there is a need to promote the effective adoption of AI in CLs and to implement education and training programs on AI at certified centers that guarantee ethical use, transparency, and security in the use of AI algorithms. It is also necessary to design public policies and regulations that promote the effective integration of AI tools in CLs, without excluding certain social groups, in adherence with best practices and the common good [36-38]. There is a need for Mexican CLs to obtain specific national and international accreditations to operate AI technologies, including those with or without ML. In CLs, decisions will have to be made about the type of technology and specific applications to use according to the identified needs [20,39].

**Opportunities in Education**

According to the responses provided by our sample, and as reported by Cadamuro et al. [32] and Jafri et al. [33], there is a need to implement regulation and to standardize ethics and norms for AI via training courses in Mexico, since only 4.7% of our respondents had received a formal education in AI. AI is not currently included in the syllabuses of healthcare-related undergraduate courses in Mexico; as such, formal education on this topic should be initiated, accompanied by modification of the syllabuses of healthcare-related courses and incorporation of continuous professional development courses for professionals aligned with international standards such as ISO 15189, the official standard for medical laboratories, setting out the requirements for quality and competence [40]. In addition, certification that ensures that CL staff fully understand the functioning of AI tools and correctly interpret the results should be actively pursued. The gap between end users and developers identified in medicine could be narrowed via the contributions and participation of CL professionals and other healthcare professionals, with experience and formal education in AI for the implementation and adaptation of these technologies in specific clinical contexts [41].

**Ethical challenges**

The rapid advance in the use of AI in the healthcare sector is accompanied by significant ethical challenges, including concerns about reliability, transparency, bias, and data privacy, as well as the risk of AI replacing CL professionals, as identified in this study. It has been proposed that assuaging these concerns and mitigating these risks requires a proactive approach, including determining the minimum data required for a specific purpose and limiting collection to what is strictly necessary, obtaining informed consent from patients for AI tools to use their data through clear communication, anonymizing personal data, incorporating privacy considerations into AI design ("privacy by design"), ensuring transparency in AI

decision-making, regularly monitoring AI-related practices, and evaluating data models for emerging risks. From an ethical perspective, it is particularly important to ensure that rather than replacing humans, AI should complement their expertise and enhance their work in professional practice [42].

In the field of AI, the term "integrated ethics" refers to the ongoing practice of prioritizing ethics in the entire AI development process in a collaborative and interdisciplinary manner. This involves the systematic promotion of explicit and robust normative analysis and the use of ethical reasoning to justify or question a particular position or course of action [43]. Among the concerns associated with the use of AI in the healthcare field are regulations, meta-ethics, epistemology, medical practice, medico-legal concerns, the need to uphold patients' and physicians' rights, and the potential risks posed by predictive analytics [44], which must be explicitly considered in initiatives to legislate on this topic.

**Conclusions and perspectives**

Based on the findings of the present study and given the rapid development of AI in this sector, the appropriate incorporation and utilization of AI must involve the implementation of ex post surveillance systems to detect problems or errors in its daily use. There is also a need for the reporting of adverse events related to AI tools, protocols that identify and correct biases in the data on which algorithms are trained, and validation that AI tools are effective for diverse populations and do not generate healthcare inequalities in diagnosis or treatment.

It is also necessary to propose and ratify complementary laws and regulations that establish an appropriate framework to guide the development, implementation, and use of AI in CLs; to promote collaboration between academic institutions and industry; to develop regulations that can adapt to technological advances; and to participate in global initiatives to standardize the regulation of AI in the healthcare sector and promote the exchange of best practices and knowledge internationally. Moreover, there is a need to create procedures for clearly establishing where responsibility lies (e.g., with the AI tool developer, with the laboratory, or with the professionals who operate the system) in cases of diagnostic errors or problems arising from the use of AI in CLs.

The findings of this study can serve as a basis for defining opportunities and priority areas to promote public policies that encourage the adoption of AI in the CL sector, along with the implementation of appropriate training strategies. This work can also help foster the more extensive, regulated, ethical, and effective implementation of these technologies, with the aim of improving the quality and accessibility of services and avoiding the exclusion of particular social groups from accessing the most accurate possible clinical diagnosis.

This initial exploration of the conceptualization of AI among CL professionals in Mexico highlights an opportunity for further research. Future studies should focus on validating AI tools within specific CL contexts and employing different study designs (e.g., longitudinal or qualitative approaches) to assess the adoption and acceptance of AI over time. Additionally, future research could analyze the experiences of professionals in specific laboratory areas and facilitate cross-national comparisons to better understand the broader impact of AI in CL settings.

This should help to improve the diagnosis and prognosis of diseases and promote technological development for the benefit of patients while considering the needs and wishes of clinical doctors who order laboratory analyses, generating continuous communication among doctors, patients, and laboratory staff.

Finally, the results of this study highlight the need to implement AI more intensively in CLs in Mexico and an interest in resolving the main limitations and challenges obstructing its implementation. We conclude by stating that rather than fearing being replaced, those who are trained in the use of regulated, standardized AI, along with robust and transparent ethics, should have better opportunities for ongoing professional development in this field.

**Declaration of Conflict of interests**

The authors declare that they have no material or financial conflicts of interest relevant to the research described in this article.

**Ethical Approval**

The study was conducted with the ethical principles for medical research involving human subjects, in accordance with the Declaration of Helsinki (Oct., 2024 revision) and approved by the Ethics Committee of the National Institute of Learning, Skills, and Research in Sciences, SC (Registration number CIE-INHAIC-2024-013).

**CRediT author statement**

The contributing roles of all authors in the research, review and editing of this manuscript are named:

JMSG: Conceptualization, Methodology, Validation, Investigation, Data Curation, Visualization, Supervision, Project administration, Writing - Original Draft, Writing - Review and Editing.

MCMM: Conceptualization, Methodology, Validation, Investigation, Data Curation, Visualization, Supervision, Writing - Original Draft, Writing - Review and Editing.

AERC: Validation, Formal analysis, Data Curation, Visualization, Writing - Review and Editing.

EJRB: Validation, Formal analysis, Data Curation, Visualization, Writing - Review and Editing.

RISA: Validation, Writing - Review and Editing.

JHPG: Writing - Review and Editing.

MLR: Writing - Review and Editing

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Research Article

# From establishment to ISO15189:2012 Accreditation: the case of Hararghe Health Research Laboratory, Harar, Ethiopia

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**Abstract**

**Background**: Accreditation of laboratories offering diagnostic services improves the operation of clinical as well as research performance.

**Objective**: This case report describes the journey of Hararghe Health Research Laboratory from it's inception to the International Organization for Standardization 15189:2012 accreditation by the Ethiopian Accreditation Service.

**Methods**: An external consultant conducted a baseline audit in November 2019 following the World Health Organization African Region's Stepwise Laboratory Quality Improvement Process Towards Accreditation guideline. The follow-up internal audit was conducted in January 2021. Then, an on-site laboratory assessment was conducted by experts from Ethiopian Accreditation Service towards the end of 2022.

**Findings**: The Hararghe Health Research laboratory received multiple remarks during audit by external consultant and drew up a corrective action plan. Some of the actions were revision of quality policy manual, managerial and technical documents, participation in the United Kingdom National External Quality Assessment Scheme and implementation of the International Organization for Standardization 15189:2012 accreditation checklist. The internal audit revealed a total of 26 gaps in the microbiology and 16 in the molecular biology sections and these were filled by the end of April 2022.The laboratory was cited for nine minor non-conformities during an assessment by experts from the Ethiopian Accreditation Service. The laboratory developed a corrective action plan, cleared non-conformities by end of February 2023 and received the accreditation certificate on 3rd May 2023. The laboratory's accreditation achievement in less than five years is a significant milestone and serves as a model for other institutions to achieve it in a similar time frame.

**Background**

The quality of healthcare is directly influenced by the quality of laboratory services [1, 2]. Laboratory test results often provide information for clinical decision-makers which influence 60-70% of medical diagnoses and patient management [1-4]. To guarantee that medical testing and diagnostic laboratory tests are of the highest quality, it is crucial to have them accredited by recognized accreditation authorities [2, 5]. Accreditation requires the establishment of a quality management system (QMS) [6].

Laboratory accreditation is a voluntary scheme. It is a formal recognition by a third-party expert of a laboratory's credibility and competency in testing services. It also encourages the laboratories to enhance/motivate the management system, technical capability, and competitiveness of laboratory personnel. Continuous improvement and dedicated staff are essential for maintaining quality assurance in an accredited medical laboratory [2].

The International Organization for Standardization (ISO) 15189:2012 since updated to ISO15189:2022, is an international comprehensive standard, which covers management and technical requirements, for the accreditation of medical laboratories [7]. It is used by medical laboratories to develope their QMS and assess their competence. It can also be used to confirm or recognize the competence of medical laboratories by laboratory customers, regulatory authorities, and accreditation bodies [8-11]. Medical laboratories meeting the requirements of the accrediting body will apply for the accreditation assessment. After that, the accreditation body will review the documents and then perform an on-site audit of the applicant's laboratory [2, 5]. Each country may have its own national accreditation body (NAB) which is responsible for granting accreditation. The NAB operates according to ISO 17011, the international standard for accreditation bodies [12]. The Ethiopian Accreditation Service (EAS) Formerly Ethiopian National Accreditation Office (ENAO) provides accreditation services to laboratories, certification bodies, and inspection bodies, that operate both within the Federal Democratic Republic of Ethiopia and outside its borders in countries where either no national accreditation body exists or a national body cannot accredit in a specific field [13] The EAS achieved signatory status in the African Accreditation Cooperation (AFRAC) Mutual Recognition Arrangement (MRA) in May 2017 in testing to International Organization for Standardization/ International Electrotechnical Commission (ISO/IEC) 17025 Testing, and Medical Testing to ISO 15189:2012. In addition, the EAS is a signatory to the International Laboratory Accreditation Cooperation (ILAC) Mutual Recognition Arrangement (ILAC MRA) [14, 15]. This report summarizes the journey of Hararghe Health Research Laboratory (HHRL) from its establishment to achieving ISO15189:2012 accreditation in Microbioilogy testing.

**Description of Setting**

There was an initial funding from the Bill and Melinda Gates Foundation through Emory University to the London School of Hygiene and Tropical Medicine (LHSTM) and Haramaya University (HU) to establish a greenfield site for the Child Health and Mortality Prevention Surveillance (CHAMPS), Ethiopia [16]. The LSHTM and HU established the Hararghe Health Research Partnership (HHRP) as a collaborative program for the advancement of health sciences research through strengthening research and surveillance capacity, ensuring best research practices, and informing health policy in maternal and child health in Ethiopia. The program is domiciled at the College of Health and Medical Sciences of Haramaya University, Harar Ethiopia. A student's demonstration and teaching laboratory was allocated to the program and the renovation, refurbishment, and equipping of the laboratory named Hararghe Health Research Laboratory (HHRL) was carried out from May 2017 to December 2018. In February 2019, the laboratory started accepting and processing samples of Minimally Invasive Tissue Sampling (MITS) for isolation and identification of bacterial pathogens, and molecular detection of targeted bacterial, viral, parasites, and fungal pathogens using Real-Time PCR TaqMan Array Cards. Other research protocols such as Maternal Infections Study (MIS), Acute Febrile Illness (AFI), Mortality due to Bacterial Infections Resistant to Antibiotics (MBIRA), Epidemiolocal surveillance of anti-SARS-COV-2 antibody, Invasive Bacterial Diseases (IBD), pneumococcal and meningococcal carriage survey also came onboard.

The HHRL is well-equipped with high-tech laboratory equipment such as EZ1 adavanced XL and EZ2 connect (QIAGEN/ Japan) , QuantStudioTM 7 Flex real-time polymerase chain reaction system (Applied Biosystems/Singapore) used to run TaqMan Array Cards (Applied Biosystems/USA), including 96 and 384 well plates, GeneXpert (Cephied/USA), BACT/ALERT® 3D Microbial Detection Systems (BioMérieux/USA), MicroScan WalkAway bacterial identification and antibiotic susceptibility testing system (Beckman Coulter/USA), aerobic (Genlab,UK) and $CO_2$ (BINDER,Germany) incubators, biosafety level 2 cabinets (CAS,UK), tissue processors (Leica, Germany ), tissue slide scanner (Leica,USA), refrigerators (Lec MEDICAL, UK), ultra-low temperature freezers (Haier,China), ELISA (Biotek 50 TS microplate washer and BioTek 800TS Microplate reader) and other ancillary equipment. The operation as well as the biosafety and biosecurity of the laboratory are monitored and controlled through a closed-circuit television (CCTV) surveillance system, indoor climate control, smoke detection system, and access control systems. The laboratory is also connected to the main national electric utility through an automatic 40-kilovolt (KV) generator and uninterrupted power supply backup providing three layers of redundancy.

The laboratory recruited and has been able to retain medical microbiologists, and medical laboratory technologists. As a public service, it supports public health by identifying and reporting pathogens of public health significance. It was one of

the first laboratories selected by the Federal Ministry of Health and Ethiopian Public Health Institutes to test and provide training to testing laboratories in eastern Ethiopia during the COVID-19 pandemic. The laboratory analyzed 34,647 nasopharyngeal samples collected from eastern and western Hararghe, Dire Dawa Administration, and Somali regional states in Eastern Ethiopia. Additionally, the laboratory performs blood and cerebrospinal fluid cultures, identification and antimicrobial susceptibility testing of bacterial pathogens isolated from severely ill children admitted to the neonatal and pediatric intensive care units at Hiwot Fana Comprehensive Specialized Hospital.

**Accreditation Preparation Processes**

**Baseline assessment by external consultants**

The first step was the international competitive hiring of a Laboratory Director as the team leader who then led the hiring of the technical staff and then securing management and staff support for the accreditation process. The HHRP management was very clear about the need for a laboratory accredited to internationally accepted standards.. After this unambiguous set objective from management, the laboratory leadership then started putting in place the pillars that were necessary for the journey to accreditation. The second step was to perform a gap analysis and then subsequent ongoing monitoring through internal and external evaluation using the ISO 15189:2012 accreditation checklist [17]. The initial assessment was conducted in November 2019, by external assessors from Kenya and Nigeria, experienced in auditing medical laboratories ISO 15189:2012 and World Health Organization African Region's Stepwise Laboratory Quality Improvement Process Towards Accreditation considering management and technical processes requirements. The WHO-AFRO/SLIPTA program is used in resource-limited countries as a tool for implementing QMS [18, 19]. The team assessed by observing and reviewing documentation for sample reception and registration, general store, staff room, laboratory equipment, cleaning rooms, molecular, microbiology, and data entry sections..

The external assessment team identified areas for improvement which included: the need for laboratory staff to train on the ISO 15189:2012 standard, laboratory quality management systems and on different documents/blank forms development, internal auditing, root cause analysis, and corrective and preventive action (CAPA). The baseline score was 94/265 (35.5%) (0 stars) (Figure 3). Following the external evaluation, the laboratry strived to meet ISO 15189:2012 accreditation criteria. The WHO-AFRO SLIPTA and ISO 15189:2012 checklists are nearly similar . The ISO 15189:2012 checklist evaluated each question as either conforming or nonconforming, whereas the WHO-AFRO SLIPTA checklist evaluated each question as conformance, partial conformance, and nonconformance and made a step-by-step accreditation process from star 1 to star 5 [20, 21]. The Laboratory converted all the content and structure of SOPs to ISO 15189:2012 standards and used them for the accreditation process.

Following the hiring of a highly skilled and knowledgeable Quality Assurance /Quality Control (QA/QC) officer, the HHRL started creating management, technical, and procedural documents. The HHRL also registered for External Quality Assessment (EQA) with the United Kingdom National External Quality Assessment Scheme (UK NEQAS) for the test types conducted in the laboratory. The staff were also trained on good clinical laboratory practice (GCLP) by certified external trainers.

**Process improvement**

The following documents and blank forms were prepared between January 2021 and November 2022 and put into use after the HHRL director reviewed and approved them.

**1. Quality policy manual**

Using the document details the structure of the HHRL and its quality system policies, processes, and operating standard procedures for all the HHRL staff to carry out their job and related activities to achieve the mission of the organization/ laboratory. The QMS is described in the Quality Policy Manual. It also contains basic and simple procedures and referrals to supportive documents. The laboratory revised the quality policy manual developed in 2019 to guide the implementation of the QMS as per ISO 15189:2012 requirements.

**2. Managerial and technical requirements documents and blank form preparation**

The laboratory developed 16 standard operating procedures and 3 manuals (quality policy, Safety and laboratory client checklists) in 2019. Revision of the previously developed documents and preparation of additional ISO 15189:2012 laboratory management (4.1- 4.15) and technical processes (5.1 - 5.10) QMS [7] document and blank forms were done between January 2021 and November 2022. A total of 139 SOPs, 3 manuals, and 154 documents and blank forms were developed by the end of February 2023.

**Service maintenance and calibration of equipment**

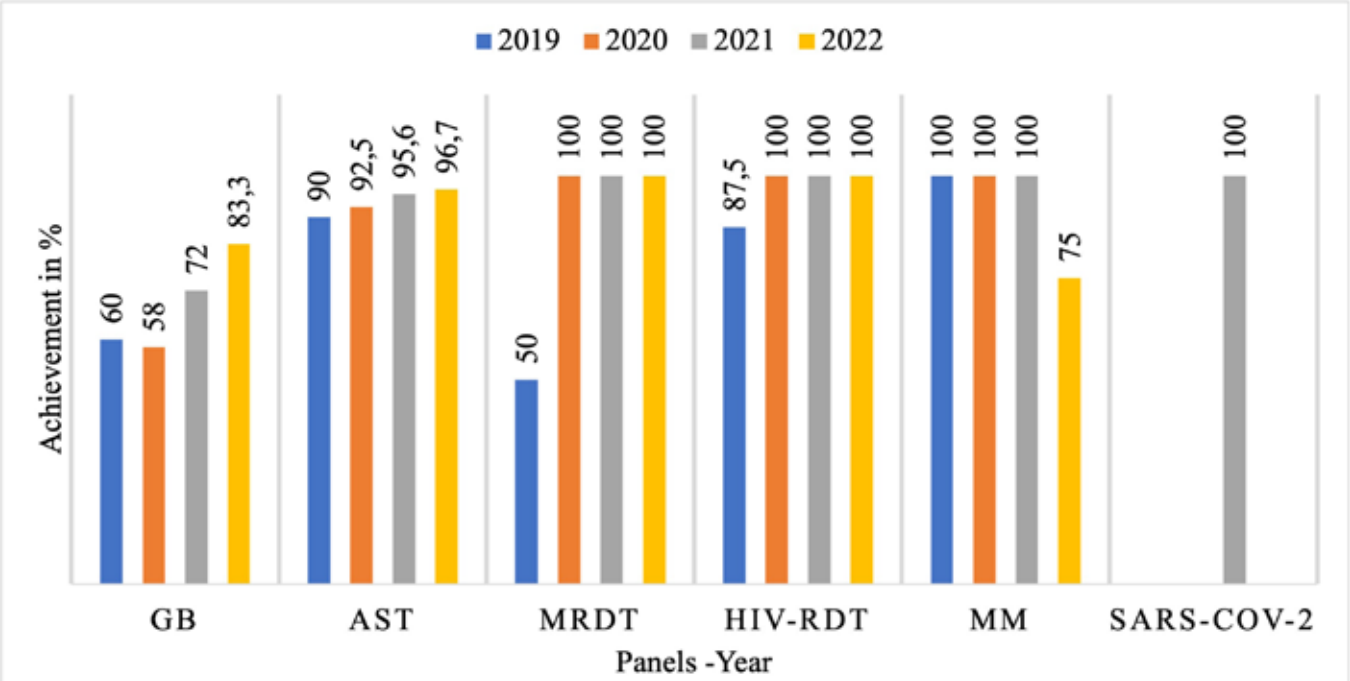
Metrological traceability is the property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty [22]. The International Laboratory Accreditation Cooperation (ILAC) and its associate member, the Ethiopian Accreditation Service (EAS) have a mandatory requirement on metrological traceability, i.e., medical laboratories are required to have an established calibration program for critical equipment that directly or indirectly affects examination results [23, 24]. The HHRL outsourced the calibration of major equipment by experts from traceable organizations in Ethiopia like the National Metrology Institute of Ethiopia (NMIE), and other regional companies for each of the various equipment like biosafety cabinets, pipettes, ultra-low temperature freezers, refrigerators, incubators, weighing balances, thermometers and water-baths. More than 50 pieces of equipment were serviced

and calibrated in the period 2021-2022.

**Proficiency testing**

Accredited test methods must be validated. Personnel, instruments, reagents, and other factors affecting the test results must be checked. The laboratory must prove the correctness of the test results. Proficiency testing programs' test results must be ready for evaluation by assessors [2]. In early 2019, the HHRL enrolled and has been participating in 6 United Kingdom National External Quality Assessment Scheme (UK NEQAS) proficiency testing panels including general bacteriology, antimicrobial susceptibility testing, malaria rapid test using First response malaria parasite lactate dehydrogenase (pLDH) (PF/HRP2)(OBELIS S.A./Belgium) , HIV rapid test using HIV ½ STATPAC (CHEMBIO Diagnostic System inc /USA), molecular detection of Mycobacterium tuberculosis (Xpert® MTB/RIF Ultra Cepheid/Sweden) and molecular detection of SARS-CoV-2 using TaqMan Array Cards(Applied Biosystems/ USA) . The HHRL quality policy manual defines the attainment of at least 80% or an equivalent score in EQA in each panel as verification of personnel competence and appropriateness of methods used. The laboratory made a commitment in its QMS quality plans for the achievement of these objectives to meet the customer and ISO 15189:2012 requirements. The change processes are implemented in a manner that will not compromise these requirements [25]. The progress in EQA participation of HHRL is shown in Figure 1 below.

**Figure 1:** The UK NEQAS performance of HHRL for the period 2019 to 2022.




GB: General Bacteriology ; AST: Antimicrobial Susceptibility Test; MRDT: Malaria Rapid Test; HIV-RDT: HIV rapid test; MM: Mycobacterium molecular; SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2

Note: The laboratory did not participate in SARS COV 2 testing after 2021

**Training and implementation of the ISO 15189:2012 documents and records**

Laboratory staff competence and understanding of the rationale behind accreditation are crucial for effective compliance with ISO 15189:2012 [10, 26]. Accordingly, the HHRL designed a QMS based on its quality policy manual and ISO 15189:2012 standard. The implementation of QMS would result in a well-organized structure, smooth work relations, and efficient services. The training of staff between October 2021 and February 2022 ensured the full and successful implementation of the system. The staff received training both in-person and online. The first face-to-face training covered all Standard Operating Procedures (SOPs), management and technical requirements, and manuals. It was provided by experienced senior staff in the laboratory who have training certificates. The other face-to-face training, which covered guidelines for auditing management systems, ISO 19011:2018 and Ethics, and ISO 15189:2012, was provided by a consulting company from Kenya. All staff received both free and purchased online training on Good Clinical Laboratory Practice (GCLP), Saf -T pack for Shipping Infectious Substances and Related Materials, Bloodborne Pathogens, Firefighting, Ergonomics, and Ethics.

**Internal audit and action plan**

The initial gap analysis and then the continuous monitoring through internal assessment provided invaluable tools for root cause analysis, corrective and preventative actions, and the eventual successful accreditation to ISO 15189:2012 [27]. The internal audit was conducted in January 2021 using the 15189:2012 checklist. A total of 26 gaps in microbiology and 16 in molecular biology sections were identified. The non-conformity reduced from 64.5% during baseline assessment by an external consultant to 9.8% (Figure 2). An action plan was developed after the internal audit and a final correction completion date of April 2021 was put in place. The progress, challenges, and suggested solutions in the accreditation process were discussed in the weekly laboratory staff meetings and implemented.

The sustainability of the implementation of QMS was routinely monitored via selected quality indicators ensuring the continuous regulation of the entire laboratory process from the pre-analytical, analytical, and post-analytical phases.The Laboratory management draws up annual quality objectives in line with its policy statement. The quality objectives are reviewed and revised during the annual management review meeting to ensure their suitability. The Turnaround times (TAT) to generate at least 85% test results within the set TAT, External Quality Assessments to attain at least 80% or equivalent score, and customer satisfaction levels, to maintain above 80% based on regular feedback, are the current quality objectives for the laboratory in 2023/2024 calendar year.

The HHRL recognizes that any feedback from service users is vitally important to achieving continual quality improvement of the diagnostic services. To monitor user experiences and satisfaction levels the HHRL undertakes regular user satisfaction surveys annually. This is done through the distribution of questionnaires to customers which contains courtesy staff, TAT, errors corrected promptly, professionalism of staff, reporting of critical results, service conformance with requirements, and past complaints if applicable . The customer concerns are evaluated and provided to the management for remedial actions. The customer satisfaction was 84% and 86% in 2021 and 2022, respectively. General positive feedback was provided by customers regarding the services provided by the HHRL. The customers gave negative feedback about delays in results reporting and communication. This was solved through awareness creation and distribution of the laboratory client handbook to customers, particularly health-care personnel. The customers were encouraged to refer to the handbook for information on the nature and time required for processing and reporting microbiology and molecular biology samples..

**Submission of Application Document, Assessment and Accreditation**

**Phase 1: Scope of accreditation and documents review**

It is a requirement for the candidate laboratory to determine the 'Scope of Accreditation' and the areas and names of tests, which will be specifically examined by expert assessors [2]. Our laboratory requested to be accredited for bacteriological culture tests and molecular detection of pathogens using TaqMan Array cards. These were based on the clinical and research testing requirements of the HHRP.

In April of 2022, the HHRL made a formal accreditation application to the EAS and submitted the following documents: the quality policy manual, managerial and technical requirement procedures, checklists, forms, proficiency test (PT) participation plan and recent results, a summary of internal audit, non-conformity clearance audit report, laboratory legal entity and risk assessment and mitigation reports. The EAS reviewed the documents and provided feedback, which helped us prepare for the physical in-person audit.

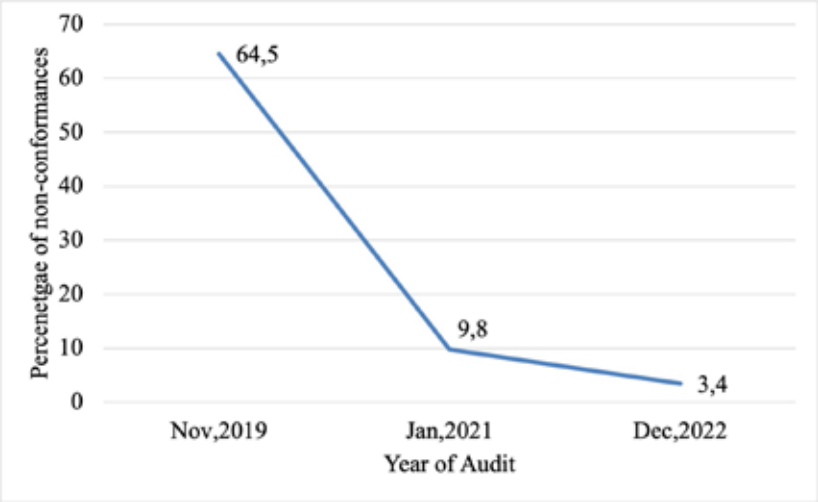
**Phase 2: Accrediting body assessment physical visit**

The ISO 15189:2012 accreditation involves an independent third-party assessment of the medical laboratory that includes an examination of personnel qualifications and competence, equipment, reagents, and supplies, quality assurance, pre-analytical, analytical, and post-analytical factors. Qualified assessors perform an in-depth evaluation of all factors that influence the production of test data [28].

An on-site assessment of the HHRL was conducted by EAS from November 30th to December 3rd , 2022. Three assessors, comprising a technical expert, a managerial expert, and an observer were assigned by EAS. The assessment process started with an opening meeting with laboratory staff to provide information about the objective and activities of the assessment process. After visiting the laboratory, the assessors began the assessment process. They verified the implementation of all technical and managerial documents in the submitted application to ensure their availability and utilization by the laboratory staff. The assessors reviewed the workflow process, including sample reception, processing, result dispatch, and waste disposal, using laboratory request forms that were previously reported. Additionally, the assessors also verified the competence of the laboratory director, and QA/QC officer on management and the laboratory staff/technical signatories through observance performing tests according to approved SOPs and reviewing personnel files for evidence of required staff training.

The assessors found nine minor non-conformances, and the laboratory was given four months to clear these non-conformances. The reduction of non-conformity between the initial assessment in 2019 and the final audit in 2022 showed significant improvement (Figure 2).

**Figure 2:** The percentage of non-conformances by year of audit.




**Phase 3: Non-conformities clearance , action plan development and accreditation.**

The non-conformites were reviewed by the laboratory director and an action plan was developed. The HHR laboratory team worked tirelessly to clear all the cited non-conformities by March 2023 and submitted a report with accompanying documents to EAS. The laboratory was accredited for medical microbiology on May 3rd, 2023. The EAS did not have the capacity to review and accredit the molecular testing section of our laboratory. The sustainability of the accreditation of the laboratory is maintained through active participation of staff in detecting and presenting non-conformities, in the weekly staff and monthly quality improvement (QI) meetings followed by prompt rectification. Figures 3 and 4 show the summary process followed by HHRL towards being accredited by EAS and the accreditation certificate.

**Figure 3:** Summary process followed by Hararghe Health Research Laboratory for accreditation from October 2021 and February 2023.


**Figure 4**: Accreditation certificate awarded to Hararghe Health Research Laboratory by Ethiopian Accreditation Service.

ETHIOPIAN ACCREDITATION SERVICE

ACCREDITATION CERTIFICATE

Hararghe Health Research Laboratory

Harar, Ethiopia

Facility Accreditation No: M3075

is accredited by the Ethiopian Accreditation Service (EAS) to perform tests in accordance with the attached *Scope of Accreditation* in the field of

Medical Testing

The facility is accredited in accordance with the requirements of ISO/IEC 15189:2012, General requirements for the competence of Medical laboratories. The accreditation demonstrates technical competence for a defined scope and the operation of a laboratory quality management system. While this certificate remains valid, the Accredited Facility named above is authorized to use the Combined ILAC-EAS accreditation mark/symbol to issue test reports and/or certificates.

Effective Date: 29 December 2023

Certificate Expires: 2 November 2027

Mr. Bonsa Bayissa
Director General
Ethiopian Accreditation Service

**Benefits accruing to the HHRL due to this accreditation**

The HHR laboratory is reportedly one of only five microbiology-accredited laboratories in Ethiopia, and this accreditation process has helped the HHRL in the standardization and traceability of its test processes which continues to contribute to improvement in the quality of laboratory services and patient care. The accreditation showed a significant improvement in the motivation and job satisfaction of laboratory staff, as evidenced by their bench work and weekly and monthly quality improvement meetings/discussions. The laboratory's accreditation has led to increased visibility at both national and international levels, establishing it as a reputable research center of excellence. This recognition has enabled the laboratory to attract research grants and protocols, allowing them to address community health issues in eastern Ethiopia. The data collected from these efforts will provide valuable insights to both the national and international public health communities, aiding in informed decision-making.

**Challenges, limitations and solutions**

The adoption and utilization of different documents, records, or quality standards was costly and labor-intensive [29]. The HHRL had challenges with the calibration and maintenance of laboratory equipment because of the limited availability of qualified biomedical engineers, spare parts, and traceability of organizations in Ethiopia. This challenge was overcome by utilizing the services of companies external to Ethiopia. The other challenge was low staff commitment and cooperation on the implementation of the ISO 15189:2012 standard, but this was solved through continuous training, continuous awareness creation, motivation, mentoring, and supervision. Another challenge was the difficulty of HHRL staff in accessing Continuous Professional Development programs. This was resolved by conducting training by using experienced and certified senior staff, procurement of online training and invited trainers from outside Ethiopia. The other limitation was the non assessment of the molecular testing section due to the absence of technical ability by the EAS at the time of the assessment.

**Discussion**

The accreditation of clinical laboratories enhances laboratory processes by reducing errors in the pre-analytical, analytical, and post-analytical phases, aiding in accurate and prompt diagnostics, supporting faster and more effective treatments, and encouraging ongoing improvement efforts [5, 30].

The ISO 15189:2012 serves as a universal benchmark for accrediting medical laboratories.[7]. Adherence to this standard showcases laboratories' capability to consistently deliver top-tier service, resulting in enhanced patient safety and better clinical outcomes[8-11].

The initial gap analysis and continuous monitoring, both through internal and external assessment, provided us with invaluable tools for successful accreditation [17]. This approach significantly reduced the accreditation process timeline. The World Health Organization (WHO) introduced the Strengthening Laboratory Management Towards Accreditation (SLMTA) and Stepwise Laboratory Quality Improvement Process Towards Accreditation (SLIPTA) programs to facilitate working towards accreditation in African medical laboratories. Under this scheme, the laboratories gradually go through an assessment process, starting from 0 stars and working their way up to 5 stars, before applying for the ISO 15189:2012 accreditation [31, 32]. Due to the availability of resources and strong management commitment, we were able to shorten the time required to complete these stages.

Effective implementation of accreditation standards and day-to-day compliance with ISO 15189:2012 requirements are significant challenges. It demands effort, active involvement, and commitment from all levels of laboratory staff and management. Maintaining full conformity to the specified objectives within time and cost constraints is crucial [10, 26, 33, 34]. To ensure the smooth implementation of the processes, the HHR partnership and laboratory management have demonstrated their commitment by monitoring and implementing the ISO 15189:2012 standard through the provision of necessary human and equipment resources and vital support.

Even though the laboratory experienced significant improvements in the quality of performance throughout the entire process, it faced challenges in obtaining in-country services for calibration and maintenance of equipment, as well as staff continuous professional development. These challenges are not unique to this laboratory, as others in Ethiopia and other countries have also faced similar issues during the accreditation process [29, 35, 36]. Consequently, these challenges resulted in extended equipment downtime, service interruptions, and delays in service delivery, leading to an increase in complaints from clinicians and customers [29]. However, thanks to the HHR partnership and laboratory management's remarkable efforts, the laboratory was able to sustain its services and achieve ISO 15189:2012 accreditation.

**Conclusion**

The Hararghe Health Research Laboratory successfully completed the ISO 15189:2012 accreditation process in just 36 calendar months, which is a relatively short time frame compared to similar or larger institutions. This achievement was made possible due to the strong support of management and the dedication of the staff. The accreditation serves as evidence that it is indeed possible to establish and accredit a greenfield laboratory within a relatively short time. This publication provides valuable insights for medical and research laboratories, as well as federal, local health, and other related stakeholders who are working towards accreditation.

**Lessons learned, recommendation and working to align with ISO15189:2022 requirements**

**Lessons learned**

Most laboratories rely on mentors from their baseline assessment

until they receive an accreditation certificate. However, HHRL did not have a mentor because it used senior staff members who have extensive experience in ISO 151189:2012 and the College of American Pathologists accreditation schemes. The laboratory management demonstrated readiness and commitment by identifying gaps through internal and external audits and resolving them through a development action plan. Another valuable lesson we learned was the importance of utilizing available human resources, both domestically and internationally, to enhance the capacity of our staff, calibration, and maintenance of equipment. Lastly, we evidenced the fact that the accreditation process necessitates willingness, financial support, and material resources from the management of the facility.

**Recommendation**

Laboratories should view both internal and external audits as valuable opportunities to address non-conformities, strengthen their systems, and ensure optimal efficiency and effectiveness in their operations. Therefore, they should actively participate in the auditing process and use it to identify areas for improvement. Additionally, this practice highlights the importance of involving facility management in the laboratory accreditation process, emphasizing the need for dedicated financial resources, supplies, and effective leadership. Laboratories should also evaluate their internal capacity and actively seek external opportunities for their accreditation process.

**Working to align with ISO15189:2022 requirements**

The laboratory management has received the revised and updated iso15189:2022 documents and we are in the process of performing gap analysis and aligment with the new requirements which we have set a timeline to be completed by May of 2025.

**Author contributions**

ZT conceptualization and design, write up the manuscript. DM, DB, MB: data extraction, write-up, review, and editing manuscripts. MD, FA, DD, ES, MB and MN: reviewing the manuscript. JO: conceptualization, critical revision, and editing of the manuscript. LM and NA: critical revision and editing of the manuscript. All authors read and approved the submitted version manuscript.

**Conflict of Interest**

The authors didn't have any conflict of interest.

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Research Article

# Monoclonal gammopathy presenting with pseudo biclonal pattern in serum protein electrophoresis – An interesting perspective of case series

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**Keywords**

Monoclonal gammopathy, blood protein electrophoresis, Multiple myeloma, M protein, 2D Immunoelectrophoresis, Immunoglobulins

**Abstract**

**Introduction**: Monoclonal gammopathy (MG), arising from aberrant clonal proliferation of plasma cells, is diagnosed through the identification of an M-band via serum protein electrophoresis (SPEP), subsequently confirmed by immunofixation electrophoresis (IFE). The presence of two M-bands in SPEP is designated as double or biclonal gammopathy. Conversely, a pseudo-biclonal pattern is characterized by two M-bands on SPEP that resolve into a single immunoglobulin clone upon IFE. This case series delineates three instances of pseudo-biclonal patterns observed on SPEP in the absence of true biclonality.

**Methodology**: Following ethical approval, a retrospective analysis was conducted on SPEP reports from Sri Ramachandra Medical College & Research Institute, Chennai, spanning 2022 to 2024. Capillary electrophoresis was employed for SPEP, followed by immunofixation.

**Results**: Three cases exhibiting pseudo-biclonal patterns were identified. Case 1 involved a 73-year-old male with acute kidney injury, hypercalcemia, osteopenia, multiple fractures, and a reversed albumin/globulin ratio, where a biclonal pattern on SPEP resolved to IgA Lambda on IFE. Case 2 concerned a 57-year-old male with nodal marginal lymphoma evaluated for myeloma, whose SPEP biclonal pattern resolved to IgM Kappa with elevated polyclonal immunoglobulins on IFE. Case 3 was an 86-year-old female with acute-on-chronic kidney disease and urosepsis, where SPEP showed a biclonal pattern resolving to IgG Lambda and an additional Lambda isomer on IFE.

**Conclusion**: This case series highlights pseudo-biclonal patterns stemming from monoclonal gammopathy, polyclonal elevations secondary to tumor lysis syndrome, and double gammopathy due to excess free light chains. The integration of SPEP with immunofixation, serum free light chain, and serum immunoglobulin assays enhances the detection of pseudo-biclonal patterns.

## Introduction

Monoclonal gammopathy (MG) arises from the overproduction of immunoglobulins due to the aberrant clonal proliferation of B-lymphocytes or plasma cells. Diagnosis of MG is established by the presence of monoclonal protein (M-protein) in serum or urine. The diagnostic workup for M-protein detection commences with the identification of an M-band on serum protein electrophoresis (SPEP), subsequently followed by serum and urine free light chain assays, immunofixation electrophoresis (IFE), beta-2 microglobulin assessment, and other relevant markers [1]. Upon performing SPEP, monoclonal immunoglobulins typically manifest as a single, intense, discrete band on the electrophoretic gel and a sharp peak on the densitometer tracing. Immunofixation electrophoresis (IFE) serves as a definitive method for identifying monoclonal proteins and characterizing the secreted heavy and light chains [2].

Based on established literature, the presence of a single monoclonal band (M-band) in SPEP predominantly indicates monoclonal gammopathy, although exceptions exist. In these less common exceptions, IFE results may reveal double bands, potentially indicative of either double gammopathy or true biclonal gammopathy contingent upon the nature of the heavy and light chains involved [2]. Double gammopathy can present as two distinct M-bands/peaks or as a single M-band on SPEP that subsequently resolves into two separate bands of either heavy or light chains upon IFE. In contrast, true biclonal gammopathy involves two distinct immunoglobulin clones, each with its own heavy and light chain, detectable as two separate bands/peaks in both SPEP and IFE. The reported incidence of double gammopathies among various cohorts in the literature is approximately 2–6% [5]. Double gammopathies are known to be associated with multiple myeloma, certain lymphoproliferative disorders, as well as with cases of monoclonal gammopathy of undetermined significance [4,5].

Interestingly a pseudo-biclonal pattern can happen in rare cases and is characterized by the presence of two M-bands on SPEP (suggesting a biclonal pattern) that resolve into a single immunoglobulin clone upon IFE [6]. The published literature on such cases remains sparse. Accurate identification of double gammopathy or pseudo-biclonal patterns is crucial for timely diagnosis and appropriate therapeutic intervention. This case series presents three cases exhibiting pseudo-biclonal patterns on SPEP, where the subsequent IFE revealed either double gammopathy or monoclonal gammopathy, thus highlighting the absence of true biclonality.

## Materials and Methods

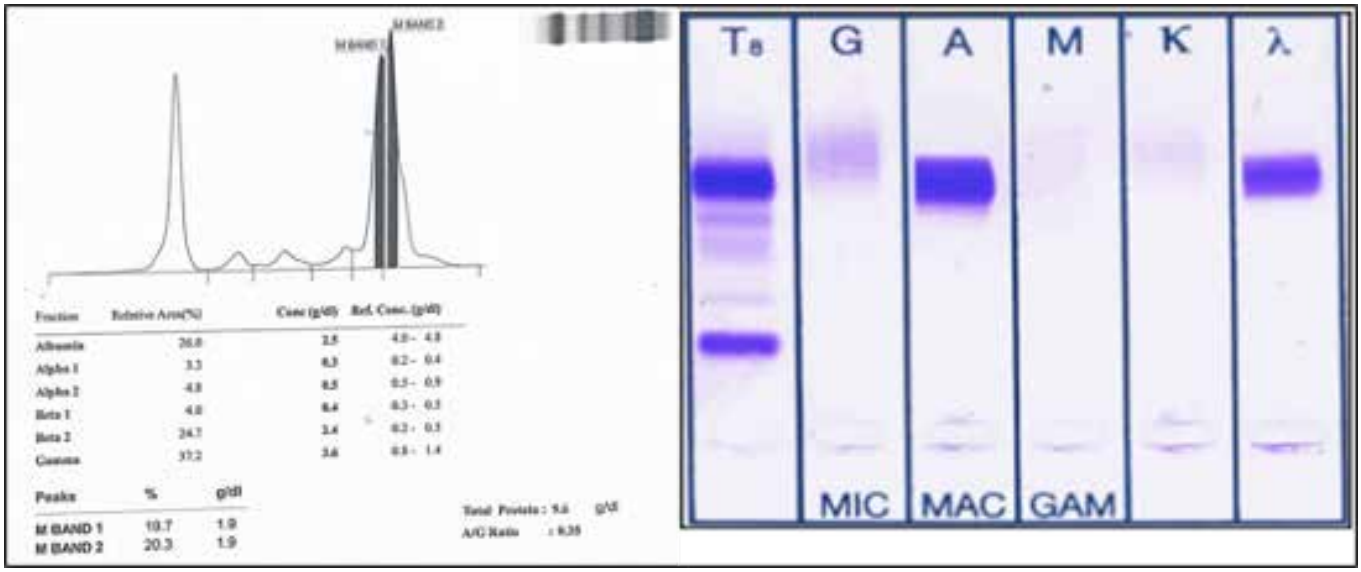
This retrospective case series was conducted using SPEP reports obtained from the Department of Biochemistry at Sri Ramachandra Medical College & Research Institute, Chennai, Tamilnadu, India. Ethical approval, with a waiver of consent due to the retrospective nature of the study, was granted by the Institutional Ethics Committee. SPEP was performed using capillary electrophoresis, followed by immunofixation electrophoresis. The study period encompassed the years 2022 to 2024, and included reports demonstrating a biclonal pattern on SPEP that did not correspond to true biclonality on IFE, instead revealing double gammopathy or monoclonal gammopathy.

## Results

### Case 1

A 73 year old male presented with slurred speech, decreased appetite and generalized fatigue. His baseline investigations revealed Hemoglobin-7.9 g/dl (peripheral smear- normocytic normochromic anemia with neutrophilia), Total count – 11,780 cells/cu.mm; BUN – 26 mg/dl; Creatinine – 2.3 mg/dl and Calcium – 14.5 mg/dl. LFT showed Total protein – 9.7 gm/dl; Albumin - 2.5 g/dl; Globulin – 7.2 g/dl with reversal of A:G Ratio along with diffuse osteopenia and multiple fractures of varying degree in CT scan of thorax. In view of AKI, hypercalcemia, osteopenia with multiple fractures and A:G ratio reversal, Myeloma workup was performed. Bone marrow biopsy showed myelomatous marrow with 27% plasma cells. Serum protein electrophoresis detected hypoalbuminemia along with two distinct M-bands in the gamma region (biclonal pattern) that subsequently resolved into Ig A and Lambda in IFE (monoclonal gammopathy) as depicted in Figure 1. These findings were further corroborated by elevated levels of IgA - 4666 mg/dl; Lambda - 198 mg/l; Beta-2-microglobulin - 9234 ng/ml confirming the diagnosis of Multiple myeloma. The Patient was started on VCD (Bortezomib, cyclophosphamide, Dexamethasone) regimen chemotherapy.

**Figure 1:** shows the SPEP & IFE reports of Case 1.

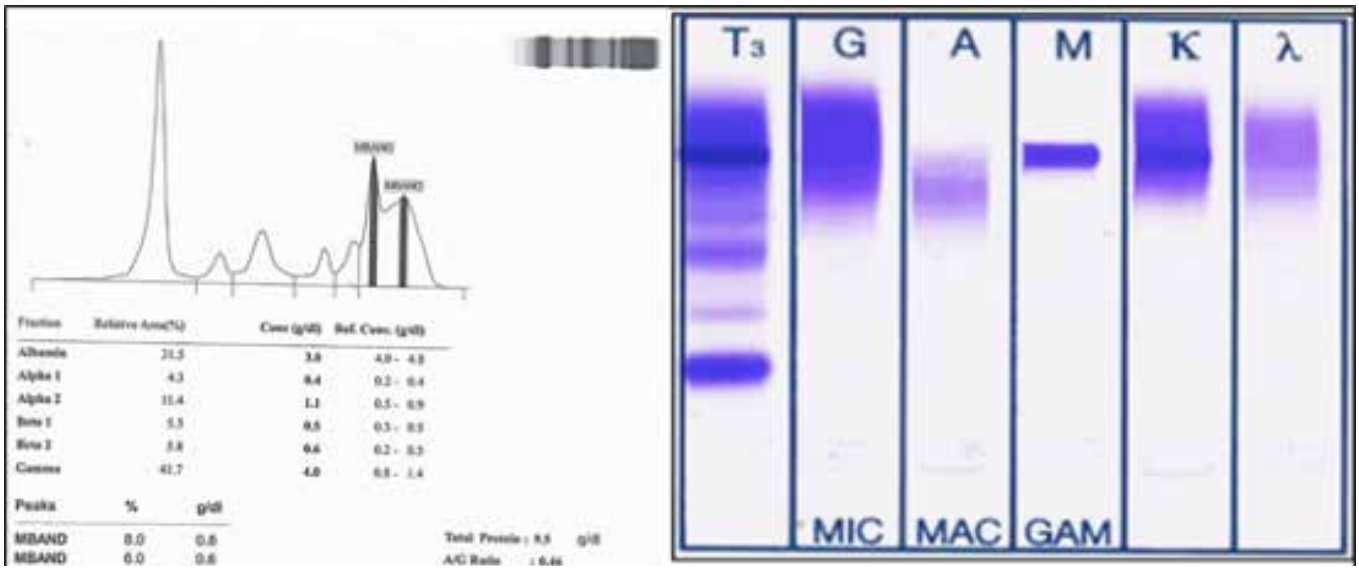



In Case 1 the above SPEP (left) demonstrates two distinct M bands in the gamma region which upon IFE (right) resolved into IgA- Lambda.

**Case 2**

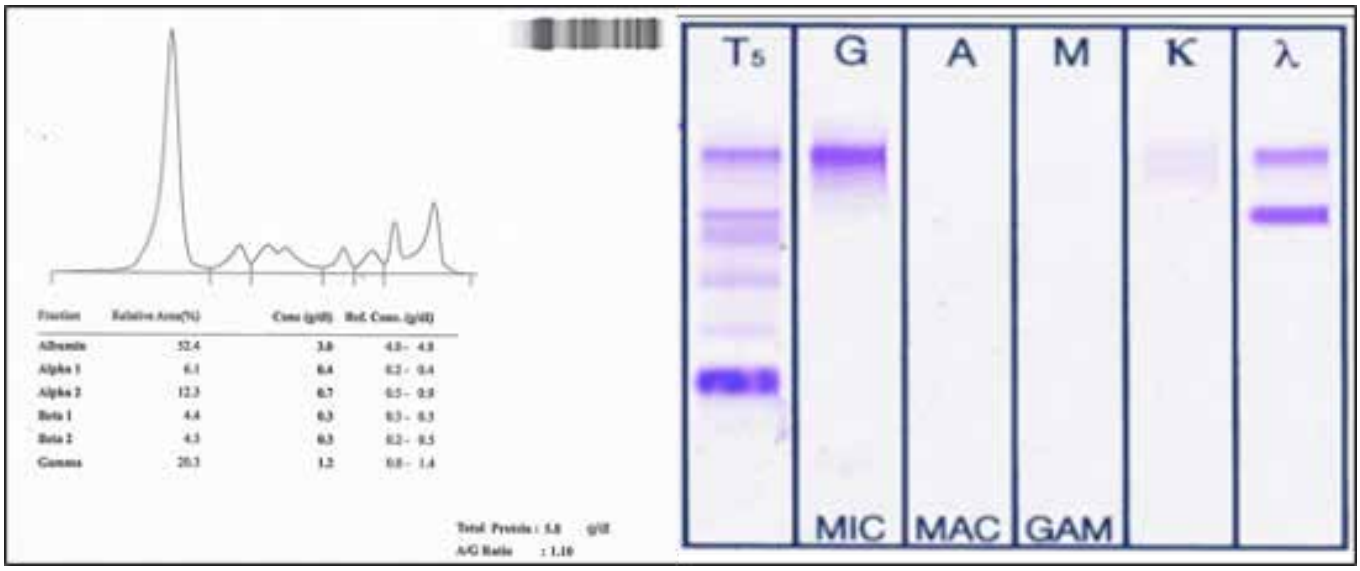
A 57-year-old male was referred with a provisional diagnosis of Nodal marginal lymphoma / lymphoplasmacytic cell lymphoma. His basic investigation revealed Hemoglobin-7.4 g/dl; total count-7,700 cells/cu.mm; platelets- 4.25 lakhs; calcium- 7.90 mg/dl; BUN-28 mg/dl; creatinine-1.21 mg/dl; and albumin-2.50 g/dl. PET CT scan reported multiple enlarged nodes in the abdomen, neck, axilla, mediastinum, pelvis and inguinal region. Histopathology of cervical lymph nodes and bone marrow aspiration biopsy showed features suggestive of lymphoproliferative disorder. In view of lymphoma, serum protein electrophoresis was done. SPEP showed hypoalbuminemia and elevated levels of alpha1, alpha2, beta1, beta2 globulins. Two discrete M bands were observed in the gamma globulin region (biclonal pattern), which further resolved into IgM and Kappa (monoclonal gammopathy) upon IFE, along with elevation of other immunoglobulins as depicted in Figure 2. These findings were further confirmed by quantifying serum immunoglobulins, which revealed elevated IgG - 3928 mg/dl; IgM - >3300 mg/dl; IgA - 365.80 mg/dl; Kappa - 280 mg/l; Lambda - 174 mg/l and Beta-2-microglobulin - 10884 ng/ml. The patient was started on chemotherapy cycles with Bendamustine.

**Figure 2:** shows the SPEP & IFE reports of Case 2.




In Case 2 the above SPEP (left) shows two M-Bands in Gamma region which resolved upon IFE(right) as IgM-Kappa along with increase in other Ig lanes.

**Figure 3:** shows the SPEP & IFE reports of Case 3.




In Case 3 the above SPEP (left) shows two M-Bands in the Gamma region which resolved upon IFE (right) as IgG-Lambda-Lambda.

**Case 3**

An 86-year-old woman, with history of Acute on chronic kidney disease, coronary artery disease and systemic hypertension was admitted for Urosepsis with persistent hyperkalemia. Baseline investigations revealed Hemoglobin - 7.8 gm (Peripheral smear - normocytic normochromic anemia); total count – 3350 cells/cu.mm; platelet – 2.47 lakhs. LFT showed total protein – 6.6 g/dl; Albumin – 3.6 g/dl; Globulin -2.9 g/dl; A:G ratio- 1:2 and Serum calcium-9 mg/dl. CT scan of the abdomen revealed multiple lytic lesions with an unknown primary. Serum protein electrophoresis showed the presence of two discrete bands in the gamma region (biclonal pattern) which further resolved into IgG Lambda and one more Lambda isomer in IFE as depicted in Figure 3. These findings were further confirmed by elevated levels of serum Immunoglobulins IgG - 1045 mg/dl; Lambda - 10297.4 mg/l and beta 2 microglobulin - 17142 ng/ml, confirming the diagnosis of multiple myeloma with excess free light chains.

**Discussion**

A pseudo-biclonal pattern, as observed in SPEP, is characterized by the presence of two discrete or non-discrete peaks that subsequently resolve upon IFE into either monoclonal gammopathy or double gammopathy, without exhibiting true biclonality [5,6]. The terms Double gammopathy (DG) and Biclonal gammopathy (BG) are often used interchangeably in the literature. True biclonal gammopathy may arise from the division of a single B-lymphoid cell clone into two distinct clones following antigenic selection or from the neoplastic proliferation of two independent malignant plasma cell lines [4]. In contrast, double gammopathies can originate from two or more distinct, yet clonally related, plasma cell lines, resulting in the production of multiple monoclonal proteins [4,6]. Double gammopathies have been observed in various plasma cell disorders, including monoclonal gammopathy of undetermined significance (MGUS), multiple myeloma (MM), and Waldenström's macroglobulinemia. They are also frequently associated with leukemia, lymphoproliferative disorders, primary amyloidosis, cryoglobulinemia, solitary plasmacytoma, POEMS syndrome (a rare condition), and certain infections, such as Hepatitis C virus (HCV) infection [6].

The literature provides limited elucidation regarding the underlying causes of pseudo-biclonal patterns. A common misinterpretation involves the presence of a fibrinogen band in the beta-gamma region, resulting from inadequate clotting of the blood sample. As IFE does not employ specific antisera for fibrinogen, this band will not be apparent upon immunofixation [2,3]. Another recognized cause is the propensity of immunoglobulins, originating from the same plasma cell lines, to polymerize and form aggregates. IgA and IgM are the immunoglobulins most commonly implicated in forming such atypical electrophoretic patterns [7]. The pseudo-biclonal pattern observed in Case 1 (Figure 1) can likely be attributed to the polymerization of IgA, leading to the appearance of an additional M-band near the beta region, which subsequently resolved as IgA Lambda upon IFE. The quaternary structure of IgA allows for polymerization, with or without subsequent light chain (LC) production due to the sequestration of LC epitopes. Owing to their low isoelectric pH, these aggregates can exhibit more anodal migration, appearing as separate bands in proximity to the beta region. IgA can also form dimers, potentially resulting in two M-bands due to variations in electrophoretic mobility [7] . However, IFE in such instances reveals a single band in the IgA and its corresponding light chain lanes, consistent with monoclonal gammopathy. In rare cases, Polymerized IgA structure hinders the reaction between LC epitopes and its antibodies, producing a condition termed

as 'IgA with no apparent light chain attached' [6,8]. Clinically, the polymerization of IgA may contribute to hyperviscosity syndrome and potentially lead to the overestimation of serum calcium levels or underestimation of the hemoglobin levels. This spurious increase in calcium occurs due to its binding to the secreted paraprotein, necessitating careful evaluation [9]. If the polymerization effect is due to IgM (pentamer), it can result in the appearance of two or more bands on SPEP [7].

Polyclonal increase in Immunoglobulins can occur in various conditions such as chronic inflammation, chronic liver disease, systemic lupus erythematosus, rheumatoid arthritis, cystic fibrosis etc. [10]. Such polyclonal elevations are usually depicted in SPEP as hypoalbuminemia alongside elevation of alpha, beta and gamma globulins without a discrete monoclonal band. Interestingly, a Polyclonal elevation of Immunoglobulins may coexist with a monoclonal gammopathy in the context of metastatic hematological malignancy, underlying chronic infection, inflammation or autoimmune disorders [11]. In our Case 2, the patient had chronic cervical lymphadenopathy, multiple lymph node metastasis along with features of Tumor lysis syndrome. These changes have contributed to the elevation of all globulin fractions (alpha, beta and gamma). The observed pseudo-biclonal pattern in Case 2 is thus attributed to the polyclonal immunoglobulin elevation combined with increased acute phase reactants due to Tumor lysis syndrome complicating splenic marginal zone lymphoma. This was confirmed by IFE revealing IgM kappa and elevated serum levels of IgG, IgA, and free light chains (kappa and lambda). Tumor Lysis Syndrome (TLS) is a common complication in hematological malignancies occurring either during disease progression or following chemotherapy. TLS can cause an increase in serum free light chains, acute phase reactants and also hyperuricemia, which can influence the migration of proteins during SPEP [12,13]. These metabolic changes can induce Pseudo-biclonal patterns as seen in Case 2.

In our Case 3, the biclonal pattern resolved into IgG-Lambda-Lambda (LC isotype-matched). This finding is probably because of the asynchronous production of excess free light chains (serum Lamba-10297.4 mg/L), which can migrate faster and produce an additional M-band on SPEP. Double gammopathy (DG) occurs only in clonally related plasma cells, of which IgG-IgA, IgG-IgM and IgG-IgG are reported in various literatures [12,13]. These can occur with either heavy chain or light chain isotype matching, attributable to two primary mechanisms. The first mechanism involves the molecular process of antigenic diversity that allows clonally related plasma cells to undergo class switch recombination. This can be seen during disease progression or following treatment. The levels of such Double M proteins can rise or fall either concordantly or discordantly [16]. Molecular analysis of these DGs has frequently demonstrated isotype switches in heavy chains, where different heavy chains can be encoded from the same IGHV, IGHD, and IGHJ genes with varying degrees of somatic mutations, resulting in identical immunoglobulin heavy chain variable regions (identical amino acid sequences) [17]. Yet such class switch was not observed in the kappa and lambda chains, rather low abundance of Plasma cells for light chain proliferation was reported in molecular analysis [16,17]. The second common cause is the asynchronous production of excess free light chains along with Ig heavy chains. This excess free LC migrates faster than HC producing a biclonal pattern [18]. In addition to IFE, the measurement of serum free light chains and serum IgA, IgG, and IgM levels is valuable in the diagnosis of these DGs. In case 3, the serum lambda levels (serum lambda - 10297.4 mg/l) was disproportionally elevated compared to the heavy chains (serum IgG - 1045 mg/dl). Other less common factors that can lead to biclonal patterns include renal impairment, which can cause elevated serum free light chains, and oligoclonality following stem cell transplantation [19]. Among these causes renal failure was also observed in this patient. Renal impairment can lead to decreased clearance of free light chains and an increased molecular half-life of light chains. The increase in free light chains secondary to renal failure has been identified as a poor prognostic factor in myeloma patients [20,21].

SPEP alone exhibits limited sensitivity in detecting pseudo-biclonal patterns. The diagnostic accuracy for pseudo-biclonal patterns can be enhanced by the combined use of SPEP with immunofixation electrophoresis, serum free light chain assays, and serum immunoglobulin assays. In limited resource settings or as a cost-effective strategy, reducing agents like beta-mercaptoethanol and dithiothreitol can be used to differentiate between true biclonality and polymeric forms [2,3]. Pre-treatment of serum with beta-mercaptoethanol prior to SPEP depolymerizes polymeric immunoglobulins by disrupting their disulfide bonds, resulting in a single M-band [4]. However, it is important to note that reducing agents cannot distinguish between true biclonal gammopathy and biclonal patterns arising from excess free light chains, necessitating IFE for definitive confirmation.

## Conclusion

While no major differences may exist in the clinical presentation, treatment strategies, or outcomes among biclonal/double or monoclonal gammopathy, the accurate identification of these atypical patterns is crucial for improving diagnostic precision and laboratory quality indices. Double gammopathy/ Biclonal gammopathy are commonly associated with leukemia and lymphomas other than multiple myeloma, for which early diagnosis can help in timely treatment. Similarly, in cases where multiple myeloma is diagnosed based on double gammopathy or biclonal gammopathy, monitoring the synchronous response of all clones to treatment is critical, as it may necessitate modifications to the treatment regimen. The identification of double gammopathies, particularly those involving different heavy chains, may provide valuable insights into the molecular mechanisms underlying these conditions, potentially shedding light on the genetic evolution and genomic alterations in multiple myeloma. Our case series has primarily focused on pseudo-biclonal patterns arising from monoclonal gammopathy,

polyclonal elevation due to TLS and Double gammopathy due to excess free light chains. Although literature exists regarding polymerization and excess free light chains, the significance of our series lies in highlighting the rarely reported TLS-induced pseudo-biclonal pattern. SPEP followed by IFE remains a necessary approach for the accurate identification of such pseudo-biclonal patterns.

**Author contributions**

Conceptualization: Kanmani N, Karthick E, Sathya Selvarajan, K Sowmya; Methodology: Kanmani N, Karthick E, Sathya Selvarajan, K Sowmya; Material preparation, data collection: Kanmani N, Karthick E, Sathya Selvarajan, K Sowmya; Formal analysis and investigation: Kanmani N, Karthick E, Sathya Selvarajan, K Sowmya;Writing - original draft preparation: Kanmani N, Karthick E, Sathya Selvarajan, K Sowmya; Writing - review and editing: Kanmani N, Karthick E, Sathya Selvarajan, K Sowmya; Resources: Kanmani N, Karthick E, Sathya Selvarajan, K Sowmya; Supervision and final approval: Kanmani N, Karthick E, Sathya Selvarajan, K Sowmya; Accountability for the research: Kanmani N, Karthick E, Sathya Selvarajan, K Sowmya.

**Ethical approval**

The study was approved by Sri Ramachandra Institute of Higher Education and Research Institutional Ethics Committee (CSP-MED/24/AUG/107/270). Waiver of consent was provided pertaining to the nature of study. This study was conducted in compliance with the ethical principles for medical research involving human subjects, in accordance with the Declaration of Helsinki.

**Data availability**

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

**Conflict of interest**

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

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Research Article

# Exploring Genetic Variability in VDR FokI and BsmI Polymorphisms and Their Association with Rheumatoid Arthritis

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**Keywords**

Rheumatoid Arthritis, Vitamin D Receptor, Polymorphism, RFLP, Gene Association

**Abstract**

**Background**: Rheumatoid Arthritis (RA) is a chronic, inflammatory autoimmune disease that mainly affects small joints and progresses to larger joints. It impacts approximately 1% of the global population, with women being three times more likely to develop it than men, typically between ages 40 and 50. Genetic and environmental factors, including vitamin D deficiency, contribute to RA development. The vitamin D receptor (VDR) gene plays a crucial role in regulating metabolism and inflammation, making it a key candidate in RA.

**Aims**: This study determines the relationship between VDR FokI (rs10735810) and VDR BsmI (rs1544410) gene polymorphisms in RA patients and healthy controls.

**Methods**: A total of 400 participants were included in the study, consisting of 200 RA patients and 200 healthy controls. Genetic varations of VDR genes was performed using the PCR-RFLP technique.

**Result**: Among 200 RA patients, 42 (21%) were male and 158 (79%) were female, while in the control group, 40 (20%) were male and 160 (80%) were female. The average age of RA patients was 43±17 years and a mean disease onset at age 30.3±19.3 years. The Ff and ff genotypes of the FokI polymorphism were significantly more frequent in RA patients (OR=1.8, p=0.011 and OR=2.8, p=0.004, respectively). For the BsmI polymorphism, the Bb genotype showed a significant association with RA (OR=1.5, p=0.049), while the bb genotype did not (OR=1.4, p=0.241). Gender-based analysis revealed higher frequencies of the Ff, ff, and bb genotypes in females, with significant associations for Ff (OR=3.0, p=0.009), ff (OR=4.5, p=0.002) for Bsml genotype only bb (OR=3.9, p=0.006) was significantly increase the risk of RA.

**Conclusions**: This study concluded that the VDR FokI (rs10735810) gene polymorphism was associated with RA, while the VDR BsmI (rs1544410) polymorphism did not appear to have a significant association with the disease.

## Introduction

Rheumatoid Arthritis (RA) is a chronic, systemic autoimmune disorder that primarily targets the synovial joints, particularly affecting the small joints of the hands and feet [1]. The hallmark features of RA include persistent inflammation, progressive joint destruction, and deformities, which can lead to significant functional disability and impaired quality of life [2,3]. The global prevalence of RA is estimated to be approximately 1%, with a notable disparity between the sexes, women are disproportionately affected at a ratio of 3:1 compared to men [4,5]. Although the disease most commonly presents between the ages of 40 and 50, it can occur at any age, including in children and the elderly, indicating that both genetic and environmental factors contribute to its onset.

The pathogenesis of RA is multifactorial, involving a complex interplay of genetic predisposition, environmental triggers, and immune system dysregulation [2,4]. In genetically predisposed individuals, environmental factors such as infections, smoking, and vitamin D deficiency may contribute to the initiation and progression of the disease [6]. Autoimmunity plays a central role in RA, with the immune system mistakenly targeting the body's own tissues, particularly the synovial membrane, leading to the chronic inflammation that characterizes the disease [7,8]. As the disease progresses, inflammation leads to cartilage and bone destruction, resulting in deformities and loss of joint function.

Among the genetic factors influencing the development of RA, the Vitamin D Receptor (VDR) gene has attracted significant attention due to its critical role in regulating immune function and inflammation [9]. Vitamin D, through its receptor VDR, plays a pivotal role in modulating immune responses by influencing the differentiation and activation of T cells, B cells, and macrophages, which are central players in autoimmune diseases such as RA [10]. Vitamin D deficiency has been associated with an increased susceptibility to autoimmune diseases, including RA, suggesting that alterations in VDR signaling may contribute to the disease's pathogenesis [11].

The VDR gene is located on chromosome 12q13 and encodes the vitamin D receptor, which mediates the biological actions of vitamin D [12,13]. Several polymorphisms in the VDR gene have been identified, and these genetic variations are thought to influence the receptor's ability to bind to vitamin D and regulate gene expression. Two of the most studied VDR polymorphisms in relation to autoimmune diseases are the FokI (rs10735810) and BsmI (rs1544410) polymorphisms [13-15].

The FokI polymorphism is located in the translation initiation site of the VDR gene and leads to the production of a variant receptor with a different length compared to the wild-type receptor [16]. The shorter form of the receptor, resulting from the presence of the F allele, is believed to be more transcriptionally active, potentially leading to altered immune responses. This polymorphism has been shown to affect the receptor's ability to regulate inflammatory cytokines, which are key mediators in RA [17-18]. The BsmI polymorphism, located in the 3' untranslated region of the VDR gene, has been associated with variations in VDR expression levels, though its functional significance remains less well understood [19,20]. Both of these polymorphisms have been implicated in various autoimmune conditions, including rheumatoid arthritis, suggesting that they may play a role in modulating susceptibility to the disease.

In this study, we aimed to examine the relationship between the VDR FokI and VDR BsmI gene polymorphisms and the susceptibility to RA. We hypothesized that certain genotypes of these polymorphisms could be associated with an increased risk of developing RA.

## Material and Methods

### Study Population

This cross-sectional study was conducted from November 2019 to October 2021 in the Department of Biochemistry and the Department of Medicine at Uttar Pradesh University of Medical Sciences (UPUMS), Saifai, Etawah. The sample collection and rhematoid markers (RA Factor, hs-CRP, Anti-CCP, and vitamin D) were done in the UPUMS, Saifai and the genotype study was done in VMMC & Safdarjung Hospital, New Delhi, India. The study encompassed 400 subjects, comprising 200 Rheumatoid Arthritis (RA) patients and 200 healthy controls who were matched for age and sex, all from the same ethnic group.

The diagnosis of RA was established based on the Revised American College of Rheumatology's 2010 clinical criteria. Patients were accommodated in the rheumatology clinic's inpatient wards and outpatient departments within the Department of Medicine. A comprehensive oral questionnaire was administered to each participant after obtaining their consent. This questionnaire included a detailed history and a clinical examination based on the Clinical Disease Activity Index (CDAI). This rigorous approach ensured a thorough evaluation of each participant's condition.

### Subject selection criteria

The study included patients who met the 2010 Revised American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) criteria for Rheumatoid Arthritis [21]. However, individuals taking Vitamin D supplements or the hypolipidemic drug HAART (Highly Active Antiretroviral Therapy) were excluded. Additionally, subjects with chronic conditions such as Diabetes, Hypertension, Familial Hypercholesterolemia, Chronic Kidney Disease, and Tuberculosis were not considered for the study.

### Sample Collection

Blood samples were collected by venipuncture into labelled plain & EDTA vials. An EDTA sample was used to estimate for molecular study. The plain sample was centrifuged at 4500

rpm for 20 minutes for serum separation and then the serum was used for the analyses of rheumatoid markers (RA factor, hs-CRP, Anti-CCP, and vitamin D estimation.

**Molecular Analysis**

Reagent purchased

DNA Isolation Kit (CatLog No.: 51104, Qiagen, USA), PCR master mix (CatLog No. RR310A, Takara BioInc, Japan, FokI and BsmI enzymes (CatLog No.: R01095 and R01345, New England Biolabs Inc. New England).

**DNA Extraction**

Genomic DNA was extracted from peripheral blood samples using a commercially available DNA isolation kit. The DNA extraction kit is designed to efficiently isolate high-quality genomic DNA from human blood samples. This method ensures the removal of contaminants that could interfere with downstream applications, such as polymerase chain reaction (PCR). Briefly, whole blood was collected in EDTA tubes, and the extraction process followed the manufacturer's protocol. The purified DNA was quantified using a spectrophotometer and stored at -20°C until further use. The extracted DNA served as the template for subsequent genotyping of VDR gene polymorphisms.

**Genotyping**

Genotyping of the VDR FokI (rs10735810) and VDR BsmI (rs1544410) polymorphisms was performed using the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) technique. PCR amplification of specific gene regions was followed by restriction enzyme digestion to detect the presence of specific alleles at each polymorphic site.

**Genotyping of VDR (FokI) (rs10735810)**

For the genotyping of VDR FokI polymorphism (rs10735810), PCR amplification was performed using specific primers: forward primer 5‟AGCTGGCCCTGGCACTGACTCTGCTCT3‟ and reverseprimer5‟ATGGAAACACCTTGCTTCTTCTCCCTC3‟. This amplification targeted a fragment of 265 base pairs. The amplified product was then digested using the FokI Fast Digest enzyme. The PCR conditions were as follows: an initial denaturation at 94°C for 5 minutes, followed by 35 cycles consisting of denaturation at 94°C for 1 minute, annealing at 61°C for 1 minute, and extension at 72°C for 1 minute, with a final extension step at 72°C for 7 minutes. The PCR products were separated on a 2% agarose gel under UV light for visualization.

The genotypes were identified based on the banding pattern: the FF (homozygous wild-type) genotype yielded a single 265 bp band, the Ff (heterozygous) genotype produced three bands at 265, 200, and 65 bp, and the ff (homozygous mutant) genotype produced two bands at 200 and 65 bp (Figure 1A).

**Genotyping of VDR (BsmI) (rs1544410)**

For the VDR BsmI polymorphism (rs1544410), PCR amplification was performed using the following primers: forward primer 5‟CAACCAAGACTCAAGTACCGCGTCAGTG3‟ and reverse primer 5‟AACCAGCGGAAGAGGTCAAGGG3‟. This reaction amplified a fragment of 825 base pairs, which was subsequently digested with the MvaI restriction enzyme. The PCR amplification profile consisted of an initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, and extension at 72°C for 1 minute, with a final extension at 72°C for 7 minutes. The amplified products were analyzed on a 2% agarose gel under UV light.

Genotypes were determined based on the digestion pattern: the BB (homozygous wild-type) genotype produced a single band at 825 bp, the Bb (heterozygous) genotype resulted in three bands at 825, 650, and 175 bp, and the bb (homozygous mutant) genotype produced two bands at 650 and 175 bp (Figure 1B).

**Figure 1:** Electrophoresis gel image of genes

**Figure 1A**

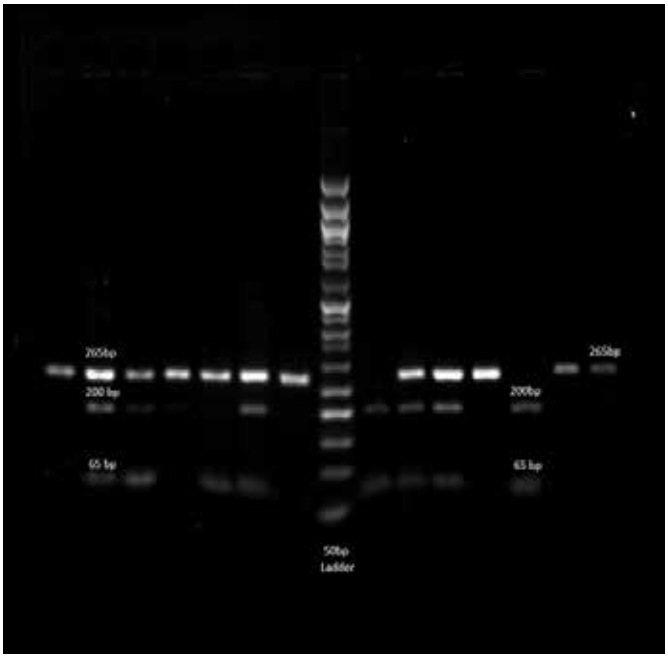
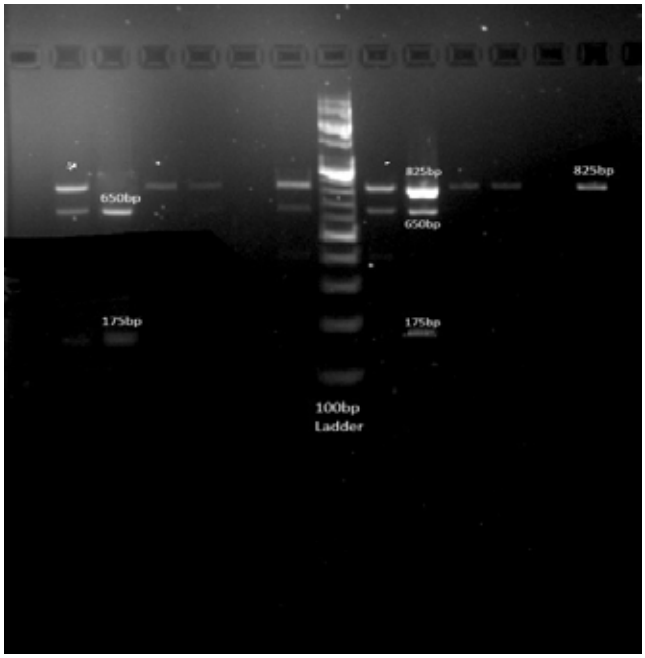



**Figure 1B**




1A FokI: Wild: 265bp, Heterozygous: 265bp, 200bp, 65bp, and Mutant: 200bp, 65bp.

1B BsmI: Wild: 825 bp, Heterozygous: 825bp, 650bp, 175bp, and Mutant: 650bp, 175bp.

### Statistical analysis

Data analysis was done using the Statistical Package for the Social Sciences (SPSS) version 21 software. Genotype and allele frequencies were calculated, and Chi-square analysis was applied to compare allele frequencies between the patient and healthy control groups. Odds ratios were computed to evaluate genotype distributions. A p-value of less than 0.05 was considered statistically significant.

## Result

### Demographic Characteristics

The demographic data of the study participants are summarized in Table 1. The average age of RA patients and healthy controls was comparable, with no significant difference between the two groups. There were also no significant differences in the gender distribution between the RA patients and the control group.

**Table 1:** Demographical characteristics of study population.

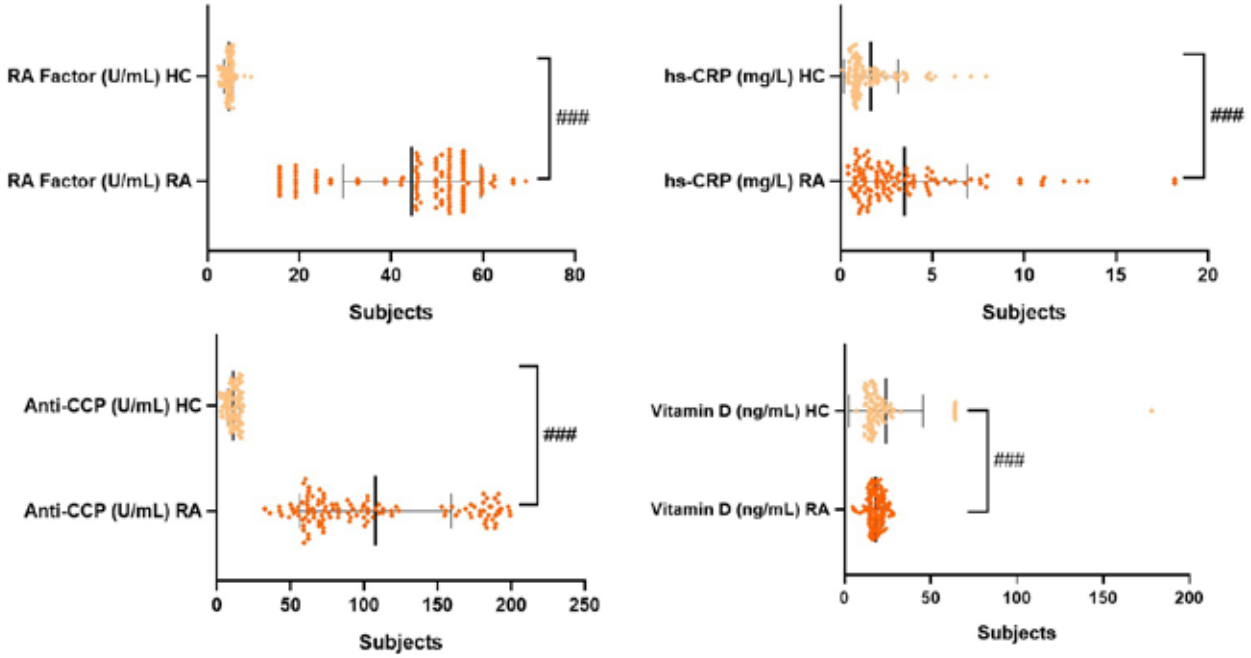
| Parameters | RA Patients (n=200) | Healthy Controls (n=200) | p-value |
|---|---|---|---|
| Male n (%) | 42 (21%) | 40 (20%) | 0.804* |
| Female n (%) | 158 (79%) | 160 (80%) | |
| Age (mean ± SD) | 43 ± 17 | 44 ± 16 | 0.545* |
| Age at Disease Onset (mean ± SD) | 30.3 ± 19.3 | - | |

The chi-square test was used calculate the p-value. *p<0.05 statistically significant.

**Comparison of rheumatoid markers among study groups**

Figure 2 represents the levels of hsCRP, vitamin D, Rh Factor, and anti-CCP among study groups. All the markers were significantly elevated in rheumatoid patients than the control except vitamin D. Vitamin D was significantly reduced in cases than the healthy controls.

**Figure 2:** Levels of RA Factor, Hs-CRP, Anti-CCP, and Vitamin D in Rheumatoid patients and healthy control.




Abbreviations: RA: Rheumatoid arthritis, HC: Healthy control, hs-CRP: high-sensitive C reactive protein, Anti-CCP: Anti-cyclic citrullinated peptide, ###: p<0.0001

**Genotypic and Allelic Frequencies of VDR FokI Polymorphism**

The genotypic distribution of the VDR FokI polymorphism in RA patients and healthy controls is presented in Table 2A. The Ff and ff genotypes were significantly more frequent in RA patients compared to controls. Specifically, the Ff genotype had an odds ratio (OR) of 0.3 (95% CI: 0.1–0.8, p=0.004), and the ff genotype had an OR of 4.9 (95% CI: 2.1–11.55, p=0.0001), suggesting a strong association with RA. The F allele was more frequent in RA patients (OR=15.5, p=0.000), further supporting the role of the FokI polymorphism in RA susceptibility.

**Table 2A:** VDR FoKI genotype polymorphism in the study population.

| Genotype | RA Patients (n=200) | Healthy Controls (n=200) | OR (95% CI) | p-value |
|---|---|---|---|---|
| FF | 40 (20%) | 66 (33%) | 1 | |
| Ff | 102 (51%) | 90 (45%) | 1.8 (1.1–3.0) | 0.011* |
| ff | 58 (29%) | 34 (17%) | 2.8 (1.6–5.0) | 0.004* |

Abbreviations: 1: Reference group, OR: Odd Ratio, CI: Class Interval, RA: Rheumatoid arthritis,

*p<0.05 considered as statistically significant.

**Genotypic and Allelic Frequencies of VDR BsmI Polymorphism**

The genotype distribution analysis between RA patients (n=200) and healthy controls (n=200) revealed that the Bb genotype was significantly associated with RA, with an odds ratio (OR) of 1.5 (95% CI: 1.0–2.4) and a p-value of 0.049. However, no significant association was observed for the bb genotype, with an OR of 1.4 (95% CI: 0.8–2.5) and a p-value of 0.241. The BB genotype showed similar frequencies between RA patients (27%) and healthy controls (26%), indicating no significant difference. Thus, the Bb genotype may have a potential role in RA susceptibility, while the bb genotype does not appear to be significantly associated (Table 2B).

**Table 2B:** VDR BsmI genotype polymorphism in the study population.

| Genotype | RA Patients (n=200) | Healthy Controls (n=200) | OR (95% CI) | p-value |
|---|---|---|---|---|
| BB | 54 (27%) | 72 (36%) | 1 | |
| Bb | 106 (53%) | 90 (45%) | 1.5 (1.0–2.4) | 0.049* |
| bb | 40 (20%) | 38 (19%) | 1.4 (0.8–2.5) | 0.241 |

1: Reference group, OR: Odd Ratio, CI: Class Interval, RA: Rheumatoid arthritis.

*p<0.05 considered as statistically significant.

**Genotypic and Allelic Frequencies of VDR FokI Polymorphism**

The analysis of VDR gene polymorphisms (FokI and BsmI) revealed significant gender-based differences in genotype distributions. For the FokI polymorphism, males were more likely to carry the Ff and ff genotypes, with odds ratios of 3.0 (1.3-6.9, p = 0.009) and 4.5 (1.7-11.7, p = 0.002), respectively, compared to females. For the BsmI polymorphism, the bb genotype was significantly more common in males (33%) than females (7%), with an odds ratio of 3.9 (1.5-10.5, p = 0.006). These findings suggest a notable gender-related difference in the distribution of these VDR polymorphisms, with females showing higher frequencies of the Ff, ff, and bb genotypes (Table 3).

**Table 3**: Genetic variations of VDR genes based on gender among RA Patients.

| Genes | Genotypes | Male (n=42) n (%) | Female (n=158) n (%) | OR | p-value |
|---|---|---|---|---|---|
| VDR (FokI) | FF | 10 (24%) | 82 (52%) | 1 | |
| VDR (FokI) | Ff | 20 (48%) | 54 (34%) | 3.0 (1.3–6.9) | 0.009* |
| VDR (FokI) | ff | 12 (28%) | 22 (14%) | 4.5 (1.7–11.7) | 0.002* |
| VDR (BsmI) | BB | 18 (43%) | 71 (45%) | 1 | |
| VDR (BsmI) | Bb | 13 (31%) | 76 (48%) | 0.7 (0.3–1.5) | 0.324 |
| VDR (BsmI) | bb | 11 (26%) | 11 (7%) | 3.9 (1.5–10.5) | 0.006* |

1: Reference group, OR: Odd Ratio, CI: Class Interval, RA: Rheumatoid arthritis.

*p<0.05 considered as statistically significant.

**Discussion**

In this study, we aimed to explore the relationship between two specific Vitamin D Receptor (VDR) gene polymorphisms: FokI (rs10735810) and BsmI (rs1544410)—and the risk of developing Rheumatoid Arthritis (RA). Our findings revealed a significant association between the VDR FokI polymorphism and RA, while the VDR BsmI polymorphism showed no such association. These results support the growing body of evidence suggesting that VDR gene variants may influence susceptibility to autoimmune diseases like RA.

The VDR gene encodes the receptor for Vitamin D, a hormone that plays a central role in regulating immune system function [5]. Vitamin D deficiency has been linked to increased susceptibility to autoimmune diseases, including RA [5,11]. The VDR is expressed on a wide range of immune cells, including T-cells, B-cells, dendritic cells, and macrophages, where it modulates immune responses. The activation of the VDR by Vitamin D leads to the suppression of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-17, which are central to the pathogenesis of RA [2-4]. Moreover, Vitamin D regulates the differentiation and activation of T-helper cells, balancing the immune response between Th1/Th17 and regulatory T-cells (Tregs), both of which are implicated in RA pathogenesis.

Rheumatoid arthritis (RA) is characterized by an overactive immune response that targets the synovial joints, leading to chronic inflammation, pain, and irreversible joint damage. The VDR's ability to regulate immune responses and inflammatory pathways makes its genetic variants an important area of investigation in RA research. Among the two polymorphisms studied, the FokI polymorphism, located in the translation initiation site of the VDR gene, results in a variant protein with a different functional profile. Individuals with the ff genotype express a shorter VDR protein, which may affect the receptor's ability to regulate immune responses effectively. This shorter VDR may result in a reduced capacity to control inflammatory processes, potentially contributing to autoimmune diseases like RA [16,17].

Our study found that the Ff and ff genotypes were significantly associated with RA, with the ff genotype showing a particularly strong association with increased risk. This observation aligns with previous studies that have suggested that the FokI polymorphism could alter the receptor's function and contribute to the development of autoimmune conditions. For example, similar associations between the FokI polymorphism

and systemic lupus erythematosus (SLE) have been reported by Zhang et al. (2025), and studies by Shirai et al. (2022) have highlighted its role in multiple sclerosis (MS), suggesting that VDR gene variants may serve as common genetic risk factors for autoimmune diseases [22,23]. The FokI polymorphism may therefore influence the receptor's efficiency in modulating inflammatory cytokine levels, skewing the immune response in a way that favors the development of RA. Moreover, Vitamin D deficiency, often prevalent in RA patients, may exacerbate the effects of these polymorphisms, further promoting disease onset and progression. A study by Raftery et al. (2012) emphasized that VDR gene polymorphisms could modulate disease activity in RA, underlining the importance of Vitamin D in managing autoimmune diseases [24].

While the FokI polymorphism showed a clear association with RA in this study, the BsmI polymorphism did not. The BsmI polymorphism, located in the 3' untranslated region of the VDR gene, has functional consequences that are less well-understood compared to FokI. The lack of an association in our study is consistent with findings from other studies, such as those by Tang et al. (2020), which have failed to establish a significant link between BsmI and RA [19,20,25-27]. One possible explanation for the absence of an association is the specific role of the BsmI polymorphism, which may influence VDR expression or its stability rather than directly altering the receptor's structure or function. This could result in a more subtle or context-dependent effect. Additionally, environmental factors, such as Vitamin D levels and geographic location (e.g., sunlight exposure), may modify the impact of the BsmI polymorphism, potentially explaining the variability in findings across studies.

Interestingly, while the BsmI polymorphism did not show an association with RA in this study, it has been implicated in other autoimmune diseases, such as Crohn's disease and psoriasis, suggesting that its effect might vary depending on the disease context [21]. Furthermore, the presence of other VDR polymorphisms or interactions with other genetic factors may modify the effect of BsmI on RA susceptibility. These complex interactions emphasize the need for further research to understand the broader genetic and environmental landscape that influences autoimmune disease susceptibility.

This study provides valuable insights into the role of VDR polymorphisms in RA. However, there are some limitations to be considered. The sample size, particularly for the BsmI polymorphism, may limit the generalizability of our findings. Further studies with larger sample sizes are needed to confirm these results and explore potential interactions with other genetic and environmental factors. Moreover, this study focused only on two polymorphisms within the VDR gene. Given that the VDR gene contains several other variants that could contribute to RA susceptibility, a comprehensive analysis of additional VDR polymorphisms—especially those that affect receptor binding affinity or downstream signaling pathways—could provide a more complete understanding of how Vitamin D signaling contributes to RA pathogenesis.

Finally, the role of Vitamin D deficiency and other environmental factors, such as smoking or diet, should be explored in more detail. These factors may modulate the effects of VDR polymorphisms, making it crucial to account for them in future studies. As the interplay between genetic predisposition and environmental influences becomes increasingly apparent, future research must aim to identify how these factors collectively contribute to the onset and progression of RA.

## Conclusions

In conclusion, this study provides strong evidence that the VDR FokI gene polymorphism is significantly associated with an increased risk of developing Rheumatoid Arthritis. The association of the Ff and ff genotypes with RA, along with the higher frequency of the f allele in RA patients, suggests that this polymorphism may influence disease susceptibility through altered immune regulation. In contrast, the VDR BsmI polymorphism did not show a significant association with RA. These findings highlight the importance of genetic factors in the pathogenesis of RA and suggest that VDR polymorphisms could serve as potential biomarkers for disease risk and therapeutic response. However, further research is needed to clarify the precise mechanisms by which these genetic variants contribute to RA and to explore the potential for personalized treatments based on VDR genotypes.

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## Declaration

This original article has not been published before and is not currently under consideration for publication elsewhere. All authors have read and approved the study. The authors declare no conflict of interest.

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Case report

# A Case of Myelodysplastic Syndrome-Induced Acquired Sideroblastic Anemia

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Sideroblastic, anemia, hematological, myelodysplastic

**Abstract**

Hematological disorders are frequently encountered at the doctor's office and in the emergency room. Sideroblastic anemia, a rare hematological malady, is characterized by ring sideroblasts in the red blood cells due to accumulation of poorly transported and underutilized iron. Unlike primary sideroblastic anemia which can be conclusively diagnosed through genetic testing, the more common secondary form of the disease requires intricate cascading of several preliminary and confirmatory laboratory testing to determine the accurate etiology and provide the appropriate management regimen. The scope of laboratory medicine is broadening; clinical chemists and other laboratorians are tasked with directing broader clinical pathology sections, including Core lab hematology. Understanding the testing dynamics, diagnostic criteria and management of hematological diseases is fundamental to attaining success in consulting with providers to manage diseases such as sideroblastic anemia. Via a relevant case study, we explore the etiology, workup, symptoms, and treatment of myelodysplastic syndrome-induced sideroblastic anemia.

## Introduction

Sideroblastic anemia is a rare hematological disorder involving disrupted mitochondrial iron metabolism and of iron transport which inadvertently impacts heme synthesis [1]. By classification, rare diseases affect less than 200,000 individuals in the United States. Primary sideroblastic anemia is usually caused by genetic mutations with three modes of inheritance (Autosomal recessive, maternal and X-linked) [2], while environmental factors are the main features in the acquired form of the disease. Myelodysplastic syndromes with ring sideroblasts (MDS-RS), driven by dynamic alterations in the bone marrow are responsible for the most common forms of acquired sideroblastic anemia. A subtype of MDS-RS, Refractory anemia with ring sideroblasts (RARS) is a low-grade MDS characterized by dyserythropoeises, anemia and at least 15% ring sideroblasts, and the significant involvement of ring sideroblasts is what differentiates RARS from Refractory anemia (RA). RARS has a 3-12% chance of progression to acute myeloid leukemia, even though the elevated ring sideroblasts diminishes the risk of progression to leukemia [3]. Here, we present a clinical case of MDS-RS to highlight the diagnostic criteria, laboratory investigation, and the management rudiments of the disease. This case report is a great resource for clinical chemists, clinical pathologists, and hematologists in understanding the distinct and collective roles of laboratory and clinical teams in working up and resolving a typical secondary sideroblastic anemia involving myelodysplasia in the bone marrow.

## Case presentation

A 78-year-old male patient was admitted at a large tertiary academic hospital for cough and weakness for one week. He tested negative for COVID and Influenza. On admission, the patient's hemoglobin level and hematocrit were 4.7 g/dL and 14.1 respectively. His other lab results were white blood cells count (WBC) 1.9 (4.5 to 11.0 ×109/L), absolute neutrophile count (ANC) 0.72 (2.0–7.5×109/L), red blood cells count (RBC) 1.53 x106/uL, red cell width distribution width, 19.2 (11.5-14.5 %) and platelet count was 3,000/µL. The patient had a history of hyperlipidemia and hypertension and was also diabetic, suggesting metabolic syndrome. He had reported being a former smoker but was not currently using alcohol.

The patient had thrombocytopenia. Bone marrow biopsy was consistent with refractory anemia with ringed sideroblasts and complex cytogenetics were noted. Chest X-ray revealed left lower lobe infiltrate with effusion and enlarged heart. Additional techniques performed to unravel the patient's predominant diagnosis were bone marrow biopsy with Fluorescence In Situ Hybridization (FISH), and neoplastic bone marrow chromosome study with specific neoplastic interphase EGR1, D7S522 with D7Z1, D8Z2 and D20S108 FISH studies performed. The results of the cytogenetic studies showed chromosome 7q locus copy number loss. The patient had been on a hypomethylating agent with Vidaza but demonstrated resistance to the medication. Consequently, he was started on one cycle of Dacogen and was also receiving Procrit but with no response. The rest of the tests performed are itemized in Table 1. He is now receiving supportive care at a hospice facility.

**Table 1:** Results of tests performed on patient at the time of admission.

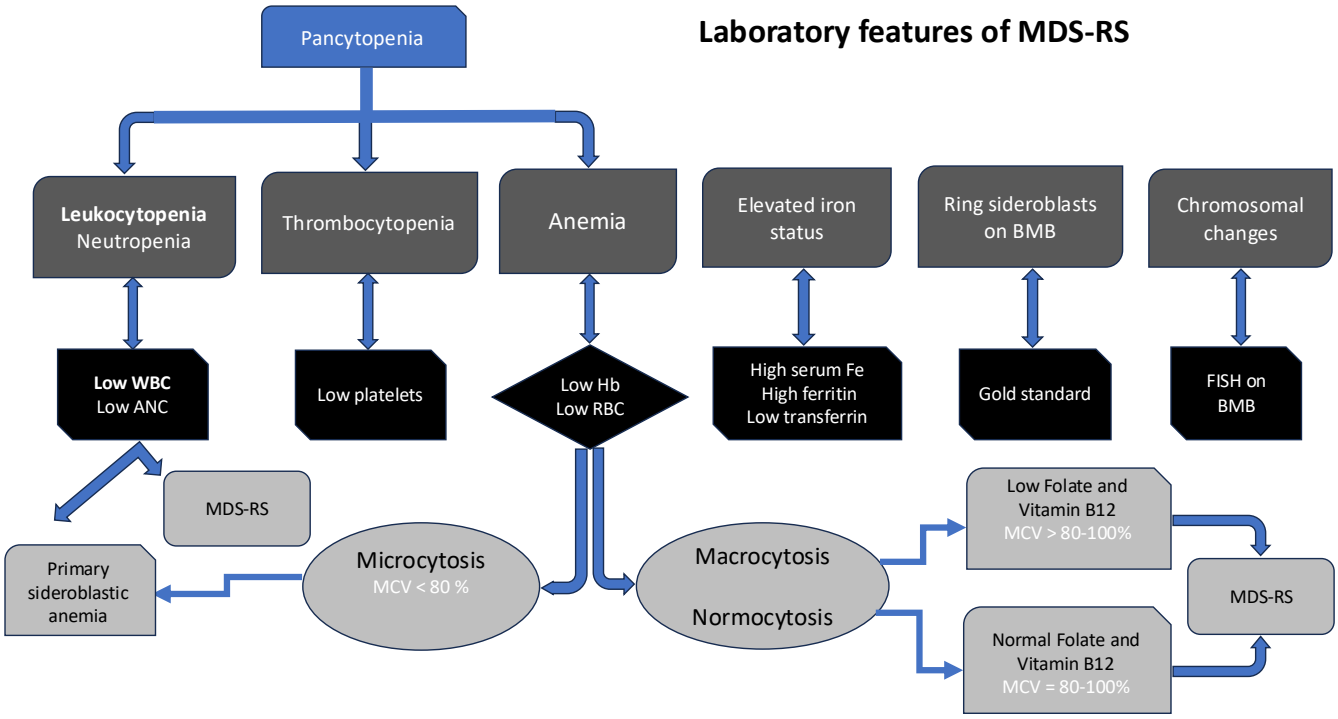
| Test | Result | Reference interval |
|---|---|---|
| Hemoglobin | 4.7 | 13.9–16.3 g/dL |
| RBC | 1.53 | 4.30–5.90 x 10^6/µL |
| WBC | 1.9 | 4.50–11.90 x 10^3/µL |
| Platelets | 3 | 150–400 x 10^3/µL |
| Hematocrit | 14.1 | 39–55% |
| MCV | 92.2 | 80–100% |
| MCH | 30.7 | 23.4–34.6 pg |
| MCHC | 33.3 | 31.0–37.0 g/dL |
| ANC | 0.72 | 2.0–7.5 x 10^3/µL |
| RDW | 19.2 | 11.5–14.5% |
| Absolute lymphocytes | 0.26 | 1–3.4 x 10^3/µL |
| Serum iron | 184 | 65–175 µg/dL |
| Iron saturation | 97 | 11–46% |
| Transferrin | 133 | 200–360% |
| Ferritin | 1425 | 8–388 ng/mL |
| TIBC | 190 | 260–400 µg/dL |
| Serum folate | >20 | 3.1–17.5 ng/mL |
| Vitamin B12 | 536 | 193–986 pg/mL |

Normal result, low result, high results.

**Case discussion**

The present case explores the laboratory features of MDS-RS (Figure 1). The initial investigation of anemia begins with an order for a complete blood count (CBC). Often associated with low hemoglobin, confirming anemic state is low RBC. The results for hemoglobin and RBC of the patient under review were very low, strongly indicating an anemic state. It's been reported that sideroblastic anemia patients have high RDW as it was in this scenario [2]. Neutropenia is a notable feature of sideroblastic anemia, particularly the primary form. On the other hand, myelodysplastic syndromes are also known to cause diminished neutrophils. The patient's low ANC which typifies neutropenia is therefore consistent with his sideroblastic anemia and potentially the extensive myelodysplastic involvement [4, 5].

**Figure 1:** Laboratory features of MDS-RS.




Routine CBC is the preliminary laboratory test panel in the work up of sideroblastic anemia. Pancytopenia is a common feature in MDS-RS and corresponds to low RBCs, low platelets, low WBCs. If CBC is ordered with differential, neutropenia, indicated by low ANC may be seen in either primary sideroblastic anemia or myelodysplastic syndromes. Normal to elevated iron status is a key component of sideroblastic anemia since in this condition, iron is present, but the body has a usage problem due to a broken mechanism. It is therefore common to find normal or high iron and ferritin, low transferrin and TIBC. In most cases of acquired sideroblastic anemia like MDS-RS, folate and vitamin $B^{12}$ are normal or may be slightly elevated. On the other hand, microcytosis is frequently associated with primary rather than secondary sideroblastic anemia. Ring sideroblasts on BMB is a key feature in MDS-RS. If 15% or more ring sideroblasts are involved, RARS is often more accurately the diagnosis. Patients with acquired myelodysplastic syndrome with ring sideroblasts often show chromosomal structural changes on BMB through FISH. MDS-RS: Myelodysplastic syndrome with ring siderobasts; BMB: Bone marrow biopsy; ANC: Absolute neutrophil count; MCV: Mean corpuscular volume; CBC: Complete blood count; FISH: Fluorescence In situ Hybridization; RARS: Refractory anemia with ring sideroblasts.

The MCV result of the patient propounds normocytic anemia, often associated with the acquired form of sideroblastic anemia. Microcytic anemia is a common feature of congenital sideroblastic anemia while macrocytic or normocytic anemia is often seen in acquired forms of the disease [6] (Figure 1). The high serum folate and normal vitamin B12 further rule against other causes of macrocytosis. The patient's high serum iron and ferritin are consistent with his normocytic anemia. Further, the patient's markedly elevated iron saturation, low transferrin and low TIBC confirm the overall elevated iron status of the patient. Microcytosis or macrocytosis is not a measure of discrimination to determine if a patient has sideroblastic anemia or not. This suggests that a routine CBC alone is inadequate to untangle the disease.

The gold standard diagnostic criterion for sideroblastic anemia is the visualization of ringed sideroblasts following a bone marrow biopsy examination. Of note, for this patient, three types of samples were retrieved from the bone for the diagnosis: bone marrow core biopsy, bone marrow clot section and bone

marrow aspiration smears. The results from all these specimens were accurately comparable: Myelodysplastic syndrome with features most compatible with refractory anemia with ring sideroblasts. A common complication of myelodysplastic syndromes is thrombocytopenia [7] {Basood, 2018 #4547}, and this is supported by the patient's extremely low platelet count. Myelodysplastic syndrome is the underlying cause of the patient's sideroblastic anemia. By contrast, thrombocytopenia is a rare clinical feature of copper-induced sideroblastic anemia [6]. Linezolid-induced thrombocytopenia in which ringed sideroblasts form has been reported [6], but the drug was not used for the patient. The confirmation of the patient's MDS, in addition to denying alcohol abuse, rules out alcohol as the secondary cause.

FISH is a technique used to evaluate the complete set of chromosomes by using a fluorescent dye-tagged probe that identifies and binds to specific complementary sequences on the chromosomes [8]. Interpretation of the FISH results revealed an abnormal cell population containing an isolated clonal chromosome 7q (long arm) deletion (breakpoints of 7q22 and 7q34), among ten of twenty metaphase cells examined by conventional cytogenetic techniques. A copy number loss for a chromosome 7q31 locus specific-probe signal, consistent with the chromosome 7q deletion as identified, was also demonstrated in cells examined by interphase and metaphase FISH methods. A loss of chromosome 7 long arm material (e.g., del(7q) or monosomy) is among the most frequently reported solitary aberrations observed in AML (3% of cytogenetically abnormal cases) but is also common in MDS and chronic myeloproliferative disorders. In addition, a loss of chromosome 7 long arm material is a frequent observation in therapy-related disease and as a secondary chromosomal aberration, especially in the setting of when an alkylating agent is used in the chemotherapy regimen or with radiation treatment affecting the bone marrow [9].

All the laboratory tests described here (Figure 1) are useful to evaluate the initial, differential and final diagnosis of both types of sideroblastic anemia (congenital/primary and acquired/secondary). But contrary to acquired sideroblastic anemia, the diagnosis of the congenital form of the disease almost always requires genetic testing to identify the specific mutation responsible for the pathological consequences. The different genetic tests are not described here since the current article is focused on acquired form of the disease. Most types of anemia including, but not limited to iron deficiency anemia, anemia of chronic disease, macrocytic and even megaloblastic anemia have many common clinical manifestations. Hence, these may constitute differential diagnosis of sideroblastic anemia and can pose minimal diagnostic conundrum. Care must therefore be taken in the work up of sideroblastic anemia in order to distinctively decipher the disease from other forms of anemia. Identification of ring sideroblast in bone marrow is a key that dichotomizes sideroblastic anemia from all other forms of anemia, and chromosomal testing with FISH isolates MDS-RS from other acquired forms.

Treatment of the patient commenced with 10 mL oral guaiFENesin-codeine, and opioid cough suppressant, to relieve his acute cough, being among the primary symptoms he presented with at the time of admission. The patient was treated with 2g of intravenous cefepime, a broad-spectrum cephalosporin antibiotic which functions by preventing the growth of both gram positive and negative bacteria [10]. This medication was important because of the patient's neutropenia which restricts the body's ability to fight infections. The patient's neutropenia may also have been partly responsible for his acquisition of Pseudomonas pneumonia infection. The patient benefited from blood transfusion, which, as noted earlier, is a standard approach for patients with severe cases of sideroblastic anemia [2, 11]. Transfusion is essential for patients with pancytopenia and severe anemia such as the current patient. The geriatric patient was also prescribed a multivitamin (1 tablet orally daily). The multivitamin treatment was essential to provide some necessary cofactors (of particular interest being pyridoxine-Vitamin B6) for the normal enzymatic activity in heme synthesis within the mitochondria. Critical to the patient's management is the administration of epoetin alfa (3000 units intravenously), an effective medication for bone marrow failure-induced anemia [12] to activate erythropoiesis in the bone marrow. The patient is currently in geriatric hospice care and is being closely followed and monitored.

**Learning points**

Thorough understanding of the work up of sideroblastic anemia is essential, not only for diagnostic and treatment purposes, but also pertinent to deciphering the specific etiology of the disease. The existence of many other types of anemia with convergent symptoms further complicates investigation of sideroblastic anemia and meticulous diagnostic process cannot be overemphasized. Some patients may end up in the emergency room with complications from sideroblastic anemia, augmenting the need for time-sensitive management. In addition, up to 12% of patients with RARS may develop leukemia, making laboratory-testing-based disease monitoring paramount. Complete blood count with differential is probably the most important preliminary test panel. Pancytopenia, normocytosis, macrocytosis or microcytosis are discernable on the CBC and these inform the need for further work up of the disease. Identification of ring sideroblasts on a peripheral blood smear or more effectively, on bone marrow biopsy is the definitive diagnosis for sideroblastic anemia but it still falls short of determining the actual etiology. Measurement of analytes such as copper, zinc, lead and certain drugs can exclude causes of such acquired forms of the disease. If clinically indicated, iron studies are recommended, since RARS, a form of MDS-RS is usually refractory to iron. FISH technology identifies chromosomal changes and thus, confirms MDS-RS.

Laboratory medicine is evolving and the need for clinical chemists to cover core laboratory and hematology is ever increasing. It is therefore imperative for chemists to thoroughly familiarize themselves with the work up and diagnosis of hematological diseases such as sideroblastic anemia. This will enhance proper stewardship and effective clinical consultation between lab directors and providers or hematologists, who will also benefit from precise test selection.

**Declaration of conflicts**

The authors declare no conflict of interest.

**Ethical Approval**

The University of Florida waves ethical approval if the manuscript involves only one clinical case.

**Author contributions**

Bremansu Osa-Andrews: Conceptualization, writing, reviewing, editing.
John O. Ogunbileje: Reviewing, editing.
Neil Harris: Reviewing, editing.
Tung Wynn: Reviewing, editing.

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Case report

# Unmasking MHC Class II Deficiency: A Novel Mutation in a Child with Pediatric ARDS due to Pneumocystis jirovecii Pneumonia

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**Keywords**

genetics, immune, respiratory tract, treatment, diagnosis

**Abstract**

An 8-month-old boy, the first child of a fourth-degree consanguineous couple with an uneventful past medical history, presented with fever and respiratory distress. He was intubated and managed with high-frequency ventilation. Chest radiography revealed bilateral white-out lungs, and his oxygenation index was 31. Pneumocystis jirovecii was identified through polymerase chain reaction of respiratory secretions. The child was treated with cotrimoxazole and systemic steroids. Due to the severity of the infection caused by an atypical organism, an underlying immunodeficiency was suspected. Genetic analysis revealed a novel homozygous mutation in the RFXANK gene, consistent with major histocompatibility complex class II deficiency. This case represents a rare inborn error of immunity with survival following a severe infection.

## Introduction

Major histocompatibility complex (MHC) class II deficiency, also known as 'bare lymphocyte syndrome,' is a rare inborn error of immunity (IEI) [1]. It is characterized by the absence of MHC class II molecules on the surfaces of immune cells, resulting in severe impairment of both cellular and humoral immune responses. Patients with this condition are highly susceptible to infections caused by a broad spectrum of bacterial, viral, fungal, and protozoan pathogens, with the respiratory and gastrointestinal systems being most commonly affected.

We report the case of an infant in whom Pneumocystis jirovecii pneumonia (PCP) led to the diagnosis of MHC class II deficiency with a novel mutation. Although a few cases of MHC class II deficiency have been reported from India, this is the first known child to survive pediatric acute respiratory distress syndrome (pARDS) caused by PCP [2].

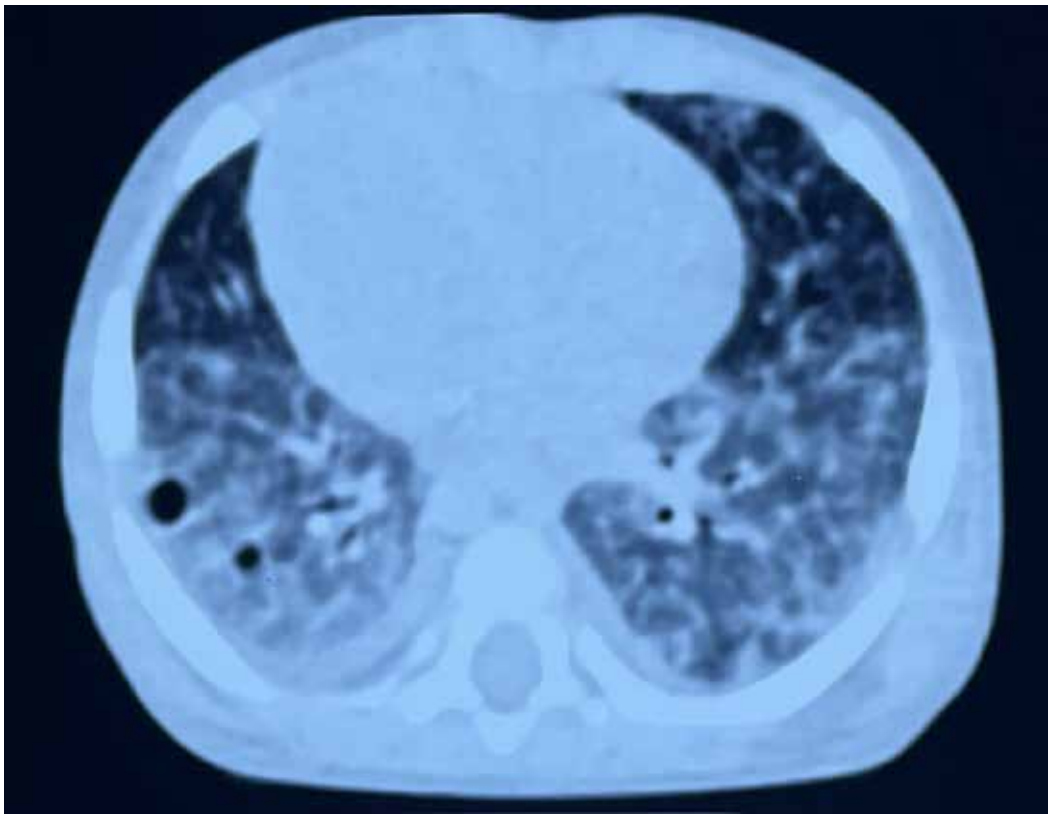
## Case Report

An 8-month-old male infant presented with complaints of fever, cough, and fast breathing for five days. He was the firstborn child of a fourth-degree consanguineous couple. His past and birth history were uneventful. He was developmentally normal and had been completely immunized for his age. His anthropometric parameters were within the normal range.

At the time of admission, the child exhibited tachycardia (heart rate: 142/minute), tachypnea (respiratory rate: 78/minute), severe intercostal and subcostal retractions, an audible grunt, central cyanosis, and an oxygen saturation of 71% despite receiving oxygen through a non-rebreathing mask. Chest auscultation revealed diffusely decreased air entry with crepitations. Examination of other systems was unremarkable.

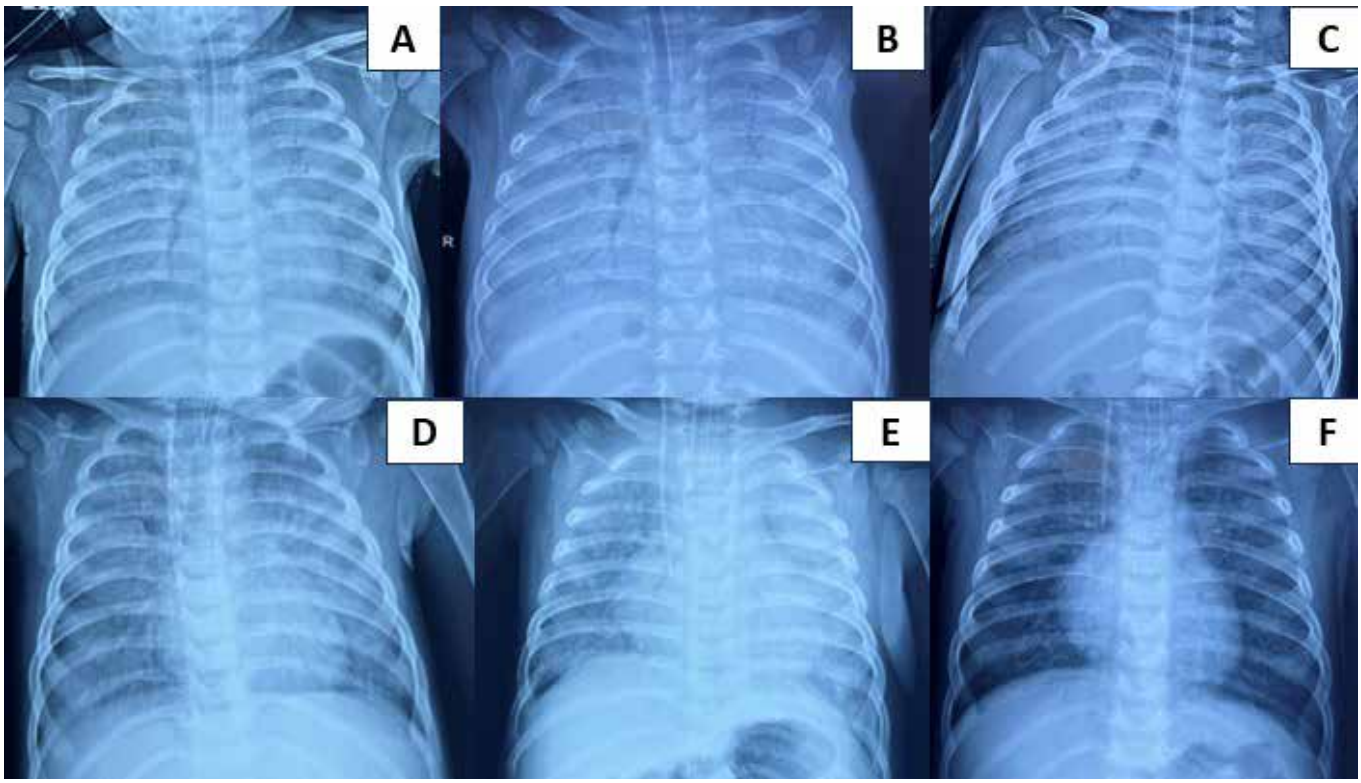
The child was intubated and placed on mechanical ventilation. Arterial blood gas analysis revealed hypoxemia and mixed acidosis (pH: 7.0, pO2: 42 mmHg, pCO2: 79 mmHg, HCO3: 18 mEq/L) with an oxygenation index (OI) of 31. A diagnosis of acute respiratory distress syndrome (ARDS) was made. Due to the failure of conventional ventilation, the child was transitioned to high-frequency ventilation (HFV). Empirical intravenous (IV) antibiotics- piperacillin-tazobactam (300mg/kg/day), and vancomycin (60mg/kg/day) were initiated. The hemogram revealed eosinophilic leucocytosis (total count: 34 × $10^3$/L, with differential counts of neutrophils 68%, lymphocytes 8%, and eosinophils 18%). Peripheral smear examination showed no atypical cells. Inflammatory markers, including C-reactive protein (CRP: 68 mg/dL) and erythrocyte sedimentation rate (ESR: 25 mm/hour), were elevated, as was serum lactate dehydrogenase (LDH: 874 U/L). Renal and liver function tests were normal. Blood and urine cultures were sterile. Chest radiography revealed bilateral homogeneous opacities, consistent with a white-out lung. Contrast-enhanced computed tomography (CT) showed diffuse confluent areas of airspace opacifications with surrounding ground-glass attenuation in the bilateral lung parenchyma, interspersed with multiple cystic lesions (Figure 1).

**Figure 1:** Computed tomography of thorax depicting diffuse, confluent areas of air space opacifications with surrounding ground-glass attenuation affecting both lungs, along with multiple interspersed cystic lesions.



Polymerase chain reaction (PCR) of respiratory secretions identified Pneumocystis jirovecii. Consequently, IV cotrimoxazole (Trimethoprim at 20mg/kg/day) and systemic corticosteroid (prednisolone at 1mg/kg/day) were initiated. The child's clinical and radiographic parameters gradually improved. Serial chest radiographs are shown in Figure 2.

**Figure 2:** Serial chest radiographs showing the gradual clearance of parenchymal infiltrates.




After 9 days on HFV, he was transitioned to conventional ventilation and extubated on day 12. He was discharged after completing 21 days of IV cotrimoxazole.

The occurrence of a severe atypical infection raised suspicion of an underlying immunodeficiency. Serum immunoglobulin (Ig) levels were measured during a follow-up visit and revealed the following: IgG 92.2 mg/dL (normal range: 217–904), IgM 17.7 mg/dL (normal range: 34–126), IgA 1.1 mg/dL (normal range: 11–90), and IgE 22.2 IU/mL (normal range: 0.76–7.31). Whole exome sequencing identified a homozygous single-base pair deletion in exon 6 of the RFXANK gene (chr19:g.19197565del; Depth: 33x), resulting in a frameshift and premature truncation of the protein 76 amino acids downstream of codon 128 (p.Leu128SerfsTer76; ENST00000303088.9) leading to a diagnosis of MHC class II deficiency. Parental genetic testing was recommended. The parents were counseled on the importance of prenatal genetic testing for subsequent pregnancies. The child was referred for hematopoietic stem cell transplantation (HSCT). He had non-specific viral infections twice in the three-month follow-up. Respiratory symptoms did not recur after the discharge.

## Discussion

MHC Class II deficiency is classified under combined cellular and humoral immunity as per the latest IEI classification [1]. MHC Class II molecules, also known as human leukocyte antigens, present exogenous peptides to the T-cell receptors on CD4+ T helper cells, thus playing a crucial role in developing a normal adaptive immune response. Mutations in the genes encoding the MHC class II transactivator (CIITA), regulatory factor X-associated protein (RFXAP), regulatory factor X-5 (RFX5), and ankyrin repeat-containing regulatory factor X (RFXANK) have been linked to the bare lymphocyte syndrome phenotype [3]. It is inherited in an autosomal recessive manner and is predominantly seen in the Mediterranean basin. This form typically has a milder course than other severe combined immunodeficiency syndromes, with the immune system retaining some functionality. This is evidenced by the presence of lymphocytes, positive lymphocyte proliferation in response to phytohaemagglutinin, and, in some cases, detectable levels of immunoglobulins [4]. The presentation begins in infancy with recurrent infections of the respiratory and gastrointestinal tracts along with septicemia. Frequently isolated organisms are Pseudomonas, Salmonella, E. coli, Streptococcus species, enterovirus, cytomegalovirus, herpes simplex virus, Candida

species, Giardia lamblia, and Cryptosporidium parvum [5].

pARDS is a clinical condition resulting from the disruption of the alveolar endothelial barrier, unrelated to cardiogenic pulmonary edema. Direct injury to the alveolar epithelium by infectious agents causing pneumonia is considered as the most common cause. The mortality rate of pARDS is as high as one in four cases [6]. PCP is an opportunistic fungal infection in MHC-II deficiency associated with high mortality. Diagnosis of PCP is multifactorial and may include clinical suspicion, patient risk factors, laboratory evaluation especially elevated LDH, chest computed tomography and definitive diagnosis requires detecting and identifying the organism by PCR assays of respiratory specimens.

Although MHC-II deficiency is less severe than severe combined immunodeficiency, most patients do not survive beyond early childhood. Supportive care, including IVIG and antibiotic prophylaxis, may extend survival only until early childhood. HSCT is regarded as the only curative treatment for this condition. Patients who do not undergo HSCT typically have a median survival of four to five years. Unfortunately, MHC class II deficiency patients seem to be at increased risk for developing post-transplant graft vs. host disease [7,8].

In a case series of 5 patients reported from India, all children exhibited disease onset before 6 months of age, with common manifestations including failure to thrive, sepsis, pneumonia, diarrhea, or candidiasis. Only two children had undergone HSCT. The reported mortality was 80% with respiratory failure being the commonest cause [9]. In contrast, our index case remained asymptomatic during the first half of infancy with normal growth and development. Additionally, presentation with PCP pARDS is noteworthy, as it is unreported in Indian children, although it has been documented globally [10].

Managing rare genetic disorders like MHC Class II deficiency carry significant challenges, particularly in resource-limited countries like India. The limited availability and high cost of genetic diagnostic facilities and definitive treatments like HSCT, and lack of newborn screening programs contribute to delayed diagnosis and poor outcomes. Many families face financial constraints leading to difficult access to specialized care and long-term IVIG therapy. Additionally, counseling regarding consanguinity and genetic risks is often limited, further perpetuating the incidence of such disorders. Policies promoting newborn screening, accessible diagnostic facilities, subsidised treatment costs, early genetic counseling for IEI along with awareness among healthcare providers can improve outcomes and reduce the disease burden in resource-constrained settings.

## Conclusion

Severe infection with Pneumocystis jirovecii can be the first manifestation of an IEI in an apparently healthy infant with normal growth and development. Detection of such infections should alert clinicians to evaluate for suspected IEI, enabling early diagnosis and intervention in the index case, as well as offering prenatal genetic testing for subsequent pregnancies.

## Declaration by authors

Manuscript has been read and approved by all the authors, the requirements for authorship as stated earlier in this document have been met, and that each author believes that the manuscript represents honest work and the information is not provided in another form.

## Conflict of interest

None.

## Acknowledgement

None.

## Contributions

AKS, DS, and ABT managed the case, did literature search and drafted the manuscript.

## Declarations

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Nil.

## Conflicts of interest

None declared.

## Availability of data and material (data transparency)

Not applicable.

## Code availability (software application or custom code)

Not applicable.

## Consent to participate

Signed informed consent obtained from patient.

## Consent for publication

Signed informed consent obtained from patient.

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Case report

# Navigating the challenge: Selecting the optimal assay for serum albumin measurement

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**Keywords**

serum albumin, nephrotic syndrome, Bromocresol green, Bromocresol purple

**Abstract**

**Background**: Photometric techniques are the most common methods for measuring serum albumin, where albumin binds with an organic dye to form a complex. These methods are popular due to their simplicity, automation potential, speed, and cost-effectiveness, with bromocresol green (BCG) and bromocresol purple (BCP) being the most frequently used dyes.

**Case presentation**: A 2-year-old boy presented with facial swelling, starting around the eyes in the morning and gradually spreading to the face, improving by evening, and accompanied by reduced urine output. He was diagnosed with nephrotic syndrome and treated with a high-calorie, high-protein diet, oral prednisolone, furosemide, and intravenous albumin. Despite treatment, his local laboratory's serum albumin level remained consistently low. However, tests from a private lab showed levels higher than the state lab. The nephrology team was informed of this discrepancy and chose to rely on the private lab's results.

**Conclusion**: A comparison study between BCP and BCG methods found a good correlation overall, but Bland-Altman analysis revealed that BCG had a significant positive bias compared to BCP, explaining the lab discrepancies. The study underscored the importance of clinician awareness of different serum albumin measurement methods, noting that BCP is more specific and does not overestimate albumin in kidney disease patients like BCG does.

**Introduction**

Serum albumin is a key biomarker for assessing nutritional status, liver function, and kidney health [1,2]. Accurate measurement is vital for proper diagnosis and management of various conditions. Photometric methods, using dyes like Bromocresol Green (BCG) and Bromocresol Purple (BCP), are widely used due to their efficiency and cost-effectiveness [3,4]. While both dyes are commonly employed, the choice between BCG and BCP can affect result accuracy, particularly in patients with conditions such as kidney disease [5]. Understanding these methods helps ensure precise diagnosis and treatment.

**Case presentation**

A 2-year-old male patient presented with facial swelling, first noticed around the peri-orbital region upon waking. The swelling gradually spread across the face throughout the day but lessened by evening. In addition, the child had a marked decrease in urine output, suggesting potential renal pathology. Aside from the facial swelling, other general and systemic examinations were normal. Based on the clinical presentation and investigative findings, a diagnosis of nephrotic syndrome was made, and the child was admitted for further assessment and treatment.

Upon admission, the child was started on a comprehensive treatment plan aimed at addressing the nephrotic syndrome. This regimen included a high-calorie, high-protein diet to support his nutritional needs, along with oral prednisolone to reduce inflammation and manage the disease process. In addition, furosemide, a diuretic, was prescribed to help alleviate fluid retention, and intravenous albumin was administered to replenish the low serum albumin levels commonly associated with nephrotic syndrome.

Despite this aggressive treatment approach, the serum albumin levels, as measured by the local laboratory, remained critically low at 6 g/L (with a normal reference range of 35–50 g/L). This persistently low reading was concerning, as it suggested ongoing hypoalbuminemia despite therapeutic interventions. To investigate further, serum albumin levels were also assessed at a private laboratory, where the results indicated significantly higher levels, ranging between 15–20 g/L (35–50 g/L).

The nephrology team carefully reviewed the discrepancies between local and private lab results and, noting the impact of inaccurate albumin measurements on treatment, opted to rely on the private lab's results, which aligned with the clinical picture. A comparison study of the BCP and BCG methods was conducted to address this discrepancy.

However, based on the findings of the comparative study, the patient was provided with an additional albumin supplement, which resulted in improvements in oedema, proteinuria, and the overall clinical condition, that helped the nephrologist to decide on accurate treatment.

**Differential Diagnosis**

Given the diagnosis of nephrotic syndrome and the persistently low serum albumin levels, two main differential diagnoses were considered:

was excluded, as the prescribed dosages were appropriate and aligned with the standard treatment protocols.

**Serum Albumin Assay Discrepancies**

The other concern was the accuracy of the serum albumin measurements. The significant difference between the local laboratory and the private laboratory results led to the consideration of assay discrepancies. This was likely due to the use of different methods for measuring albumin, with the local lab potentially using a more specific but less reliable method for hypoalbuminemia cases (BCP), and the private lab using a method (BCG) that was more consistent with the clinical picture. After careful review, this assay discrepancy was deemed the likely explanation for the low serum albumin levels reported by the local lab.

Thus, the assay discrepancy was identified as the primary cause of the differing albumin results, rather than a suboptimal treatment regimen.

**Diagnostic Workup**

To evaluate the situation, the following steps were undertaken:

A total of 59 anonymized serum samples were randomly selected and analyzed using three analyzers: Dimension EXL 200 (BCP assay), Cobas c 311 (BCG assay), and Beckman Coulter AU 480 (BCG assay). The results were compared using linear regression and Bland-Altman methods. Additionally, 16 more samples were tested with the Dimension BCP method and compared with results from an external lab using the Abbott Architect Plus C8000 (BCP assay). The comparison was analyzed through linear regression, with statistical analysis performed in Excel (version 1808).

**Results**

Before testing patient samples, quality control checks were performed, with all results falling within acceptable ranges. The BCP method, serving as the reference standard, was compared with the BCG method to evaluate its accuracy.

The analytical measurement ranges for serum albumin are 6–80 g/L on the Dimension EXL 200, 2–60 g/L on the Cobas c 311, and 15–60 g/L on the Beckman AU 480. Our serum albumin samples ranged from 4–50 g/L. The comparison plots showed that these samples covered the entire analytical measurement range, demonstrating a linear relationship between the BCP and BCG methods within this range (Figure 1 and 2).

Bland-Altman plot (Figure 4) indicated that the Cobas c 311 BCG assay was positively biased compared with Dimension EXL 200 BCP method; producing results that were approximately 6 g/L higher.

Linear regression analysis showed good agreement ($y = 0.9065x + 1.315$, $R2 = 0.9848$) between the Dimension EXL 200 BCP results and external laboratory Abbott Architect Plus C8000 BCP results (Figure 3).

**Figure 1:** The Comparison Plot between BCP and BCG method between Cobas c 311 and Dimension EXL 200

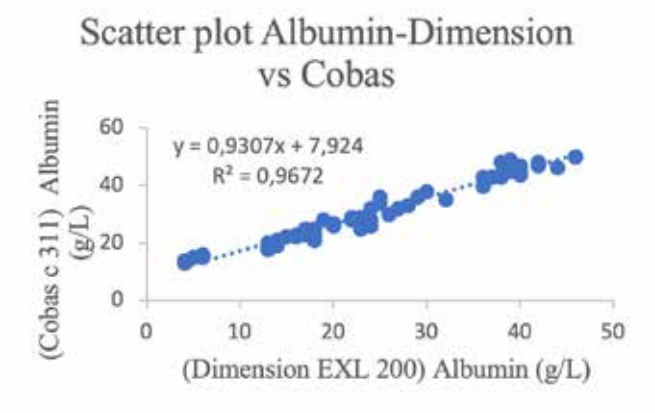



**Figure 2:** The Comparison Plot between BCP and BCG method between Beckman Coulter AU480 and Dimension EXL 200

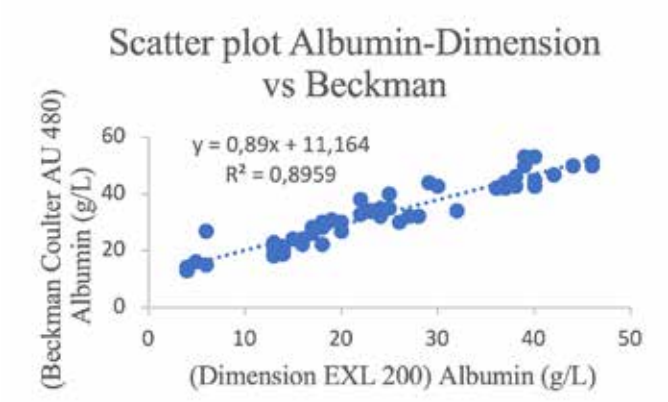



**Figure 3:** The Comparison Plot between Dimension and Abbot Architect BCP method

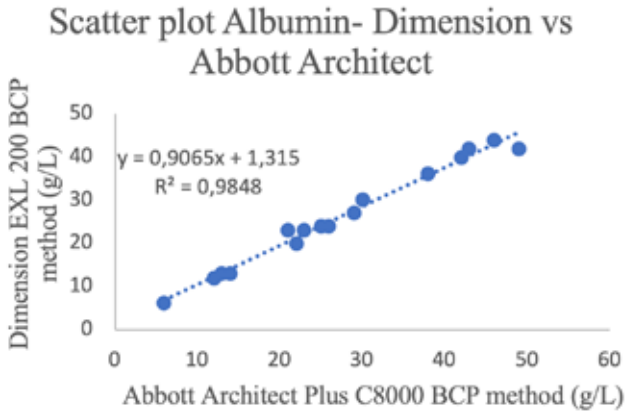


**Figure 4:** Bland-Altman plot comparison of albumin results for Dimension EXL200 BCP and Cobas c 311 BCG methods.

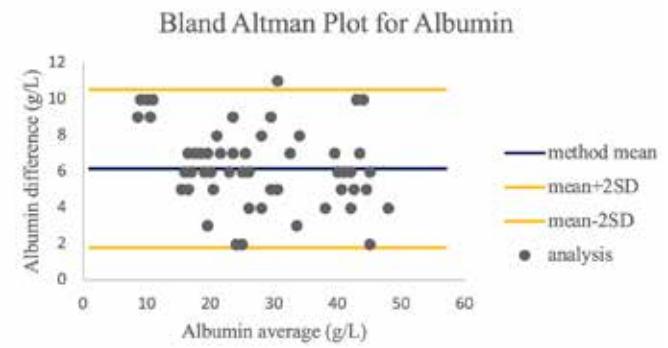
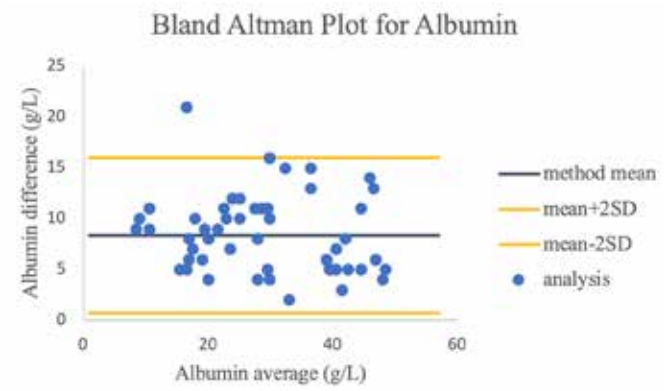



**Figure 5:** Bland-Altman plot comparison of albumin results for the Dimension BCP and Beckman Coulter AU480 BCG methods.




The Bland-Altman plot (Figure 5) showed that the Beckman AU 480 BCG assay had a positive bias, yielding results approximately 8 g/L higher than the Dimension EXL 200 BCP method. Most results fell within the 95% confidence interval.

## Discussion

Albumin is a crucial biomarker in kidney disease, as low serum albumin levels often indicate nephrotic syndrome or other forms of kidney dysfunction. Monitoring albumin levels helps assess the severity of kidney damage and guides treatment decisions to prevent further complications [6,7].

In this study, we compared serum albumin measurements obtained from three different analyzers in one laboratory: Dimension EXL 200, Cobas c 311, and Beckman AU 480. Values of serum albumin samples, which ranged from 4–50 g/L, effectively covered the concentration ranges of analyzers.

The comparison plots revealed a linear relationship between the BCP and BCG methods, indicating that both methods performed comparably within the tested concentration range. This linearity suggests that both methods can be used effectively within their respective ranges for measuring serum albumin levels.

Linear regression analysis showed a strong correlation between the Dimension EXL 200 BCP results and the external lab's Abbott Architect Plus C8000 BCP method, with an equation of $y = 0.9065x + 1.315$ and $R^2 = 0.9848$, confirming the accuracy of the Dimension EXL 200 analyzer.

Bland-Altman analysis revealed a positive bias in the Cobas c 311 and Beckman AU 480 BCG assays, with albumin results approximately 6 g/L and 8 g/L higher, respectively, than the Dimension EXL 200 BCP method. While most results fell within the 95% confidence interval, the BCG method tended to overestimate serum albumin levels compared to the BCP method, indicating consistent but clinically significant discrepancies.

This case report provides value in three key areas: clinical presentation and management of nephrotic syndrome, analytical challenges with serum albumin measurement, and the importance of statistical comparisons in laboratory medicine. It serves as an important lesson in ensuring the alignment of clinical signs with laboratory findings and recognizing the limitations of different diagnostic assays in medical practice [8,9].

## Conclusion

This study highlights significant differences between serum albumin measurement methods, particularly between BCP and BCG techniques [3,4]. The BCP method showed excellent agreement across analyzers, supporting its reliability as a reference standard. In contrast, the BCG method exhibited positive biases, leading to higher albumin readings. Clinicians should be mindful of these biases when interpreting results, as accurate serum albumin measurement is critical for diagnosing and managing conditions like nephrotic syndrome [8,10]. The clinical team chose the BCP method to ensure more accurate patient assessments and better management outcomes.

There are certain limitations need attention. First, it focuses

on a single patient, which restricts the generalizability of its findings to broader populations or settings. The absence of a larger dataset further limits the robustness of the statistical analyses. Additionally, while the study compares the BCG and BCP methods, it does not include electrophoresis as a reference technique, which could have provided a more comprehensive evaluation of the methods' biases.

The lack of access to electrophoretic analysis, which is considered a more precise method for evaluating serum albumin, restricts the ability to provide a more comprehensive assessment of the biases between the two methods. Finally, the study was conducted in a single laboratory setting, which may limit the applicability of the findings to other laboratories with different equipment, reagents, and standard practices. Addressing these limitations in future research will provide a more comprehensive understanding of serum albumin measurement discrepancies and their clinical implications.

**Learning Points**

Importance of serum albumin in diagnostics: Serum albumin is a key biomarker for assessing nutritional status, liver function, and kidney health, crucial for diagnosing and managing various conditions.

Photometric methods: These are widely used for serum albumin measurement due to their efficiency, simplicity, and cost-effectiveness, relying on the interaction of albumin with a dye.

BCG vs BCP: The choice between BCG and BCP methods can impact accuracy, particularly in patients with kidney disease, as both are commonly used but may yield different results.

Clinical implications: Discrepancies in lab results, as seen in the case study, can complicate diagnosis and treatment, underscoring the need for accurate measurements.

**Perspective of Laboratory Input**

The clinical team recognizes the importance of understanding the differences between BCG and BCP methods for serum albumin measurement. The study's findings of a positive bias in BCG highlight potential inaccuracies that could affect patient management.

Discrepancies in lab results raise concerns about over- or under-treatment, especially in kidney disease management. As a result, the team is considering shifting to more reliable methods like BCP and exploring external lab verification for critical tests. They emphasize the need for collaboration with labs to ensure accurate diagnostics through regular reviews and protocol updates.

The team supports ongoing research to improve diagnostic methods and monitoring of serum albumin measurement practices. They believe further studies could refine current techniques and lead to more accurate tools, enhancing care for patients, especially those with conditions like nephrotic syndrome.

**Conflicts of Interest**

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript.

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