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INTERNATIONAL JOURNAL OF
MEDICAL BIOCHEMISTRY

ABOUT JOURNAL

AIM AND SCOPE

International Journal of Medical Biochemistry (IJMB) is a double-blind peer-reviewed, open-access journal of the Association of Clinical Biochemistry Specialists Türkiye.

International Journal of Medical Biochemistry (IJMB) publishes articles relating to clinical and experimental chemistry, molecular biology, genetics, therapeutic drug monitoring, toxicology, immunology, hematology and laboratory medicine with the focus on analytical and clinical investigation of laboratory tests used for diagnosis, prognosis, and monitoring of diseases.

BASIC PUBLICATION RULES

Authors are responsible for the accuracy of data. The Editorial Board of the International Journal of Medical Biochemistry and the Publisher adheres to the principles of the International Council of Medical Journal Editors (ICMJE), the World Association of Medical Editors (WAME), the Council of Science Editors (CSE), the Committee on Publication Ethics (COPE), the US National Library of Medicine (NLM), the World Medical Association (WMA) and the European Association of Science Editors (EASE).

DISCLAIMER

Statements or opinions expressed in the manuscripts published in IJMB reflect the views of the author(s) and not the opinions of the editors, the editorial board and the publisher; the editors, the editorial board and the publisher disclaim any responsibility or liability for such materials.

JOURNAL FREQUENCY

The International Journal of Medical Biochemistry published three issues per year from its establishment until 2024. The journal has started publishing four issues per year since 2025. The publication months are January, April, July, and October.

ABSTRACTING AND INDEXING

International Journal of Medical Biochemistry is indexed in TUBITAK TR Index (2019), CNKI (2019), TurkMedline (2019), Open Ukrainian Citation Index (2019), ProQuest (2019), CABI (2021), CEEAS (2021), EBSCO (2022), CAS (American Chemical Society) (2022), Directory of Open Access Journals - DOAJ (2022), Scopus (2023), Gale Cengage (2023), Sherpa Romeo (2024), ASCI (2024) and Web of Science - ESCI (2025), EZB (2025), OpenAlex (2025), Sudoc (2025), ZDB (2025).

ABBREVIATION

Int J Med Biochem

PUBLICATION FEE

Submission Fee

As of January 1, 2026, authors are required to pay a submission fee of USD 120 (including VAT) at the time of manuscript submission to the International Journal of Medical Biochemistry (IJMB). The submission process will not be initiated for manuscripts for which the submission fee has not been paid. This fee is charged to contribute to the costs of editorial handling. The submission fee is applied during the manuscript submission process and is non-refundable, regardless of the final editorial decision (acceptance or rejection). Kindly note that any bank transaction fees must be covered by the author.

Article Processing Charge (APC)

As of January 1, 2026, manuscripts submitted to the International Journal of Medical Biochemistry (IJMB) that are accepted for publication will be subject to an Article Processing Charge (APC) of USD 120 (including VAT). The publication process will not proceed unless the APC has been fully paid. This fee is collected to support the production, and publication of the accepted manuscripts. Kindly note that any bank transaction fees must be covered by the author.

Additional Information

Authors based in Türkiye may pay the above fees in Turkish Lira (TRY), calculated based on the USD–TRY exchange rate applicable at the time of payment. For any questions regarding payments, please contact us at kare@karepb.com.

ACCESS TO JOURNAL CONTENT

The abstracts and full texts of published articles can be accessed free of charge at www.internationalbiochemistry.com.

INTERNATIONAL JOURNAL OF
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INSTRUCTIONS FOR AUTHORS

AIMS & SCOPE

International Journal of Medical Biochemistry publishes articles relating to clinical chemistry, molecular biology and genetics, therapeutic drug monitoring and toxicology, hematology, immunology and laboratory medicine with the focus on analytical and clinical investigation of laboratory tests used for diagnosis, prognosis, treatment and therapy, and monitoring of disease. The official language of the Journal is English. The journal will be published 4 times a year in print and electronically.

Main Topics

- Clinical Biochemistry
- Molecular Biology
- Clinical Haematology
- Clinical Immunology
- Drug Monitoring and Analysis
- Diagnostic Biomarkers
- Disease-Oriented Topics (Cardiovascular Disease, Cancers, Diabetes, Obesity, Genetic Disorders, Neurodegenerative Disease e.g..)
- Pediatric Biochemistry
- Inherited Metabolic Disorders
- Newborn Screening: Congenital and Genetic Disorders
- New Reagents, Instrumentation, Technologies and Methodologies
- Laboratory Medicine; Quality, Safety, translational laboratory
- Clinical Metrology

INSTRUCTIONS FOR AUTHORS

International Journal of Medical Biochemistry is published in accordance with the principles of independent, unbiased, and double-blind peer review. Research articles, review articles, short communications, case-reports, opinion papers, letter to editor, technical notes, editorials and article-commentaries that have not been published elsewhere, are published in the journal. **Four issues are released every year in January, April, July, and October**.

The journal evaluates only the manuscripts submitted through its online submission system on the web site http://www.internationalbiochemistry.com Manuscripts sent by other means will not be accepted.

The primary conditions for the acceptance of manuscripts for publication are originality, scientific value and citation potential.

The manuscripts should be prepared in accordance with ICMJE Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals (updated in May 2022 - https://www.icmje.org/recommendations/).

Authors are required to prepare manuscripts in accordance with the international guidelines* below.

Randomized Controlled Trial **	CONSORT (Consolidated Standards of Reporting Trials)
Non-Randomized Trial **	TREND (Transparent Reporting of Evaluations with Non-randomised Designs)
Trial Protocol	SPIRIT (Standard Protocol Items Recommendations for Interventional Trials)
Observational Epidemiologic Studies (cohort, case-control cross-sectional)	STROBE (the STrengthening the Reporting of OBservational studies in Epidemiology)
Systematic Reviews and Meta-Analyses	PRISMA (the Preferred Reporting Items for Systematic Reviews and Meta-Analyses)
Systematic Reviews and Meta-Analyses Protocol	PRISMA-P (the Preferred Reporting Items for Systematic Reviews and Meta-Analyses - Protocol)
Qualitative Research	-SRQR (the Standards for Reporting Qualitative Research) -COREQ (COnsolidated criteria for REporting Qualitative research: interviews and focus groups
Methodological Studies (Translating and Adapting Tests)	ITC (International Test Commission) Guidelines for Translating and Adapting Tests
Methodological Studies (Developing Tests)	COSMIN (COnsensus based Standarts fort the selection of Health Measurement Instruments-Study Design for Patient-reported outcome measurement instruments)
Case Report	CARE (CAse REport)

* Enhancing the QUAlity and Transparency of Health Research (equator network).
** The International Journal of Medical Biochemistry encourages the registration of all clinical trials (randomized and non-randomized) via ClinicalTrials.gov or one of the registries of the WHO's International Clinical Trials Registry Platform (ICTRP). The name of the trial registry and the registration number together should be provided at the end of the abstract.

PEER REVIEW PROCESS

International Journal of Medical Biochemistry utilizes a double-blind peer review process where both the identities of reviewers and authors are anonymous. The Editor-in-Chief is responsible for the editorial and scientific content of the journal and makes the final decision.

Submitted manuscripts that pass preliminary control are evaluated by Editor-in-Chief for their originality, methodology, the importance of the subject covered and compliance with the journal scope. For the manuscripts that are considered for publication an unbiased, double-blind peer-review process is initiated by associate editors. The manuscripts are sent to two or more independent peer reviewers who are expert in the field. The reviewers are asked to provide detailed, constructive comments that will help the editor to make a decision, and the authors to improve their manuscript. The requested changes are made by the authors using the online manuscript submission system. Associate editor sends the original manuscript, the revised manuscript and all the reviewers' comments together with his recommendation to the Editor-in-Chief.

Editor-in-Chief reviews the manuscript and makes the final decision. He might accept the manuscript, reject the manuscript or request further revisions.

In case of manuscripts submitted by editors or by the editorial board members of the journal, the editorial board will invite an external, independent editor to manage the evaluation processes of these manuscripts. The reviewers

are required to notify the editor of any kinds of conflicts of interest with respect to the research, the authors and/or the research funders. They must ensure that all the information related to submitted manuscripts is kept as confidential and must report to the Editor-in-Chief if they are aware of copyright infringement and plagiarism on the author's side.

International Journal of Medical Biochemistry takes into consideration the Committee on Publication Ethics - COPE guidelines and flow charts for peer review process.

PUBLISHING FEE

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PREPARATION AND SUBMISSION OF MANUSCRIPTS

Manuscript files should be prepared with Microsoft Office Word. The online submission system will direct authors during all stages of submission and provide necessary support for accelerating the submission process. A list of the files that should be supplied through the online submission system is provided below.

- Title Page
- Main Text
- Tables, Graphs and Figures
- Copyright Transfer Form
- Author Contribution Form

ICMJE Uniform Disclosure Form for Potential Conflicts of Interest.

MANUSCRIPT TYPES

Contributions may be in the form of clinical and basic Original Research articles, Reviews, **Short communications**, Case reports, Letters to the Editor, **Opinion papers**, Technical notes and **Editorial Comment**. The Journal also publishes brief reports on original studies or evaluations, book reviews, and proceedings of scientific meetings.

Original Research: Consists of Title, Authors, their addresses, Abstract, Key Words, Introduction, Materials and Methods, Results, Discussion, Ethical Considerations (if necessary), Acknowledgements, Conflict of Interest, References, Figure Legends, Figures, and Tables. It should not exceed 5000 words excluding the references. The manuscript should have no more than 50 references and a total of 6 tables and/or figures.

Review: Reviews prepared by authors with extensive knowledge on a particular field, which has been reflected in international literature by a high number of publications and citations, are evaluated. The authors may be invited by the Editor-in-Chief. A review should be prepared in the format describing, discussing and evaluating the current level of knowledge or topic that is to be used in the clinical practice and it should guide further studies. A review article consists of Title, Authors, their addresses, Abstract, Key Words, Introduction, Main Sections under headings written in bold and sentence case, Subsections (if any) under headings written in italic and numbered consecutively with Arabic numerals, Conclusion, Acknowledgements, Conflict of Interest, References, Figure Legends, Figures, and Tables. The manuscript should have no more than 75 references and a total of 6 tables and/or figures. Supplemental data are permitted for Review articles. Abstract should not include subheadings and should be limited to 300 words. Keywords section should contain a minimum of three and a maximum of six items in accordance with Medical Subject Headings (MeSH) terms prepared by the National Library of Medicine (NLM) and should be provided just below the abstract. Main Text should include Introduction, other subheadings and Conclusion sections and should be limited to 5000 words excluding the references. Tables, Figures and Images should be provided after the reference list according to their order of appearance in the text.

Note: The originality of the visuals included in the reviews should be assured by submission of an accompanying letter by the authors. Appropriate citation should be done for the visuals adapted from previously published sources, in accordance with the original versions of the printed or electronic copies. The written permission obtained from the copyright holder (publisher, journal or authors) should be sent to the Editor-in-Chief's Office.

Short communications: These manuscripts are intended to concise and quick publishing of a new finding. Publishing of research articles under this concept is decided by the Editorial Board, providing that the authors adhere to the publishing format. The general format of this type of manuscript is similar to that of research articles except the word and reference limitations. For the short communications, main text should not exceed 1,500 words and number of references should not exceed 15.

Case Report: Since a limited number of case reports is published, only reports which are related to rare cases and conditions that constitute challenges in diagnosis and treatment, offer new methods or suggest knowledge not included in books, and are interesting and educational are accepted for publication. A case presentation consists of Title, Authors, their addresses, Abstract, Key Words, Introduction, Patients and Methods, Results, Discussion, Conclusion, Ethical Considerations, Acknowledgements, Conflict of Interest, References, Figure Legends, Figures, and Tables. Main Text should not exceed 1500 words excluding the references. The reference list should follow the main text and the number of references should be limited to 15. Tables, Figures and Images should be provided after the reference list according to their order of appearance in the text and should be limited to two.

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Letter to the Editor: Letters to the Editor aim to discuss the importance of a manuscript previously published in the journal. This type of manuscripts should also include a comment on the published manuscript. Moreover, articles on topics of interest to readers within the scope of the journal, especially on educational issues, can be published in the format of a Letter to the Editor. It consists of title, main text and references sections. ***Abstract, Keywords, Tables, Figures and Images, and other visuals are not included.*** Main Text should not include subheadings and it should be limited to 500 words. The reference list should follow the main text and the number of references should be limited to five. The volume, year, issue, page numbers, authors' names and title of the manuscript should be clearly stated, included in the list of references and cited within the text.

Opinion papers: Opinions on the topics within the scope of the journal that are prepared by the experts are published in this section. An opinion consists of Title, Authors, their addresses, Abstract, Key Words, Introduction, Discussion, Conclusion, Ethical Considerations (if necessary), Acknowledgements, Conflict Of Interest, References, Figure Legends, Figures, and Tables. Editorial Board decides the eligibility of an opinion with respect to its concept and language. For the opinions, main text should not exceed 1,500 words and number of references should not exceed 15.

Technical reports: Manuscripts on development and application of new methodologies are published in this category. A technical report consists of Title, Authors, their addresses, Abstract, Key Words, Introduction including the main aspects of the method involved, Materials and Methods, Results, Discussion, Ethical Considerations (if necessary), Acknowledgements, Conflict of Interest, References, Figure Legends, Figures, and Tables. Data analysis should be presented in Materials and Methods. Clinical technical reports should include and discuss the clinical significance of values and their deviations. For the technical reports, main text should not exceed 1,500 words and number of references should not exceed 10.

Editorial Comment: Authors are selected and invited by the Editor-in-Chief. This type of manuscript aims at providing a brief commentary on an article published in the journal by a researcher who is an authority in the relevant field or by the reviewer of the article. It should consist of title, main text and references sections. Main Text should not include subheadings and should be limited to 500 words. The reference list should follow the main text and the number of references should be limited by 15. Abstract, Keywords, Tables, Figures, Images and other visuals are not included in editorial comments.

PREPARATION OF MANUSCRIPTS

Manuscripts should be prepared according to the above mentioned word and reference limitations and other related information. Language: Manuscripts should be written in clear and concise English. Please have your text proofread by a native English speaker before you submit for consideration.

The manuscripts submitted to our journal are classified and evaluated according to the manuscript types stated below. During preliminary evaluation, the editors assess whether a manuscript's format and sub-headings are prepared in accordance with the journal's guidelines. Therefore, it is important that authors check the accuracy of the main text in terms of the following.

Title Page: ***Title*** should be concise and informative and reflect the content of the manuscript and should not exceed 15 words. Avoid abbreviations and formulae where possible. It should be written in sentence case; that is, first letter of the initial word should be written in capital letter and rest of the title should be typed with lower case letters except proper nouns and abbreviations.

Information about the authors and their institutions should not be included in the main text, tables and figures . Since submitted manuscripts are evaluated by the reviewers through the online system, personal identification is excluded in the interests of unbiased interpretation. Thus, only information about the manuscript as specified below should be included on the title page. For each type of manuscript, it is mandatory to upload a title page as a separate Microsoft Word document through the online submission system. The title page should include the names of the authors with their latest academic degrees, and the name of the department and institution, city and country where the study was conducted. If the study was conducted in several institutions, the affiliation of each author must be specified with symbols. The first letters of authors' names and surnames should begin with capital letter and the rest should be written in lower case letters. If there is more than one author, corresponding author should be indicated.

The correspondence address should contain the full name of the corresponding author, postal and e-mail addresses, phone and fax numbers. If the content of the manuscript has been presented before, the name, date and place of the meeting must be noted. ***Disclosure of conflict of interest***, institutional and financial support, author contributions and acknowledgments should be included on the title page.

Structured Abstract: It should be structured with Objective, Methods, Results and Conclusion subheadings and should be limited to 300 words. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided. Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself. Level of evidence has to be stated at the end of the abstract as a separate paragraph.

Keywords: This section should contain a minimum of three and a maximum of six items in accordance with Medical Subject Headings (MeSH) terms prepared by the National Library of Medicine (NLM) and should be placed just below the abstract.

Main Text: Text should be prepared with MS Word document. All text should be written with Arial font type at 12 font size and double spaced on A4 sized paper with 1 cm right margin and 3 cm other margins. Header and/or footer should not be inserted in the text except that the pages should be numbered with Arabic numerals. Main text should consist of title, introduction, materials and methods, results and discussion.

Introduction: State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results. The unnecessary details and excessive references should be avoided. Hypothesis and the aim of the study should be stated in the introduction.

Material and methods: Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described. Design and place of the study, subjects/patients, if applicable, information on the treatment procedures, statistical methods and information on the adherence to the ethical rules should be indicated.

Statistical analysis should be conducted in accordance with the guidelines on reporting statistical data in medical journals. The software used for statistical analysis must be described. The outcomes of statistical analyses and interpretation of the results must be in evidence-based scientific language. Statistics- statistical methods should be explained in detail in the Materials and Methods so that if original data is given, the results can be verified. If possible, quantitative results should be given and appropriate indicators should be used to indicate measurement error or uncertainty.

Results: Results should be clear and concise. Results must be concise and include figures and tables. Descriptive statistics should be compatible with the nature of data and statistical analyses used. The graphs should be prepared to reflect the important features of data. Please avoid excessive figures and tables.

Tables, Graphs and Figures

- Tables, Graphs, Figures and other visuals should be numbered in the order of their citation within the text and names of patients, doctors and institutions should not be disclosed.
- Tables should be prepared in a Microsoft Office Word document using the command 'Insert Table' and inserted at the end of the references in the main text.
- Tables should not be submitted in JPEG, TIFF or other visual formats. For microscopic images, the magnification ratio and staining technique used should be specified in addition to figure legends.
- Roman numbers should be avoided in tables and figures within the text and their titles. Each table should be clearly labeled and numbered consecutively with Arabic numerals (Table 1, Table 2, etc.). The abbreviations used must be defined. As with the text, reporting of concentration units consists of analyte concentrations expressed in the text in the traditional mass unit (mg/dL, ng/ml, and so forth) followed by the SI unit in parentheses
- The thickness of the lines in graphs should be sufficient to minimize loss of quality if size reduction is needed during the printing process. The width of the graphs should be 9 cm or 18 cm. Drawings should be performed by professionals. No grey colours should be used.
- Abbreviations should be explained in alphabetical order at the bottom of the tables, graphs and figures.
- Explanations should be at the bottom of the table as footnotes.
- Tables should not be divided into parts. Separate parts (Table 1A, Table 1B, etc.) will be considered separate tables and will count toward your overall table/figure count.
- Table captions should be limited to 60 words.
- Tables should be easily understandable and should not repeat the data in the main text.
- In addition to the pictures included in case reports and original images, video and movie images are published on the journal's website. These images should be prepared in MPEG format with a maximum size of 2 MB. They should be submitted to the journal with the manuscript documents. The names of patients, doctors, institutions and places should be omitted from all documents.
- All figures must be uploaded separately as image files in Tagged Image File Format (.TIFF) or Encapsulated Postscript (.EPS). Microsoft Power Point (.PPT) is also acceptable; however, the graphics must contain embedded fonts with one image per slide, one slide per file. Each image should have a resolution of 600 dots-per-inch (dpi).
- Indicate each footnote in a table with a superscript lowercase letter.

Discussion: This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions: The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices: If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Abbreviations: Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements: Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. Financial and/or technical (running of tests, evaluation of results, providing language help, writing assistance or proof reading the article, etc.) supports of the study should be mentioned following Ethical Considerations including the project number. If the study originates from a thesis or has been previously presented in a meeting, this should also be indicated in this section. Contribution of each author should be declared in this section.

Nomenclature and units: Follow internationally accepted rules and conventions: use the international system of units (SI). If other quantities are mentioned, give their equivalent in SI. Authors wishing to present a table of nomenclature should do so on the second page of their manuscript. You are urged to consult IUB: Biochemical Nomenclature and Related Documents for further information.

Footnotes: It should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many word processors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Math formulae: Please submit math equations as editable text and not as images. Present simple formulae in line with normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Special Terms and Conditions: For double-blind peer-review process, the names of the corresponding author and other authors, their affiliations and any information on the study centres should not be included in any part of the submitted manuscripts and images, except the Title Page. This information should be added to the relevant section of the online submission system and included in the Title Page.

Pharmaceutical products should be written with their generic names and brand and company names, city and country should be specified for medical equipment and devices.

For centrifugation, angular velocity (xg) should be given. Period should be used to show decimals.

Enzymes: Full name of an enzyme and its International Union of Biochemistry and Molecular Biology (IUBMB) Enzyme Commission number (EC number) should be given when that enzyme was first mentioned in the text. Its abbreviation can be used after that. For detailed information, authors can refer to the Enzyme Nomenclature of IUBMB (www.chem.qmul.ac.uk/iubmb/enzyme). Enzyme activity should be given in terms of U/L in serum or plasma and U/mg protein in tissues. Unit (U) should be given as reaction rate triggered under defined conditions. SI unit for enzyme activity is defined as katal which is the moles of substrate converted to product per second. However, it can also be defined as µmol/sec or µmol/min as long as clear and precise definitions are given.

References

- References should start on a separate page. Published manuscripts and manuscripts that have been accepted and are pending publication should be cited in the reference list. Acceptance letter of the manuscripts that are pending publication should be submitted with the manuscript. Unpublished results should be written in text in parenthesis as "(unpublished result)". If such a work is cited, permission from the responsible researcher should be obtained and submitted with the manuscript.
- The reference list containing all authors mentioned in the text, should be arranged in the order of appearance and numbered accordingly by Arabic numerals in in square brackets in line with the text. When more than one reference is cited consecutively, not all of the reference

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- References that are inaccessible and not indexed in any database should not be cited.
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Technical Reports: Surname(s) and initial(s) of the author(s), title of the technical report, name and place of the publisher, report number, if applicable, publication year. The style and punctuation of the references should be formatted as in the following examples. Journal Article

- Journal article with six or fewer authors: Bozluolcay M, Andican G, Firtina S, Erkol G, Konukoglu D. Inflammatory hypothesis as a link between Alzheimer's disease and diabetes mellitus. Geriatr Gerontol Int 2016 16(10):1161–66
- Journal article with more than six authors: Koca SS, Kara M, Özgen M, Dayanan R, Demir CF, Aksoy K, et al. Low prevalence of obesity in Behçet's disease is associated with high obestatin level. Eur J Rheumatol 2017; 4 (2):113–17.
- Abstract: Hortin GL, King C, Kopp J. Quantification of rhesus monkey albumin with assays for human microalbumin [Abstract]. Clin Chem 2000;46:A140–
- Editorial: Demers LM. New biochemical marker for bone disease: is it a breakthrough? [Editorial]. Clin Chem 1992;38:2169–70.
- Letter to the Editor: Davey L, Naidoo L. Urinary screen for acetaminophen (paracetamol) in the presence of N-acetylcysteine [Letter]. Clin Chem 1993;39:2348–9.
- Book Chapter: Rifai N, Warnick GR. Lipids, lipoproteins, apolipoproteins, and other cardiovascular risk factors. In: Burtis CA, Ashwood ER, Bruns DE, editors. Tietz textbook of clinical chemistry and molecular diagnostics. 4th Ed. St. Louis (MO): Elsevier Saunders; 2006. p. 903-81.
- Book: Personal Author Harrell FE Jr. Regression modeling strategies. New York (NY): Springer; 2001. Bailar JC III, Mosteller F, editors. Medical uses of statistics. 2nd Ed. Boston (MA): NEJM Books; 1992:449 p.
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INTERNATIONAL JOURNAL OF
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EDITORIAL

Current approaches, technological transformation, and analytical quality in clinical biochemistry and laboratory medicine

This issue of the *International Journal of Medical Biochemistry* brings together articles that reflect the expanding role of laboratory medicine in modern clinical practice. The published studies address a wide spectrum of topics, including metabolic syndrome, type 2 diabetes mellitus, hepatosteatosis, laboratory workflow monitoring, analytical performance, preanalytical variables, specimen matrix validation, and flow cytometry.

Together, these contributions emphasize that medical biochemistry is no longer limited to test measurement alone. It is a discipline that integrates biological interpretation, analytical reliability, quality management, digital tools, and clinical decision-making. Studies on oxidative stress, pharmacogenetics, vitamin D status, and inflammatory indices highlight the importance of biomarkers in understanding disease mechanisms. At the same time, articles focusing on turnaround time, HbA1c performance, blood collection tubes, and free light chain measurements underline the continuing need for standardization and quality assurance in laboratory practice.

The review on flow cytometry further demonstrates how laboratory medicine is moving toward high-dimensional and technology-driven diagnostics. Overall, this issue reflects the dynamic nature of medical biochemistry and its essential contribution to accurate diagnosis, patient monitoring, and personalized healthcare.

We would like to thank our authors for their valuable contributions to our journal, which is steadily strengthening its place in international indexes with its principles of scientific impartiality and open access, our reviewers for their meticulous evaluations that enhance our quality, and our editorial board. We hope that this issue will be beneficial to the entire medical community.

Prof. Dildar Konukoglu, MD.

Editor-in-Chief

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Research Article

Obesity-associated redox shift in thiol–disulfide homeostasis: A community-based study of metabolic syndrome

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Abstract

Objectives: Oxidative stress is a central feature of metabolic syndrome (MetS); however, the relationship between thiol–disulfide homeostasis and the clinical manifestations of MetS remains unclear. This study aimed to evaluate the association between thiol–disulfide balance and MetS, focusing on clinical and anthropometric parameters.

Methods: In this cross-sectional study, 1012 adults were categorized according to MetS status. Serum native thiol, total thiol, and disulfide levels were measured, and ratio-based indices were calculated. Anthropometric measurements and blood pressure were recorded. Group comparisons, correlation analyses, and multivariate logistic regression analyses were performed.

Results: Individual thiol parameters did not differ significantly between the groups. However, the disulfide-to-native thiol ratio was significantly higher in individuals with MetS and remained independently associated with MetS after adjustment for age, sex, and body mass index. Significant but modest correlations were observed between thiol–disulfide indices and obesity-related anthropometric measures.

Conclusion: Ratio-based thiol–disulfide indices may better reflect systemic oxidative stress in MetS than individual parameters. These findings support the potential utility of integrated redox markers as indicators of metabolic dysfunction.

Keywords: Biomarkers, clinical phenotype, metabolic syndrome, oxidative stress, redox balance, thiol–disulfide homeostasis

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Metabolic syndrome (MetS) is a widely prevalent metabolic disorder defined by the co-occurrence of central obesity, dyslipidemia, elevated blood pressure, and impaired glucose regulation, all of which collectively increase the risk of cardiovascular disease and type 2 diabetes mellitus (T2DM) [1]. Beyond these established clinical components, MetS is increasingly considered a condition characterized by heightened systemic oxidative stress, which plays a central role in the onset and progression of metabolic disturbances [2].

Oxidative stress develops as a consequence of an imbalance between the production of reactive oxygen species (ROS) and the capacity of antioxidant defense systems to neutralize them [3]. In individuals with MetS, factors such as excessive adiposity, chronic low-grade inflammation, and metabolic dysregulation contribute substantially to increased oxidative burden [2]. This disruption in redox equilibrium has been implicated in the pathogenesis of endothelial dysfunction, insulin resistance, and other metabolic complications frequently observed in this population [4].

Thiol–disulfide homeostasis represents a key component of the antioxidant defense system and reflects the dynamic equilibrium between reduced thiol groups and their oxidized disulfide forms [5, 6]. Thiol groups are essential for preserving cellular redox balance, regulating enzymatic functions,

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and protecting biomolecules against oxidative damage. The reversible conversion between thiols and disulfides enables thiol–disulfide homeostasis to function as a sensitive and dynamic indicator of systemic oxidative status [7].

Alterations in thiol–disulfide homeostasis have been documented in a variety of metabolic and cardiovascular disorders, including obesity, diabetes mellitus, and hypertension [7]. However, findings regarding individual thiol and disulfide levels in MetS are inconsistent, and their clinical implications remain incompletely clarified, particularly in population-based investigations. Furthermore, analyses focusing exclusively on individual thiol-related parameters may not adequately capture the dynamic nature of redox balance [7, 8].

To address these limitations, ratio-based indices derived from thiol–disulfide parameters—such as the disulfide-to-native thiol ratio—have been proposed as more integrative indicators of oxidative status. By simultaneously reflecting both reduced and oxidized thiol fractions, these ratios may offer a more comprehensive evaluation of redox homeostasis and more effectively characterize oxidative alterations associated with complex metabolic phenotypes [7].

Accordingly, the present community-based study was designed to investigate thiol–disulfide homeostasis in individuals with and without MetS by evaluating both individual parameters and ratio-based indices. In addition, the study aimed to explore the relationships between thiol–disulfide parameters and obesity-related anthropometric measurements and to determine whether the disulfide-to-native thiol ratio is independently associated with the presence of metabolic syndrome.

Materials and Methods

Study design and population

This cross-sectional, community-based study was conducted using data obtained from adult individuals who participated in a population-based health screening program. All participants were aged 18 years or older. Individuals were excluded if they had acute infections, chronic inflammatory disorders, malignancy, pregnancy, or missing or incomplete laboratory data. Additionally, individuals with known thyroid disease, previous stroke, established cardiovascular disease, chronic kidney disease, a history of vitamin or antioxidant supplementation, and the use of obesity-related medications were excluded from the study.

Metabolic syndrome was defined according to the National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III) criteria. Participants were classified as having metabolic syndrome when at least three of the following five criteria were present: (1) waist circumference ≥102cm in men or ≥88cm in women; (2) fasting plasma glucose ≥110mg/dL; (3) blood pressure >130/85mmHg or current antihypertensive treatment; (4) HDL cholesterol <40mg/dL in men or <50mg/dL in women; and (5) triglyceride levels ≥150mg/dL [1]. Based on these criteria, participants were categorized into two groups: those with metabolic syndrome (MetS-positive) and those without metabolic syndrome (MetS-negative).

The study protocol received approval from the Tokat Gaziosmanpaşa University Faculty of Medicine Clinical Research Ethics Committee (approval no: 21-KAEK-042; date: January 28, 2021). Written informed consent was obtained from all participants prior to inclusion. All study procedures were conducted in accordance with the ethical principles outlined in the Declaration of Helsinki.

Clinical and anthropometric measurements

All anthropometric measurements were performed by trained healthcare professionals following standardized protocols. Body weight and height were measured with participants wearing light clothing and no footwear. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2).

Waist circumference was measured at the midpoint between the lower rib margin and the iliac crest, while hip circumference was measured at the widest portion of the hips. The waist-to-hip ratio (WHR) was subsequently calculated. Anthropometric variables were interpreted as obesity-related anthropometric indicators and were not considered direct measures of adipose tissue distribution.

Blood pressure measurements were obtained after participants had rested for at least five minutes in a seated position. Measurements were performed using a calibrated sphygmomanometer, and the average of two consecutive readings was recorded for both systolic and diastolic blood pressure.

Laboratory analyses

Venous blood samples were collected from all participants following an overnight fasting period. Routine biochemical analyses, including fasting plasma glucose and lipid profile parameters, were performed using standard automated laboratory techniques with a Cobas c501 analyzer (Roche Diagnostics, Basel, Switzerland).

Thiol–disulfide homeostasis parameters were measured using an automated spectrophotometric method with a Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). Initially, disulfide bonds present in the samples were reduced to free thiol groups using sodium borohydride ($NaBH_4$). Subsequently, excess $NaBH_4$ was neutralized with formaldehyde to prevent interference with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB).

Total thiol levels were then determined using a modified Ellman's reagent, whereas native thiol concentrations were measured separately under non-reducing conditions. Disulfide levels were calculated as half of the difference between total thiol and native thiol concentrations [7].

Statistical analysis

All statistical analyses were performed using SPSS version 22.0 software (Chicago, IL, USA). The distribution of continuous variables was evaluated using both visual (histograms and probability plots) and analytical methods.

Since most variables did not follow a normal distribution, non-parametric statistical methods were applied. Comparisons between MetS-positive and MetS-negative groups were conducted using the Mann–Whitney U test for continuous variables and the chi-square test for categorical variables.

Correlation analyses were performed using Spearman's rank correlation coefficients. To avoid spurious associations arising from mathematically derived relationships among thiol-related variables, analyses were restricted to biologically independent parameters.

Multivariate logistic regression analysis was conducted to identify independent predictors of metabolic syndrome. To minimize multicollinearity, only the disulfide-to-native thiol ratio was included in the regression model as a representative marker of thiol–disulfide homeostasis, along with age and sex as covariates.

Receiver operating characteristic (ROC) analysis was additionally performed to evaluate the diagnostic performance of the investigated parameter for identifying metabolic syndrome. The optimal cut-off value, area under the curve (AUC), sensitivity, and specificity values were calculated.

A post hoc power analysis was performed using a two-independent-group comparison framework. Based on a two-sided alpha level of 0.05, 80% statistical power, and a small standardized effect size, the available sample size and observed group allocation ratio were considered adequate to detect meaningful between-group differences.

Results were expressed as odds ratios (ORs) with corresponding 95% confidence intervals (CIs). A two-tailed p-value of less than 0.05 was considered statistically significant.

Results

A total of 1,016 individuals were included in the analysis, of whom 239 (23.5%) fulfilled the diagnostic criteria for metabolic syndrome (MetS). The baseline characteristics of the study population stratified by MetS status are summarized in Table 1.

Comparison of clinical and biochemical characteristics by metabolic syndrome status

There was no statistically significant difference between participants with and without MetS in terms of age (48.05±14.68 vs. 46.57±15.38 years, p=0.189) or body weight (p=0.342). However, height was slightly greater in the MetS group (p=0.019).

Anthropometric parameters, including waist circumference (p=0.730) and body mass index (BMI) (p=0.583), were comparable between the two groups. In contrast, the waist-to-hip ratio (WHR) was significantly higher in individuals with MetS (0.90±0.09 vs. 0.88±0.08, p=0.006).

As anticipated, both systolic and diastolic blood pressure values were significantly elevated in the MetS group (both p<0.001). However, despite these higher measured blood pressure values, the prevalence of previously diagnosed hypertension did not significantly differ between groups (p>0.05). This discrepancy may reflect differences between cross-sectional blood pressure measurements and documented clinical diagnosis. The distribution of sex, obesity status (BMI≥30kg/m^2), presence of chronic disease, hypertension, and diabetes mellitus did not differ significantly between groups (all p>0.05).

Regarding thiol–disulfide homeostasis parameters, no statistically significant differences were observed between the MetS and non-MetS groups for native thiol (p=0.199), total thiol (p=0.231), or disulfide levels (p=0.959).

However, the disulfide-to-native thiol ratio was significantly higher in participants with MetS compared to those without (5.23±1.27 vs. 5.03±1.09, p=0.016), indicating a relative shift toward a more oxidized redox state in MetS. Other ratio-based indices, including the disulfide-to-total thiol ratio and the native thiol-to-total thiol ratio, did not demonstrate significant differences between the groups (p=0.284 for both).

Receiver operating characteristic (ROC) analysis was performed to evaluate the diagnostic performance of the disulfide-to-native thiol ratio for identifying metabolic syndrome. The optimal cut-off value was identified as ≥4.83, yielding an area under the curve (AUC) of 0.544, sensitivity of 62.3%, and specificity of 47.1%. Although the diagnostic performance was modest, the findings support the potential role of thiol–disulfide imbalance as a supportive biomarker associated with metabolic syndrome.

Correlations between thiol–disulfide parameters and obesity-related measures

The relationships between thiol–disulfide parameters and obesity-related anthropometric measures are presented in Table 2.

Native thiol and total thiol concentrations showed significant inverse correlations with waist circumference (r=-0.212 and r=-0.208, respectively; both p<0.001), WHR (r=-0.276 and r=-0.278; both p<0.001), and BMI (r=-0.212 and r=-0.309; both p<0.001). These findings indicate that increasing adiposity is associated with reduced circulating thiol levels.

In contrast, disulfide concentrations demonstrated significant positive correlations with waist circumference (r=0.333, p<0.001), WHR (r=0.250, p<0.001), and BMI (r=0.319, p<0.001).

Similarly, ratio-based markers reflecting oxidative stress—namely, the disulfide-to-native thiol ratio and the disulfide-to-total thiol ratio—were positively correlated with all obesity-related indices (all p<0.001). Conversely, the native thiol-to-total thiol ratio exhibited significant negative correlations with these anthropometric measures (all p<0.001).

No statistically significant associations were identified between thiol–disulfide parameters and systolic or diastolic blood pressure values (all p>0.05).

Multivariate logistic regression for predictors of metabolic syndrome

Variables considered potentially relevant to metabolic syndrome were included in a multivariate logistic regression model, as presented in Table 3.

Table 1. General features of the studied population

Variables	Total (n=1012)	MetS negative (n=773)	MetS positive (n=239)	p
Age (year)	46.92±15.22	46.57±15.38	48.05±14.68	0.189
Height (cm)	162.56±9.44	162.17±9.53	163.81±9.05	0.019
Weight (kg)	75.35±14.65	75.11±14.62	76.14±14.72	0.342
Waist (cm)	106.18±11.21	106.25±11.05	105.96±11.74	0.730
WHR	0.88±0.08	0.88±0.08	0.9±0.09	0.006
BMI (kg/m^2)	28.57±5.45	28.62±5.51	28.4±5.25	0.583
sBP (mmHg)	12.08±2.02	11.64±1.83	13.51±1.93	<0.001
dBP (mmHg)	7.73±1.17	7.51±1.06	8.43±1.22	<0.001
Native Thiol (µmol/L)	427.45±78.38	429.21±78.03	421.76±79.41	0.199
Total Thiol (µmol/L)	470.03±83.49	471.78±82.94	464.38±85.17	0.231
Disulfide (µmol/L)	21.29±5.03	21.29±4.86	21.31±5.55	0.959
(Disulfide/Native thiol)*100	5.07±1.14	5.03±1.09	5.23±1.27	0.016
(Disulfide/Total thiol)*100	4.57±0.93	4.55±0.89	4.62±1.05	0.284
(Native Thiol/Total thiol)*100	90.86±1.86	90.9±1.78	90.75±2.1	0.284
Gender				
Female	555 (54.8)	435 (56.3)	120 (50.2)	0.100
Male	457 (45.2)	338 (43.7)	119 (49.8)	
Obesity				
Absent	595 (58.8)	447 (57.8)	148 (61.9)	0.261
Present (BKI>=30)	417 (41.2)	326 (42.2)	91 (38.1)	
Chronic disease				
Absent	477 (47.1)	372 (48.1)	105 (43.9)	0.257
Present	535 (52.9)	401 (51.9)	134 (56.1)	
HT				
Absent	613 (60.6)	480 (62.1)	133 (55.6)	0.075
Present	399 (39.4)	293 (37.9)	106 (44.4)	
DM				
Present	172 (17.0)	125 (16.1)	47 (19.7)	0.209
Absent	840 (83.0)	648 (83.2)	192 (80.3)	
Waist circumference				
Normal (Men <102 or Women <88)	190 (18.7)	141 (18.2)	49 (20.5)	0.434
High (Men ≥102 or Women ≥88)	822 (81.3)	632 (81.8)	190 (79.5)	
Fasting plasma glucose (mg/dL)				
Normal (<110 mg/dL)	870 (86.0)	666 (86.2)	204 (85.4)	0.755
High (≥110 mg/dL)	142 (14.0)	107 (13.8)	35 (14.6)	
Systolic Blood Pressure (mmHg)				
Normal (≤130)	759 (75)	580 (75)	179 (74.9)	0.966
High (>130)	253 (25)	193 (25)	60 (25.1)	
Diastolic blood pressure (mmHg)				
Normal (≤85)	800 (79.1)	610 (78.9)	190 (79.5)	0.846
High (>85)	212 (20.9)	163 (21.1)	49 (20.5)	
HDL cholesterol (mg/dL)				
Normal (Men<40 or Women <50)	328 (32.4)	244 (31.6)	84 (35.1)	0.301
High (Men≥40 or Women ≥50)	684 (67.6)	529 (68.4)	155 (64.9)	

Data are shown as mean±standard devaition or frequency, percentage. Independent samples test or pearson chi-square test was used. WHR: Waist hip ratio; BMI: Body mass index; sBP: Systolic blood pressure; dBP: Diastolic blood pressure.

Table 2. Correlation analysis between thiol–disulfide homeostasis parameters and anthropometric, biochemical, and clinical variables

	Native Thiol (µmol/L)	Total Thiol (µmol/L)	Disulfide (µmol/L)	(Disulfide/Native thiol)*100	(Disulfide/Total thiol)*100	(Native Thiol/ Total thiol)*100
Native Thiol (µmol/L)						
r	1	0.994*	0.461*	-0.335*	-0.335*	0.335*
p		<0.001	<0.001	<0.001	<0.001	<0.001
Total Thiol (µmol/L)						
r	0.994*	1	0.553*	-0.235*	-0.235*	0.235*
p	<0.001		<0.001	<0.001	<0.001	<0.001
Disulfide (µmol/L)						
r	0.461*	0.553*	1	0.663*	0.664*	-0.664*
p	<0.001	<0.001		<0.001	<0.001	<0.001
(Disulfide/Native thiol)*100						
r	-0.335*	-0.235*	0.663*	1	0.999*	-0.999*
p	<0.001	<0.001	<0.001		<0.001	<0.001
(Disulfide/Total thiol)*100						
r	-0.335*	-0.235*	0.664*	0.999*	1	-1.000*
p	<0.001	<0.001	<0.001	<0.001		<0.001
(Native Thiol/Total thiol)*100						
r	0.335*	0.235*	-0.664*	-0.999*	-1.000*	1
p	<0.001	<0.001	<0.001	<0.001	<0.001	
Waist						
r	-0.212*	-0.208*	0.333*	0.242*	0.234*	-0.234*
p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
WHR						
r	-0.276*	-0.278*	0.250*	0.236*	0.211*	-0.331*
p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
BMI						
r	-0.212	-0.309*	0.319*	0.316*	0.218*	-0.219*
p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
sBP						
r	-0.035	-0.034	-0.010	0.017	0.015	-0.015
p	0.273	0.285	0.741	0.588	0.627	0.631
dBP						
r	-0.016	-0.019	-0.031	-0.020	-0.022	0.022
p	0.618	0.558	0.330	0.525	0.488	0.484

*: Significance at the 0.05 level. Pearson correlation coefficients was used. WHR: Waist hip ratio; BMI: Body mass index; sBP: systolic blood pressure; dBP: diastolic blood pressure.

Tablo 3. Multivariate logistic regression for metabolic syndrome

Variables	Univariate				Multivariate			
	p	OR	95% CI for odds ratio		p	OR	95% CI for odds ratio	
			Lower	Upper			Lower	Upper
Age	0.189	1.006	0.997	1.016	0.414	1.004	0.994	1.014
Gender	0.100	1.276	0.954	1.707	0.134	1.250	0.934	1.675
BMI	0.582	0.992	0.966	1.020	0.630	0.993	0.967	1.021
(Disulfide/Native thiol)*100	0.016	1.164	1.028	1.317	0.041	1.143	1.006	1.299

Reference categories: women for gender. BMI: Body mass index; OR: Odds ratio; CI: Confidence interval. Every one-unit increase in (Disulfide/Native thiol)*100 increases the risk of metabolic syndrome 1.143-fold and is statistically significant (p=0.041).

Table 4. ROC analysis results for Disulfide/Native thiol

Variable	Cutoff	AUC (95% CI)	Se	Sp	PPV	NPV	p
(Disulfide/Native thiol)*100	≥4.83	0.544	0.623	0.471	0.267	0.802	0.038

AUC: Area under curve; CI: Confidence interval; Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value.

After adjustment, age, sex, and BMI were not independently associated with the presence of MetS (all p>0.05).

In contrast, the disulfide-to-native thiol ratio remained a statistically significant independent predictor of metabolic syndrome (OR=1.143, 95% CI: 1.006–1.299, p=0.041). Although statistically significant, the observed effect size was modest and should therefore be interpreted cautiously in terms of clinical relevance. This finding indicates that each one-unit increase in this ratio is associated with a 1.143-fold increase in the likelihood of having MetS, independent of age, sex, and BMI.

Variance inflation factor (VIF) values were examined to assess multicollinearity, and no significant multicollinearity was detected (all VIF<3).

Consistently, each one-unit increase in the (Disulfide/Native thiol)×100 ratio was associated with a 1.143-fold higher risk of metabolic syndrome, which was statistically significant (p=0.041).

Complete summary of key findings

Metabolic syndrome was identified in 23.5% of the study population.

While most anthropometric parameters were comparable between groups, WHR as well as systolic and diastolic blood pressures were significantly elevated in individuals with MetS.

Individual thiol and disulfide concentrations did not differ significantly between MetS and non-MetS groups.

The disulfide-to-native thiol ratio was significantly higher in the MetS group (p=0.016).

Increasing adiposity (waist circumference, WHR, BMI) was associated with:

- Decreased native and total thiol levels.
- Increased disulfide levels and oxidative stress-related ratios.

Multivariate analysis demonstrated that the disulfide-to-native thiol ratio was the only independent predictor of metabolic syndrome.

ROC analysis demonstrated modest discriminative ability (cut-off ≥4.83; AUC=0.544), supporting interpretation of thiol–disulfide indices as supportive rather than standalone diagnostic biomarkers (Table 4). ROC Curve of Disulfide/Native thiol Ratio was shown in Figure 1 as well.

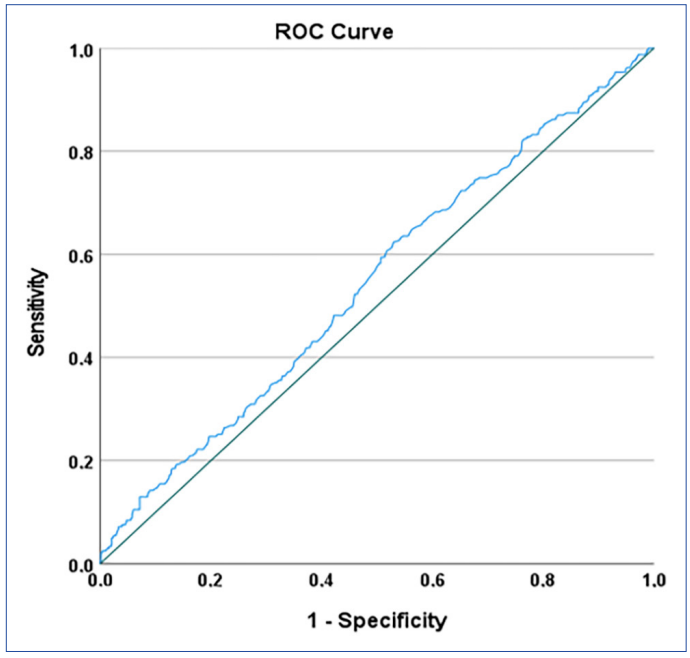

Figure 1. ROC curve of Disulfide/Native thiol ratio.
ROC: Receiver operating characteristic.

Discussion

This community-based study investigated thiol–disulfide homeostasis in relation to metabolic syndrome and associated metabolic characteristics. The principal finding of the present analysis is that the disulfide-to-native thiol ratio was significantly elevated in individuals with metabolic syndrome and remained independently associated with its presence after multivariate adjustment. In contrast, individual concentrations of native thiol, total thiol, and disulfide did not differ significantly between groups. Accordingly, interpretation of the findings focused on ratio-based redox indices rather than isolated thiol parameters. These findings emphasize the importance of ratio-based indices in capturing redox alterations that may not be evident when parameters are evaluated in isolation.

Previous studies conducted in different populations support the relationship between obesity and impaired thiol–disulfide balance. For example, investigations in obese pediatric populations have demonstrated that disruption of thiol–disulfide homeostasis is associated with increased oxidative stress and metabolic abnormalities, with more pronounced alterations observed as the severity of obesity increases [9]. Similarly, in older adults with sarcopenic obesity, a shift toward a more oxidized state—characterized by elevated disulfide-to-native thiol ratios and reduced native thiol levels—has been shown to be independently associated with sarcopenic obesity [10].

An unexpected finding of the present study was the absence of significant differences in BMI and waist circumference between individuals with and without metabolic syndrome. Since direct body composition analysis was not performed, these findings should not be interpreted as evidence regarding adipose tissue

distribution but rather as reflecting obesity-related anthropometric indicators. The similarity of anthropometric characteristics between groups may partly explain why individual thiol and disulfide concentrations did not significantly differ, while ratio-based indices remained informative. This observation further supports the concept that oxidative alterations may occur even in populations without marked anthropometric separation.

As highlighted by Erel and Neselioglu [7], thiol–disulfide homeostasis represents a dynamic redox system in which ratio-based parameters provide a more comprehensive assessment than individual thiol or disulfide measurements. The findings of the present study are consistent with this concept, as only the disulfide-to-native thiol ratio, rather than isolated parameters, was significantly associated with metabolic syndrome.

Metabolic syndrome is a complex clinical condition characterized by central obesity, metabolic dysregulation, and increased oxidative burden [2, 4]. In the current study, thiol–disulfide parameters demonstrated associations with obesity-related anthropometric indicators. Specifically, native and total thiol concentrations were inversely correlated with waist circumference, waist-to-hip ratio, and body mass index, whereas disulfide levels and oxidative ratio parameters showed positive correlations with these measures. These findings should be interpreted as associations with anthropometric indicators rather than direct measures of fat mass distribution.

Evidence from population studies has similarly demonstrated that obesity-related anthropometric indicators are associated with elevated oxidative stress levels, supporting the present findings that alterations in thiol–disulfide homeostasis may accompany increasing metabolic burden [11].

Consistent with prior evidence indicating that systemic oxidative stress leads to depletion of circulating thiols [12], the present results demonstrate a shift in thiol–disulfide equilibrium toward oxidation with increasing anthropometric burden.

These findings are further supported by mechanistic insights identifying adipose tissue as a major contributor to oxidative stress. As reviewed by Marseglia et al. [13], excessive adiposity promotes chronic low-grade inflammation and increased production of reactive oxygen species, processes that likely drive the oxidized thiol–disulfide profile observed in this study population.

An important contribution of the present study is the demonstration that the disulfide-to-native thiol ratio provides additional discriminative value beyond individual thiol measurements. Given the dynamic nature of thiol–disulfide homeostasis, assessment of single parameters may fail to detect subtle but biologically relevant changes in redox balance.

However, despite reaching statistical significance, the observed effect size and diagnostic performance remained relatively modest. ROC analysis demonstrated limited discriminative performance (AUC=0.544), indicating that the investigated parameter should not be interpreted as an independent diagnostic tool. Instead, the clinical relevance of thiol–disulfide homeostasis may lie in serving as a supportive biomarker reflecting oxidative stress and metabolic dysregulation when interpreted together with established anthropometric and biochemical indicators.

In agreement with these findings, previous studies have reported higher disulfide-to-native thiol ratios in metabolic syndrome and obesity-related conditions [14], supporting the role of ratio-based indices as sensitive indicators of oxidative alterations [10].

Several methodological considerations should be acknowledged. Due to the cross-sectional design of the study, causal relationships between thiol–disulfide homeostasis and metabolic syndrome cannot be established.

In addition, inflammatory biomarkers, including C-reactive protein (CRP), were not available in the present dataset and therefore could not be incorporated into the analyses. Since metabolic syndrome reflects both oxidative and inflammatory pathways, this limitation should be considered when interpreting the relationship between thiol–disulfide homeostasis and metabolic alterations. Future studies incorporating inflammatory biomarkers may provide a more comprehensive evaluation.

Residual confounding factors cannot be entirely excluded, and the analysis did not include a broader range of oxidative stress biomarkers beyond thiol–disulfide parameters.

Despite these limitations, the consistent associations observed across multiple analyses strengthen the overall validity of the findings.

In summary, the present study demonstrates that thiol–disulfide homeostasis is altered in individuals with metabolic syndrome, particularly when assessed using ratio-based indices. However, these findings should be interpreted as supportive evidence of oxidative imbalance rather than clinically practice-changing diagnostic information.

Conclusion

In this community-based study, thiol–disulfide homeostasis was evaluated in relation to metabolic syndrome and obesity-associated metabolic characteristics.

Although native thiol, total thiol, and disulfide concentrations did not differ significantly between individuals with and without metabolic syndrome, the disulfide-to-native thiol ratio was significantly elevated and remained independently associated with metabolic syndrome.

However, the magnitude of the association and ROC performance indicate limited standalone diagnostic utility. Therefore, thiol–disulfide indices should not be considered independent diagnostic markers. Rather, they may serve as supportive biomarkers reflecting oxidative and metabolic burden when interpreted alongside established clinical parameters.

The observed associations with obesity-related anthropometric indicators further support the interaction between metabolic dysregulation and oxidative stress.

Given the cross-sectional design, causal inferences cannot be established.

Future longitudinal and prospective studies are required to clarify the temporal dynamics, clinical utility, and potential diagnostic thresholds of thiol–disulfide parameters in metabolic syndrome.

Disclosures

Ethics Committee Approval: The study was approved by the Tokat Gaziosmanpaşa University Faculty of Medicine Clinical Research Ethics Committee (no: 21-KAEK-042, date: 28/01/2021).

Informed Consent: Written informed consent was obtained.

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Research Article

Association of *SLC22A1* gene variants with metformin response in patients with Type 2 diabetes mellitus

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Abstract

Objectives: Type 2 diabetes mellitus (T2DM) is a common chronic metabolic disorder characterized by impaired insulin secretion and insulin resistance. Metformin is the first-line pharmacological treatment for T2DM; however, substantial interindividual variability exists in therapeutic response. Organic cation transporters encoded by the *SLC22A1* and *SLC22A2* genes play a critical role in metformin transport and pharmacokinetics, and genetic polymorphisms within these genes may contribute to differences in glycemic control. The present study aimed to evaluate the association between *SLC22A1* and *SLC22A2* gene variants and metformin response in Turkish patients with T2DM.

Methods: A total of 60 patients with T2DM receiving metformin therapy were included in the study and classified into adequate and inadequate glycemic control groups according to HbA1c levels (<7.0% and >7.0%). Targeted next-generation sequencing analysis of the *SLC22A1* and *SLC22A2* genes was performed. Identified variants were evaluated under different genetic models, and genotype/allele distributions between groups were compared using Fisher's exact test.

Results: A total of 20 variants were identified in the *SLC22A1* and *SLC22A2* genes, including missense, intronic, synonymous, and one novel in-frame deletion variant. Most variants were classified as benign, whereas three variants were categorized as variants of uncertain significance. The rs683369:G>C polymorphism showed significant associations with glycemic control under the genotypic, recessive, and allelic models. The CC genotype was observed exclusively in the inadequate glycemic control group under both the genotypic (p=0.041) and recessive models (p=0.042). In addition, the C allele was significantly associated with inadequate glycemic control in the allelic model (p=0.006). Additionally, the novel c.1276+9_1276+16del variant demonstrated a significant allelic association with glycemic control (p=0.049). No significant associations were identified for the rs1867351:T>C or rs628031:A>G variants.

Conclusion: Our findings suggest that specific *SLC22A1* polymorphisms, particularly rs683369:G>C and the novel c.1276+9_1276+16del variant, may influence the glycemic response to metformin in Turkish patients with T2DM.

Keywords: Diabetes mellitus, gene sequencing, metformin, pharmacogenetic, *SLC22A1*, *SLC22A2*.

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Type 2 diabetes mellitus (T2DM) is a prevalent chronic metabolic disease affecting over 400 million individuals globally and represents a major public health concern [1]. According to 2024 data from the International Diabetes Federation, the number of people with diabetes in Türkiye is 9.6 million, with this figure expected to exceed 14 million by 2050 [2]. T2DM is characterized by persistent hyperglycemia resulting from a combination of defective insulin secretion by pancre-

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atic β-cells, progressive insulin resistance in peripheral tissues such as skeletal muscle, liver, and adipose tissue, and consequent dysregulation of glucose homeostasis [3, 4]. Over time, these pathophysiological alterations contribute not only to sustained elevations in blood glucose levels but also to additional metabolic abnormalities, including impaired lipid metabolism and chronic low-grade systemic inflammation [5]. As a result, T2DM is strongly associated with serious long-term complications such as cardiovascular disease, nephropathy, neuropathy, and retinopathy, thereby imposing a substantial healthcare burden worldwide [6]. Although no definitive cure currently exists, effective disease management and appropriate pharmacological treatment can achieve adequate glycemic control, reduce the risk of complications, and improve long-term clinical outcomes [7].

Metformin (dimethyl biguanide), first introduced into clinical practice in 1957 as a glucose-lowering agent, has since become the recommended first-line pharmacological therapy for T2DM, typically in combination with lifestyle modifications. This agent exerts its effects by preventing insulin resistance through various cellular pathways, primarily in the intestines, liver, and muscles, while lowering blood glucose levels without increasing the risk of hypoglycemia [8]. As a hydrophilic organic cation, metformin requires specific membrane transporters to enter cells and be excreted from the body [9]. Members of the organic cation transporter (OCT) family play an important role in this process and are encoded by genes belonging to the solute carrier family 22 (SLC22) [10]. OCT1 (*SLC22A1*) is mainly expressed in the liver and facilitates the uptake of metformin into hepatocytes, which is crucial for its glucose-lowering action [11, 12]. In contrast, OCT2 (*SLC22A2*), which is predominantly localized to the basolateral membrane of renal tubular epithelial cells, serves as a key mediator of metformin transport from the systemic circulation into renal cells [13]. By facilitating the uptake of metformin across the basolateral membrane, OCT2 plays a critical role in the renal handling and intracellular accumulation of the drug, thereby influencing its subsequent tubular secretion and overall pharmacokinetic profile [14].

In recent years, pharmacogenetic studies have increasingly focused on genetic variants within the *SLC22A1* and *SLC22A2* genes because of their critical roles in metformin transport and disposition [15, 16]. Despite metformin's widespread clinical use and favorable safety profile, considerable interindividual variability exists in the glycemic response to treatment [17, 18]. Emerging evidence suggests that genetic variants affecting metformin transporters may partly explain these differences in therapeutic outcomes. Identification of such genetic determinants could contribute to the development of personalized treatment strategies and improved glycemic management in patients with T2DM. Therefore, the present study aimed to investigate the association between *SLC22A1* and *SLC22A2* genetic variants and therapeutic response to metformin in Turkish patients with T2DM.

Materials and Methods

Ethics approval and consent to participate

The project was approved by İstanbul University-Cerrahpaşa, Clinical Research Ethics Committee (10.07.2024-1035272) and complied with the Declaration of Helsinki. All participants provided written informed consent before participation in the study. Peripheral blood samples were collected from all participants recruited at the Sinan Sipahi Family Health Centre of the Ministry of Health of the Republic of Türkiye (Istanbul, Türkiye) between July 2024 and February 2025.

Study population and participants

This study included 60 individuals diagnosed with T2DM who were undergoing treatment with metformin. The participants in the study consisted of patients under follow-up at the Sinan Sipahi Family Health Centre of the Ministry of Health of the Republic of Türkiye. Inclusion criteria were defined as a confirmed diagnosis of T2DM, treatment with metformin alone (1000 mg) or in combination with vildagliptin/metformin (50/1000 mg) for at least 6 months, and an age range of 18–70 years. Of the 60 patients included in the study, 32 were receiving metformin monotherapy, whereas 28 were receiving a fixed-dose combination of metformin and vildagliptin. Baseline exclusion criteria consisted of Type 1 diabetes, any form of cancer, pregnancy or breastfeeding, severe kidney or liver failure, and the use of additional medications that could significantly affect glycemic control.

Clinical and biochemical measurements

Venous blood samples were obtained from all participants following an overnight fasting period. Fasting plasma glucose (FPG) and glycated hemoglobin (HbA1c) concentrations were measured using routine laboratory procedures. Demographic and clinical data, including age, sex, body mass index (BMI), and relevant biochemical variables, were retrieved from patient medical records (Table 1). The effectiveness of metformin therapy was assessed by evaluating changes in HbA1c levels following treatment. Participants were stratified into two categories based on their HbA1c levels: those with adequate glycemic control (HbA1c<7.0%) and those with inadequate glycemic control (HbA1c≥7.0%).

DNA purification and library preparation

Peripheral blood samples were obtained in tubes containing EDTA as an anticoagulant. Samples were transported to the laboratory immediately after collection and stored at 4°C until DNA extraction. Genomic DNA was extracted from whole blood using the commercially available Abcam Genomic DNA Isolation Kit (ab65358; Cambridge, MA, USA) according to the manufacturer's instructions. The purity and concentration of the isolated DNA samples were subsequently determined using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). The isolated DNA samples were stored at −20°C until further genetic analyses were performed.

Table 1. Demographic and clinical characteristics of patients with T2DM stratified by glycemic control

Independent variables	Total population (n=60)	Adequate glycemic control HbA1c < 7.0% (n=32)	Inadequate glycemic control HbA1c ≥ 7.0% (n=28)	p
Age (years)	60.97±7.71	62.25±7.33	59.50±8.00	0.170
Sex (F/M)	37/23	21/11	16/12	0.598
HbA1c (%)	7.30±1.43	6.31±0.41	8.43±1.33	**<0.001**
BMI (kg/m^2)	30.91±4.95	30.62±4.43	31.24±5.55	0.683
Glucose (mg/dL)	141.82±50.45	111.00±16.50	177.04±53.26	**<0.001**
Total cholesterol (mg/dL)	186.40±40.84	190.63±38.64	181.57±43.41	0.396
HDL-C (mg/dL)	44.47±10.90	44.91±10.40	43.96±11.61	0.741
LDL-C (mg/dL)	108.88±33.03	114.53±33.07	102.43±32.37	0.158
TG (mg/dL)	169.20±75.99	163.16±84.66	176.11±65.53	0.211
Creatinine (mg/dL)	0.85±0.20	0.87±0.20	0.83±0.19	0.515

Variables were presented as mean±standard deviation for data. HbA1c: Glycated hemoglobin;F: Female; M: Male; BMI: Body mass index; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; TG: Triglycerides.

Long-range PCR was performed using a C1000 thermal cycler (Bio-Rad, USA) to amplify the exons and exon-intron junctions of the *SLC22A1* and *SLC22A2* genes.

Sequencing libraries were prepared using the TruSeq Custom Amplicon v1.5 Exome Library Prep Kit (Illumina, Cat. No. 20018705, San Diego, USA), and genotyping was conducted on the NovaSeq 6000 System (Illumina, San Diego, USA).

Genotyping and raw data analysis

A sequencing depth of greater than 20× was obtained for 95% of the targeted regions, following our previously published methodology [19, 20]. Before sequencing on the NovaSeq 6000 platform (Illumina, Inc., San Diego, CA, USA), the system was configured to produce FastQ files as the primary output for each sample pool, followed by the generation of CRAM files as the final aligned data format. Sequencing reads derived from the FastQ files were mapped to the human reference genome (GRCh38/hg38) using the Burrows–Wheeler Aligner (BWA) and subsequently converted into CRAM format. Variant calling was performed using the HaplotypeCaller tool from the Genome Analysis Toolkit (GATK), following GATK Best Practices [21], resulting in an unannotated Variant Call Format (VCF) file. Variants were retained for downstream analyses only if they passed all GATK quality filters (PASS status) and were supported by a minimum sequencing depth of 30×. All analyses used the GRCh38/hg38 genome assembly (http://genomereference.org) as the reference (Genome Reference Consortium, n.d.). Exome sequencing data processing and annotated variant interrogation were conducted using Ilyome© software (Ilyome©, Türkiye).

Statistical analysis

An a priori sample size calculation was performed using G*Power version 3.1. Assuming an alpha level of 0.05, a statistical power of 80%, and expected minor allele frequencies (MAFs) reported for the investigated genes, the required sample size was estimated before participant recruitment. Statistical evaluations were conducted using GraphPad Prism software (version 10.6.1; GraphPad Software, San Diego, CA, USA). Data distribution patterns were examined with the Shapiro–Wilk test to determine whether the variables conformed to a normal distribution. Comparisons of continuous variables between groups were performed using the independent samples t-test or the Mann–Whitney U test, as appropriate according to data distribution. Categorical variables were analyzed using Fisher's exact test. Allele and genotype distributions were calculated, and Hardy–Weinberg equilibrium (HWE) was assessed. Deviations from HWE were examined using an online tool developed by Michael H. Court (2005–2008). SNPs showing nominally significant associations in the initial analyses were further evaluated across genotypic, dominant, recessive, and allelic genetic models. Benjamini–Hochberg (BH) false discovery rate (FDR) correction was then applied to these multiple comparisons, and adjusted p values were reported as BH q values. A BH q value <0.05 was considered statistically significant. A multivariable logistic regression analysis was performed to evaluate the independent associations of clinical variables with glycemic control status. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. Continuous variables are expressed as mean±standard deviation (SD), while categorical variables are presented as number and percentage. p<0.05 was considered statistically significant.

Results

Baseline demographic and clinical characteristics

The inadequate and adequate glycemic control groups were comparable in terms of BMI (p=0.683), age (p=0.170), and sex distribution (p=0.597), with similar proportions of males and females in both groups.

As expected, glucose levels were significantly higher in the inadequate HbA1c group than in the adequate HbA1c group (p<0.0001). Similarly, mean HbA1c levels were markedly elevated in the inadequate glycemic control group compared with the adequate glycemic control group (p<0.0001).

Table 2. Multivariable logistic regression analysis of clinical and biochemical factors associated with glycemic control in patients with T2DM

Independent variables	β	OR	p	95% CI for OR
Age (years)	-0.037	0.964	0.320	0.892–1.036
Sex (F/M)	0.411	1.509	0.468	0.496–4.701
BMI (kg/m^2)	0.039	1.040	0.491	0.929–1.169
Total cholesterol (mg/dL)	0.065	1.067	0.377	0.950–2.075
HDL-C (mg/dL)	-0.067	0.936	0.419	0.480–1.067
LDL-C (mg/dL)	-0.078	0.925	0.256	0.474–1.040
TG (mg/dL)	-0.010	0.990	0.540	0.867–1.014
Creatinine (mg/dL)	-0.825	0.438	0.584	0.020–8.725

Variables were presented as mean±standard deviation for data. OR: Odds ratio; CI: confidence interval; F: Female; M: Male; BMI: Body mass index; HbA1c: Glycated hemoglobin; HDL-C: High-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglycerides.

Lipid parameters, including total cholesterol (p=0.396), HDL cholesterol (p=0.741), LDL cholesterol (p=0.159), and triglycerides (p=0.211), were comparable between the groups. Creatinine levels were also similar across groups (p=0.515). These findings are summarized in Table 1.

To further evaluate the independent effects of clinical and biochemical variables on glycemic control status, a multivariable logistic regression analysis was performed including age, sex, BMI, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, and creatinine as covariates. None of the investigated variables were significantly associated with glycemic control status. The estimated odds ratios were as follows: age, 0.96 (95% CI: 0.89–1.04); sex, 1.51 (95% CI: 0.50–4.70); BMI, 1.04 (95% CI: 0.93–1.17); total cholesterol, 1.07 (95% CI: 0.95–2.08); HDL cholesterol, 0.94 (95% CI: 0.48–1.07); LDL cholesterol, 0.92 (95% CI: 0.47–1.04); triglycerides, 0.99 (95% CI: 0.87–1.01); and creatinine, 0.44 (95% CI: 0.02–8.73). These findings indicate that demographic characteristics and routine biochemical parameters were not independent predictors of glycemic control status in the study population (Table 2).

Genotypes

A total of 20 variants were identified in the *SLC22A1* (RefSeq: NM_003057.3; Ensembl: ENST00000366963.9) and *SLC22A2* (ENST00000366953.8; RefSeq: NM_003058.4) genes among patients with T2DM (Table 3). The majority of the detected variants were located in *SLC22A1*, while a smaller proportion was observed in *SLC22A2*.

Most variants were classified as benign (n=15, 75%), primarily supported by high population frequencies (MAF>0.05) and fulfillment of the benign (BA1) criterion. Several variants, including c.1222A>G (p.Met408Val) and c.156T>C (p.Ser52=), exhibited relatively high allele frequencies, indicating that these are common polymorphisms in the general population. Three variants were classified as variants of uncertain significance (VUS). These included two rare variants in *SLC22A1* (c.1634A>G; p.Lys545Arg and c.412-73T>C) and one synonymous variant in *SLC22A2* (c.1506G>T; p.Val502=), all with low population frequencies (MAF<0.01) and meeting the PM2 criterion. Their potential clinical relevance remains unclear due to insufficient evidence. One variant (c.262T>C; p.Cys88Arg) was classified as likely benign, supported by its low frequency but lack of pathogenic evidence (Table 3).

An intronic deletion (c.1276+9_1276+16del, CTGGTAAGT>C) in *SLC22A1* was also identified. Although located within the splice region, its high population frequency (MAF=0.76) led to its classification as BA1, suggesting no clinical significance despite its potential proximity to splice regulatory elements. This variant was not found in dbSNP, but it has been reported in the ClinVar database. The IGV view illustrating the sequencing reads of the *SLC22A1* gene variants, including rs683369 and the c.1276+9_1276+16del variation, is presented in Figure 1.

The variations included missense (n=6), intronic (n=9), synonymous (n=4), and one in-frame deletion. Intronic variants constituted the largest group; however, most were located outside canonical splice sites and were classified as BA1.

In this study, four *SLC22A1* variants (rs1867351:T>C, rs628031:A>G, rs683369:G>C, and CTGGTAAGT>C variants) met the predefined MAF criteria and were therefore included in the genetic association analyses. All analyzed variants were in HWE. In contrast, no *SLC22A2* variant met the predefined MAF threshold required for statistical evaluation. The association between the rs683369:G>C variant and glycemic control groups was evaluated under different genetic models. The rs683369:G>C polymorphism showed significant associations with glycemic control under the genotypic, recessive, and allelic models. The CC genotype was observed exclusively in the inadequate glycemic control group under both the genotypic (OR=∞, 95% CI: 1.157–∞, p=0.041) and recessive models (OR=5.73, 95% CI: 1.40–32.19, p=0.042). In addition, the C allele was significantly associated with inadequate glycemic control in the allelic model (OR=12.06, 95% CI: 1.81–134.2, p=0.006). No significant association was identified under the dominant model (OR=6.74, 95% CI: 0.78–81.54, p=0.088) (Fig. 2). Additionally, the c.1276+9_1276+16del variant showed a significant association at the allelic level (OR=7.56, 95% CI: 1.15–87.98, p=0.049), supporting its potential role in glycemic control (Fig. 3). Genetic model analyses revealed no significant associations for the rs1867351:T>C and rs628031:A>G variations. Following BH-FDR correction, only the allelic association of *SLC22A1*

Table 3. Common and rare *SLC22A1* and *SLC22A2* genes variants detected in T2DM patients

	T2DM patients							
No	**Variant (HGVS cDNA)**	**dbSNP ID**	**Genomic position (GRCh38)**	**Variant type**	**ACMG classification**	**Amino acid change**	**Highest population MAF**	**Pathogenic criteria (Population Data)**
1	SC22A1:c.1276+9_1276+16del	NA	chr6-160139865 CTGGTAAGT>C	Splice region intron variant	Benign	-	0.76	BA1
2	SLC22A1:c.412-73T>C	rs989070852	chr6-160130031 T>C	Intron	VUS	-	<0.01	PM2
3	SLC22A2:c.1506G>T	rs316003	chr6-160224800 C>A	Synonymous variant	VUS	p.Val502=	0.48	BA1
4	SLC22A1:c.262T>C	rs55918055	chr6-160122197 T>C	Missense variant	Likely Benign	p.Cys88Arg	0.01	PM2
5	SLC22A2:c.843-59T>C	rs2279463	chr6-160247357 A>G	Intron variant	Benign	-	0.25	BA1
6	SLC22A1:c.1634A>G	rs766245985	chr6-160158551 A>G	Missense variant	VUS	p.Lys545Arg	<0.01	PM2
7	SLC22A1:c.1276+34C>T	rs9457843	chr6-160139901 C>T	Intron variant	Benign	-	0.19	BA1
8	SLC22A1:c.41C>T	rs34447885	chr6-160121976 C>T	Missense variant	Benign	p.Ser14Phe	0.03	BS1
9	SLC22A1:c.1222A>G	rs628031	chr6-160139813 A>G	Missense variant	Benign	p.Met408Val	0.31	BA1
10	SLC22A1:c.412-43T>G	rs4646272	chr6-160130061 T>G	Intron variant	Benign	-	0.49	BA1
11	SLC22A1:c.156T>C	rs1867351	chr6-160122091 T>C	Synonymous variant	Benign	p.Ser52=	0.47	BA1
12	SLC22A1:c.1260_1262del	rs72552763	chr6-160139848 CATG>C	Inframe deletion	Benign	p.Met420del	0.38	PM4, BA1,BS2
13	SLC22A1:c.955-7C>T	rs7762846	chr6-160136537 C>T	Splice region variant	Benign	-	0.18	BA1
14	SLC22A1:c.480G>C	rs683369	chr6-160130172 G>C	Missense variant	Benign	p.Leu160Phe	0.24	BA1
15	SLC22A1:c.516-26C>T	rs45584532	chr6-160132206 C>T	Intron variant	Benign	-	0.18	BA1
16	SLC22A2:c.808T>G	rs316019	chr6-160249250 A>C	Missense variant	Benign	p.Ser270Ala	0.25	BA1
17	SLC22A1:c.1599-21C>T	rs622591	chr6-160158495 C>T	Intron variant	Benign	-	0.49	BA1
18	SLC22A1:c.1390G>A	rs41267797	chr6-160155979 G>A	Synonymous variant	Benign	p.Val464Ile	0.23	BA1
19	SLC22A1:c.1498+43C>T	rs2297374	chr6-160154953 C>T	Intron variant	Benign	-	0.49	BA1
20	SLC22A1:c.1498+37G>T	rs36107168	chr6-160154947 G>T	Intron variant	Benign	-	0.20	BA1

HGVS: Human Genome Variation Society nomenclature; dbSNP ID: Database of Single Nucleotide Polymorphisms identifier; GRCh38: Genome Reference Consortium Human Build 38; ACMG: American College of Medical Genetics and Genomics; MAF: Minor Allele Frequency; VUS: Variant of Uncertain Significance; BA1: stand-alone benign evidence; BS1: strong benign evidence; PM2: moderate evidence based on rarity in population databases; PM4: moderate evidence; T2DM: Type 2 Diabetes Mellitus; NA: no dbSNP identifier assigned. MAF: Minor Allele Frequency. The highest minor allele frequency observed in any population, including 1000 Genomes Phase 3, ESP, and gnomAD. Gene variant, position, ACMG classification, amino acid change, and Pathogenic Criteria taken from Franklin Bioinformatics. Variant type and the highest population MAF are taken from Ensembl.

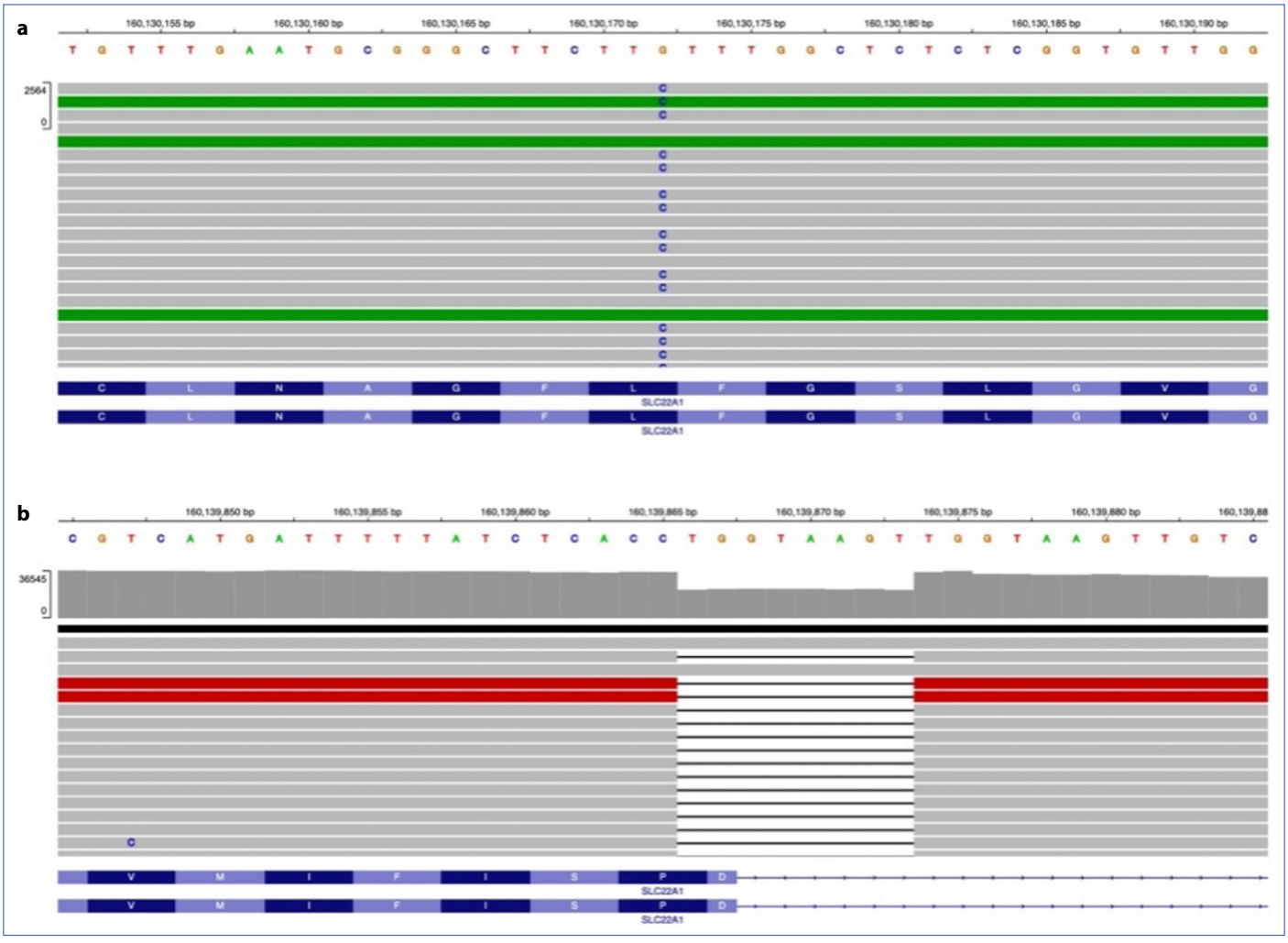

Figure 1. The IGV illustrating the reads of the *SLC22A1* gene rs683369 (a) and c.1276+9_1276+16del, CTGGTAAGT>C (b) variations.
IGV: Integrative genomics viewer.

rs683369:G>C remained significant (p=0.0058, q=0.029), whereas all other associations did not retain statistical significance after adjustment. The genotype distributions underlying these analyses are summarized in Appendix 1.

Discussion

DM, directly associated with more than 1 million deaths annually, was declared an epidemic by the World Health Organization in the early 21st century [1, 22]. The International Diabetes Federation predicts that by 2045, nearly one in eight adults will have DM, with over 90% of cases consisting of T2DM [23, 24]. Approximately 75–80% of patients with T2DM have at least one comorbid disease related to chronic hyperglycemia [23, 25]. Therefore, achieving glycemic control is the primary goal of treatment in these patients, and metformin is considered a first-line therapy for this purpose [7, 26]. Metformin, the most widely used oral antidiabetic agent, fails to achieve adequate glycemic regulation in a substantial proportion of patients [27]. Our findings suggest that genetic variations in the *SLC22A1* gene, which encodes the OCT1 protein, may contribute to interindividual differences in metformin response and glycemic control. In particular, the rs683369:G>C and c.1276+9_1276+16del intronic deletion polymorphisms in the *SLC22A1* gene may influence treatment efficacy.

The rs683369:G>C polymorphism in the *SLC22A1* gene showed a significant association with glycemic control in the genotypic (p=0.041), recessive (p=0.042), and allelic (p=0.006) models. Individuals with the CC genotype were more frequently observed in the poor glycemic control group, and the C allele was associated with reduced treatment response. These findings suggest a possible association between rs683369 and metformin response. Similarly, Jablonski et al. [28] reported that carriers of the G allele had better protection against diabetes compared with placebo in a large randomized controlled study involving prediabetic individuals. The association between the rs683369 variant and metformin response has also been investigated beyond diabetes and prediabetes populations, particularly in cohorts with polycystic ovary syndrome (PCOS), a disorder characterized by insulin resistance as a shared metabolic pathway. In a study conducted in Taiwan, PCOS patients

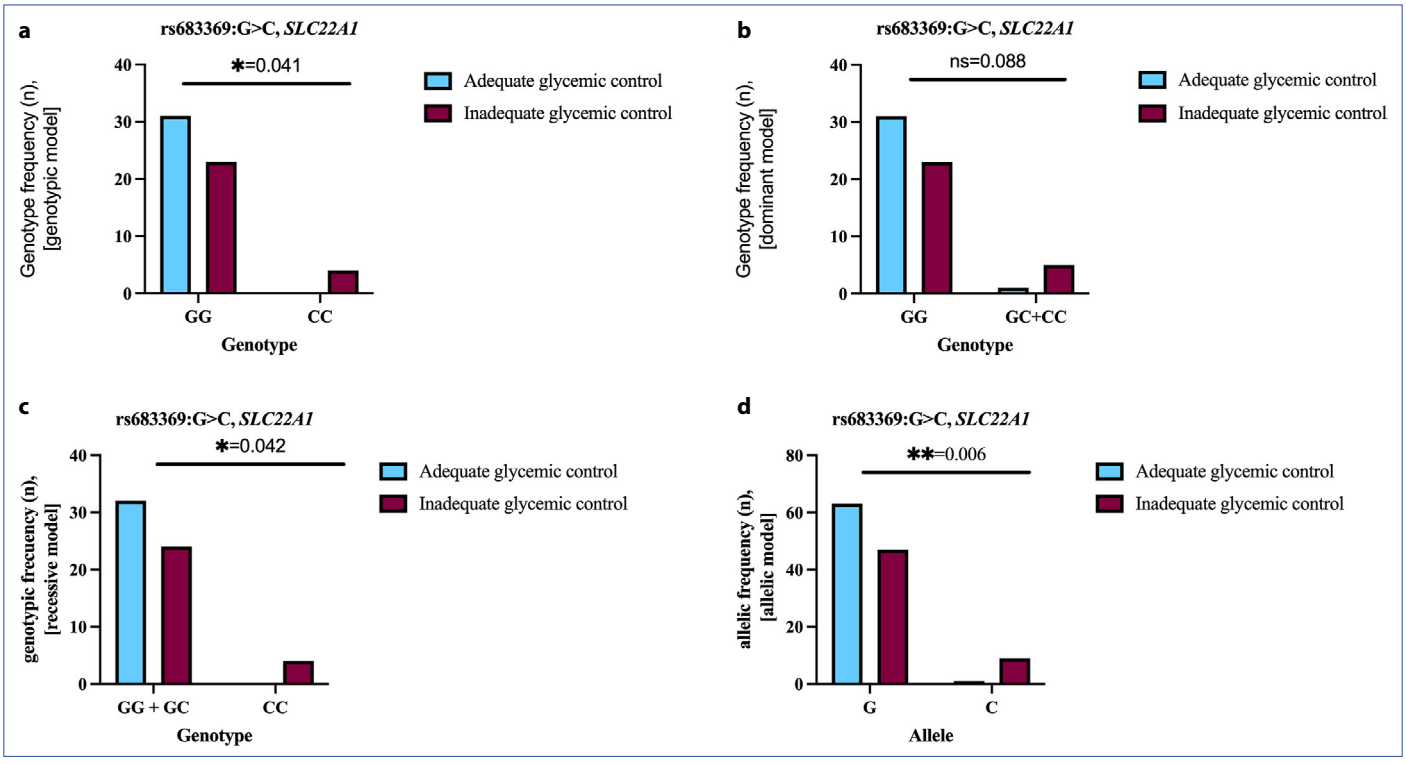

Figure 2. Association of the rs683369:G>C polymorphism in the *SLC22A1* gene with glycemic control status in metformin-treated T2DM patients. (a) Genotypic model (GG vs. CC) showing a significant association between the CC genotype and inadequate glycemic control (*p=0.041). (b) Recessive model (GG+GC vs. CC) demonstrating a significant association, with the CC genotype observed exclusively in the inadequate glycemic control group (*p=0.042). (c) Dominant model (GG vs. GC+CC) showing no statistically significant association (p=0.088). (d) Allelic model demonstrating a significantly higher frequency of the C allele in the inadequate glycemic control group compared with the adequate glycemic control group (**p=0.006).

Statistical analyses were performed using Fisher's exact test. p<0.05 was considered statistically significant.

carrying the G allele demonstrated a marked improvement in insulin sensitivity following metformin treatment, as assessed by oral glucose tolerance test (OGTT) results [29]. Similarly, Zeng et al. [30] reported that G allele carriers receiving pioglitazone–metformin combination therapy showed significantly greater reductions in testosterone levels by more effectively suppressing hyperinsulinemia-driven theca cell stimulation. The observation that the same allele is associated with comparable metabolic improvements across different diseases, populations, and environmental backgrounds highlights the potential clinical relevance of rs683369. Collectively, these findings suggest that rs683369 may influence metformin response through its effect on OCT1 transporter activity encoded by *SLC22A1*, potentially representing a conserved pharmacogenetic mechanism across different ethnic populations.

Additionally, a study of 56 Bangladeshi patients with T2DM reported that the common nonsynonymous variant rs683369:G>C was highly prevalent in both patients with T2DM and healthy populations, but in silico analyses showed no evidence of pathogenicity or splice alteration [31]. Similarly, studies conducted in Chinese (n=43), Mexican (n=69), and Jordanian (n=212) populations reported no significant association between rs683369 and metformin response [16, 32, 33].

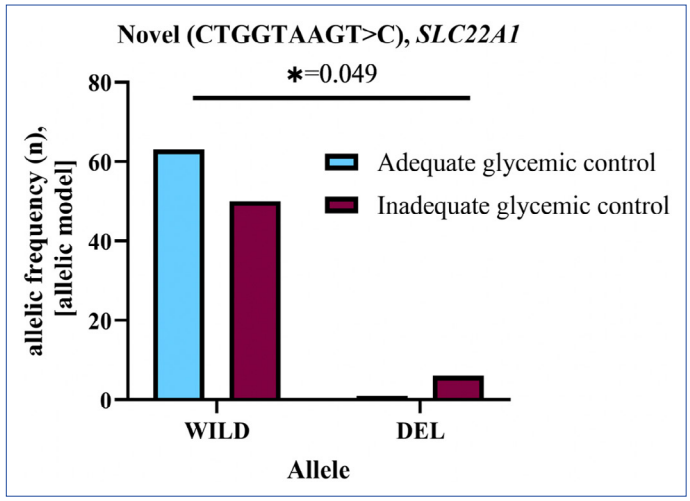

Figure 3. Association of the c.1276+9_1276+16del (CTGGTAAGT>C) variant in the *SLC22A1* gene with glycemic control status in metformin treated T2DM patients. Allelic model analysis demonstrated a significantly higher frequency of the deletion (DEL) allele in the inadequate glycemic control group compared with the adequate glycemic control group (p=0.049).

Statistical analyses were performed using Fisher's exact test. p<0.05 was considered statistically significant.

In a cohort study of PCOS patients treated with metformin, the association between the rs683369 G allele and weight loss was observed in the discovery cohort but was not replicated in subsequent cohorts [34]. Overall, such discrepancies across studies may be explained by differences in study design, sample size, and population-specific genetic backgrounds, suggesting that the observed effects of rs683369 may not be fully consistent across different cohorts. Although nominal significance was observed for several models in the unadjusted analyses, only the allelic association of *SLC22A1* rs683369:G>C remained significant after BH correction (q=0.029), supporting the potential relevance of this variant in metformin response.

Our study also identified an intronic deletion in the *SLC22A1* gene (c.1276+9_1276+16del, CTGGTAAGT>C). Despite its BA1 classification based on high population allele frequency (MAF=0.76) and prior laboratory assessment, the variant showed a nominal association with glycemic control in the allelic analysis (p=0.049), although this association did not remain statistically significant after BH correction for multiple testing (q>0.05). As shown in the data, the wild-type allele was more frequent among individuals with adequate glycemic control, whereas the deletion/variant allele was relatively more common among those with inadequate glycemic control. Although the *SLC22A1* (CTGGTAAGT>C) variant is located within a splice region and may be positioned near splice regulatory elements, its functional impact on mRNA splicing or gene expression remains uncertain. Therefore, the observed association should be considered exploratory and interpreted with caution. Nevertheless, this finding may warrant further investigation in larger cohorts and functional studies to clarify the potential impact of this intronic deletion on *SLC22A1* function and metformin treatment outcomes.

In conclusion, this study demonstrates that the glycemic response to metformin in patients with T2DM may be influenced by genetic variability in the *SLC22A1* gene. In particular, the rs683369:G>C polymorphism in *SLC22A1* showed significant associations with glycemic control, suggesting a potential role in modulating metformin efficacy. Additionally, an intronic deletion (c.1276+9_1276+16del) in *SLC22A1* was identified and found to be associated with differences in glycemic control despite its high allele frequency and previous benign classification. It should be noted that ACMG/AMP classification criteria are intended to evaluate the pathogenicity of genetic variants in relation to disease causation and are not specifically designed to assess pharmacogenetic effects. Therefore, the ACMG classification of a variant should not be interpreted as evidence for or against its potential influence on drug response. In the present study, ACMG classifications are provided to describe the clinical significance of the identified variants, whereas the observed associations relate specifically to the glycemic response to metformin therapy. This finding suggests that even common variants may contribute to interindividual variability in drug response, possibly through effects on OCT1 function or splicing regulation. Nevertheless, the clinical relevance of this variant remains uncertain and requires further validation through functional studies and confirmation in larger, independent patient cohorts. Overall, the findings support the involvement of *SLC22A1* genetic variation in metformin response and highlight the importance of pharmacogenetic factors in achieving optimal glycemic control in T2DM. Further well-designed multicenter and functional studies are needed to clarify the biological mechanisms and confirm the clinical utility of these genetic markers.

Limitations

The findings of this study provide important insights; however, several limitations should be acknowledged. First, although an a priori sample size calculation was performed during the study design phase, the final number of recruited participants was lower than the initially estimated sample size because recruitment was restricted to eligible patients who met the inclusion criteria during the study period. Therefore, the study may have had a limited ability to detect genetic variants with small effect sizes, particularly those with lower minor allele frequencies than anticipated. Second, the relatively modest sample size, single-center design, and inclusion of only Turkish patients may limit the generalizability of the findings to other populations and ethnic groups. Furthermore, although multiple testing was addressed using BH-FDR correction, the exploratory nature of this NGS-based study warrants cautious interpretation of the observed associations.

Conclusion

To more comprehensively evaluate the role of both common and rare variants in metformin response, future studies should include larger, multicenter, and ethnically diverse cohorts. Functional investigations are also warranted to clarify the biological effects of the identified variants and to validate their potential clinical relevance.

Disclosures

Ethics Committee Approval: The study was approved by the İstanbul University-Cerrahpaşa Clinical Research Ethics Committee (no: 1035272, date: 10/07/2024).

Informed Consent: Written informed consents were obtained from patients who participated in this study.

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Appendix 1. Genotype distributions of variants according to glycemic control status

Variant	Genotype	Adequate Glycemic control (n=32)	Inadequate Glycemic control (n=28)
rs1867351:T>C	TT	31 (96.9%)	24 (85.7%)
	TC	1 (3.1%)	4 (14.3%)
	CC		
		0 (0.0)	0 (0.0%)
rs628031:A>G	AA	30 (93.8%)	26 (92.9%)
	AG	0 (0.0%)	1 (3.9%)
	GG		
		2 (6.2%)	1 (3.9%)
rs683369:G>C	GG	31 (96.9%)	23 (82.1%)
	GC	1 (3.1%)	1 (3.6%)
	CC		
		0 (0.0)	4 (14.3%)
c.1276+9_1276+16del (CTGGTAAGT>C)	WILD+WILD	31 (96.9%)	24 (85.7%)
	DEL+WILD	1 (3.1%)	2 (7.1%)
	DEL+DEL	0 (0.0)	2 (7.1%)

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Research Article

Relationship between vitamin D status, blood pressure parameters, and inflammatory indices across different grades of hepatosteatosis

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Abstract

Objectives: Both vitamin D deficiency and hepatosteatosis are recognized as significant contributors to systemic inflammation and increased cardiovascular risk. This study aimed to investigate the associations between serum vitamin D concentrations, arterial blood pressure, and novel systemic inflammatory markers across ultrasonographically staged hepatosteatosis.

Methods: A total of 369 patients (188 females, 181 males; mean age: 47.92±13.10 years) with sonographically confirmed hepatosteatosis were evaluated. Participants were stratified into three groups according to the severity of liver fat accumulation: Group 1 (Grade 0; minimal), Group 2 (Grade 1; mild), and Group 3 (Grades 2–3; moderate-to-severe).

Results: The mean serum vitamin D level of the cohort was 11.70±7.97 ng/mL. Pearson correlation analysis revealed a significant inverse relationship between vitamin D levels and both systolic blood pressure (SBP) (r=-0.162, p=0.002) and diastolic blood pressure (DBP) (r=-0.155, p=0.003). Statistically significant differences among steatosis grades were observed for LDH, triglycerides, uric acid, lymphocyte count (LYM), monocyte count (MONO), and the platelet-to-lymphocyte ratio (PLR) (p<0.05). Furthermore, red cell distribution width (RDW) was positively correlated with ALP (r=0.314, p=0.001), whereas PLR was negatively correlated with ALT levels (r=-0.164, p=0.02).

Conclusion: Our findings suggest that significant variations in inflammatory markers such as PLR, LYM, and MONO across different grades of hepatosteatosis may reflect the complex interplay between metabolic dysfunction and systemic inflammation. Furthermore, lower vitamin D levels were associated with elevated blood pressure in patients with hepatosteatosis, highlighting a clinically relevant relationship between vitamin D status and cardiovascular risk in this population.

Keywords: Hepatosteatosis, hypertension, platelet-to-lymphocyte ratio, uric acid, vitamin D deficiency

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Hepatosteatosis represents the clinical starting point of a diverse pathological continuum. If left unmanaged, this condition can progress to inflammation-driven steatohepatitis and progressive hepatic fibrosis, potentially culminating in end-stage cirrhosis or hepatocellular carcinoma (HCC). A primary challenge in clinical management is the typically silent progression of the disease; consequently, its diagnosis and clinical impact are frequently recognized only after substantial liver damage has already occurred [1]. Recently renamed from non-alcoholic fatty liver disease (NAFLD) to metabolic dysfunction-associated steatotic liver disease (MASLD), this condition has become the leading cause of chronic liver disease globally,

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with an estimated prevalence of 25–30% in the general population. Current evidence emphasizes that MASLD is not merely a localized hepatic disorder but a complex multisystem disease. It is intrinsically linked to an elevated risk of cardiovascular complications and persistent systemic inflammation [2, 3].

Vitamin D, a fat-soluble vitamin with potent pleiotropic effects, plays a crucial role far beyond bone metabolism. Recent studies indexed in PubMed have highlighted its significant regulatory role in the renin-angiotensin system (RAS), suggesting that vitamin D deficiency may directly contribute to arterial hypertension and endothelial dysfunction [4]. While the association between reduced serum vitamin D concentrations and the accumulation of hepatic lipids is well documented, the precise relationship between vitamin D status and hemodynamic variables, specifically systolic blood pressure (SBP) and diastolic blood pressure (DBP), throughout the progressive stages of hepatosteatosis continues to be a focal point of contemporary clinical research [5–11].

Furthermore, the severity of hepatosteatosis is often reflected by alterations in hematological indices and inflammation-based ratios. Inflammatory indices, such as the neutrophil-to-lymphocyte ratio (NLR), which captures the balance between innate and adaptive immune signaling, and the platelet-to-lymphocyte ratio (PLR), a validated surrogate marker of subclinical systemic inflammation and prothrombotic activity, offer economical and readily available means to quantify systemic inflammatory burden. In addition, the monocyte-to-lymphocyte ratio (MLR) and the prognostic nutritional index (PNI) provide valuable information regarding the immunological profile and nutritional status of patients with metabolic dysfunction [11–16]. Nevertheless, there is a paucity of data concerning the concurrent variations of these biomarkers in relation to vitamin D status across the pathological spectrum of hepatosteatosis (Grades 0, 1, and 2–3).

Although the association between vitamin D and hepatic fat accumulation has been well established, the specific relationship between vitamin D status and hemodynamic parameters across different severities of hepatosteatosis remains insufficiently explored. The present study addresses this gap by investigating the interplay between vitamin D levels, vascular parameters, and cost-effective, readily accessible inflammatory and nutritional indices, including PLR, NLR, MLR, and PNI, across Grade 0, Grade 1, and Grades 2–3 hepatosteatosis.

Materials and Methods

Ethical considerations

This investigation was performed in strict adherence to the ethical principles outlined in the Declaration of Helsinki. The study protocol received formal review and institutional approval from the Ethics Committee of Siirt University (Date of Approval: February 26, 2026; Decision No: 168386). Given the retrospective design of the research, the requirement for written informed consent was formally waived by the ethics committee.

Study design and population

This research utilized a retrospective cross-sectional design, evaluating a cohort of patients who underwent clinical assessment at Department of Internal Medicine, Siirt University Faculty of Medicine between January 2022 and December 2025. The study population comprised 369 individuals with hepatosteatosis, as confirmed by ultrasonographic imaging. All relevant clinical, biochemical, and radiological parameters were systematically extracted from the institutional electronic medical records for retrospective analysis.

Inclusion criteria:

i) Patients aged 18 years or older.

ii) Ultrasonographically confirmed hepatosteatosis (Grade 1, 2, or 3).

iii) Patients with available data on serum vitamin D levels and complete blood count (CBC) parameters.

Exclusion criteria:

i) Chronic inflammatory diseases, active infections, or autoimmune disorders.

ii) Known history of malignancy or chronic kidney disease (Stage 3 or higher).

iii) Use of vitamin D supplements, corticosteroids, or immunosuppressive agents within the previous three months.

iv) Pregnancy or lactation.

v) Excessive alcohol consumption (defined as >20 g/day for women and >30 g/day for men).

Radiological grading assessment of hepatosteatosis

Hepatosteatosis was diagnosed and graded using high-resolution transabdominal ultrasonography performed by experienced radiologists. The radiologists were blinded to the clinical and laboratory findings of the participants. Based on parenchymal echogenicity, the presence of a "bright liver" appearance, and attenuation of the ultrasound beam, participants were categorized into three groups: Group 1 (Grade 0; minimal), Group 2 (Grade 1; mild), and Group 3 (Grades 2–3; moderate-to-severe).

Hemodynamic assessment

To ensure high precision in hemodynamic evaluation, blood pressure (BP) measurements were performed by trained healthcare professionals in a standardized clinical environment. Following a 5-minute rest period in a seated position, BP was measured using a calibrated mercury sphygmomanometer in conjunction with a validated digital oscillometric monitor (Omron HEM-907XL; Omron Healthcare, Kyoto, Japan). Blood pressure measurements were obtained at least twice, and the average values were used for statistical analysis. All measurements were performed in accordance with current international guidelines to minimize the white-coat effect and ensure intra-observer reliability.

Hormonal, biochemical, and complete blood count measurements

To minimize the influence of diurnal fluctuations, peripheral venous blood samples were collected from all participants between 08:00 and 10:00 AM following a standardized 8–12-hour overnight fast. Specimens were collected into specialized vacuum tubes containing the appropriate clot activators or anticoagulants. Subsequently, serum was separated by centrifugation at 3,000 rpm for 10 minutes under ambient temperature conditions. To ensure maximum analyte stability, all biochemical, hormonal, and CBC parameters were analyzed on the same day as sample collection.

Classification of Vitamin D status

Serum 25-hydroxyvitamin D [25(OH)D] concentrations were measured. Participants were stratified according to serum vitamin D status using the Endocrine Society guideline thresholds: deficiency (<20 ng/mL), insufficiency (20–29 ng/mL), and sufficiency (≥30 ng/mL) [17].

Hormonal and vitamin analysis

Serum concentrations of hormonal and vitamin parameters were determined using the electrochemiluminescence immunoassay (ECLIA) method on an automated platform (Beckman Coulter, UniCel DxI 600, CA, USA).

Biochemical markers, including albumin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), triglycerides, and uric acid, were measured using routine enzymatic colorimetric assays on a Cobas Integra 800 analyzer (Roche Diagnostics GmbH, Mannheim, Germany). All analyses were conducted in accordance with the manufacturer's quality assurance procedures to maintain analytical reliability and accuracy.

Hematological analysis and calculation of indices

CBC parameters, including absolute neutrophil count (ANC), absolute lymphocyte count (ALC), absolute monocyte count (AMC), and platelet count, were analyzed using an automated hematology analyzer (Sysmex XN-1000; Sysmex, Norderstedt, Germany).

Laboratory analysis and calculation of indices

The systemic inflammatory and immunological profiles were quantified using the following derived ratios and indices:

- **Neutrophil-to-Lymphocyte Ratio (NLR):** Determined by dividing the absolute neutrophil count by the absolute lymphocyte count.
- **Platelet-to-Lymphocyte Ratio (PLR):** Calculated as the quotient of the absolute platelet count and the absolute lymphocyte count.
- **Monocyte-to-Lymphocyte Ratio (MLR):** Derived from the ratio of the absolute monocyte count to the absolute lymphocyte count.
- **Prognostic Nutritional Index (PNI):** This multiparametric index was calculated to assess the intersection of nutritional status and immune competence using the following established formula:

  PNI=10×serum albumin (g/dL)+0.005×total lymphocyte count (per mm^3)

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 20.0 (IBM Corp., Armonk, NY, USA). Categorical variables are expressed as counts and percentages (n, %), while continuous variables are presented as mean±standard deviation (SD). The normality of continuous variables was evaluated using the Shapiro–Wilk test in combination with visual inspection of histograms and probability plots. Parametric statistical methods were applied for variables with a normal distribution, whereas non-parametric methods were used for variables that violated normality assumptions.

For comparisons between two independent groups, the Student's t-test was used for normally distributed variables, whereas the Mann–Whitney U test was applied for non-normally distributed variables. Associations between categorical variables were evaluated using the chi-square test or Fisher's exact test, as appropriate. For comparisons among multiple groups (hepatosteatosis grades 0, 1, and 2–3), homogeneity of variances was assessed using Levene's test before performing one-way analysis of variance (ANOVA). ANOVA was then followed by Tukey's post hoc test for pairwise comparisons.

Multivariate linear regression analyses were conducted to determine whether serum vitamin D levels were independently associated with blood pressure parameters after adjustment for potential confounding variables, including age, sex, BMI, diabetes status, lipid profile parameters, and medication use. Correlations between continuous variables were analyzed using Pearson's correlation coefficient or Spearman's rank correlation coefficient, as appropriate according to data distribution.

A two-sided p-value<0.05 was considered statistically significant. The overall study workflow is summarized in Figure 1.

Results

The study cohort comprised 369 patients with ultrasonographically verified hepatosteatosis, consisting of 188 females (50.9%) and 181 males (49.1%). The overall mean age was 47.92±13.10 years. Subgroup analysis by biological sex revealed a mean age of 48.16±0.92 years for males and 47.80±0.95 years for females, with no statistically significant age difference between the groups (p=0.790). Detailed comparisons of demographic profiles and BP characteristics across the study population are presented in Table 1.

The mean SBP and DBP values of the entire cohort were 131.43±18.11 mmHg and 83.68±13.39 mmHg, respectively, while the mean serum vitamin D concentration was 11.70±7.97 ng/mL. Pearson correlation analysis demonstrated a significant negative correlation between vitamin D levels and both SBP (r=-0.162, p=0.002) and DBP (r=-0.155, p=0.003) (Fig. 2).

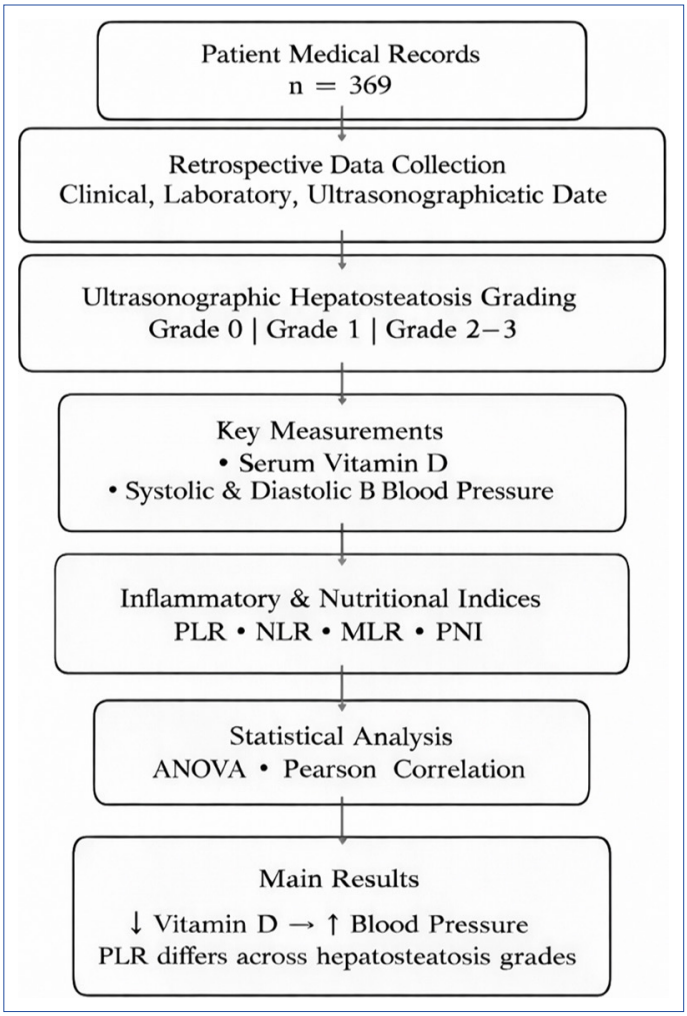

Figure 1. Study workflow illustrating retrospective data collection, ultrasonographic grading of hepatosteatosis, analytical procedures, and main outcomes.

PLR: Platelet-to-lymphocyte ratio; NLR: Neutrophil-to-lymphocyte ratio; MLR: Monocyte-to-lymphocyte ratio; PNI: Prognostic nutritional index; ANOVA: Analysis of variance.

No significant correlations were observed between vitamin D levels and liver function tests (p>0.05). A significant positive correlation was detected between RDW and ALP (r=0.314, p=0.001), whereas a significant negative correlation was observed between PLR and ALT (r=-0.164, p=0.02). No significant correlations were identified between NLR, MLR, or PNI and liver function test parameters (p>0.05).

Participants were categorized into three groups according to the severity of hepatosteatosis: Group 1 (Grade 0; minimal), Group 2 (Grade 1; mild), and Group 3 (Grades 2–3; moderate-to-severe). To identify parameters associated with hepatosteatosis severity, one-way ANOVA followed by Tukey's post hoc test was performed. Comparisons of biochemical and hormonal parameters among the study groups are shown in Table 2, while comparisons of hematological indices and inflammation-based ratios among the study groups are presented in Table 3. The analysis revealed significant differences in LDH, triglycerides, uric acid, lymphocyte count (LYM), monocyte count (MONO), and PLR among the three groups (p<0.05).

Comparisons of hemodynamic, biochemical, hormonal, and metabolic parameters according to vitamin D status groups are shown in Table 4. Significant differences were observed for SBP, DBP, TSH, and C-peptide levels among the groups (p<0.05). Post hoc analyses demonstrated that SBP and DBP values differed significantly between Group 1 and Group 2, whereas no significant differences were observed in the other subgroup comparisons. Similarly, C-peptide levels were significantly higher in Group 2 than in Group 1. In contrast, no statistically significant differences were detected regarding liver function tests, lipid parameters, ferritin, vitamin B12, insulin, uric acid, or thyroid hormone levels other than TSH (all p>0.05).

Comparisons of hematological and inflammatory indices according to vitamin D status groups are shown in Table 5. No statistically significant differences were observed among the groups regarding WBC, platelet count, MCV, RDW-C, MPV, neutrophil, lymphocyte, monocyte counts, or inflamma-

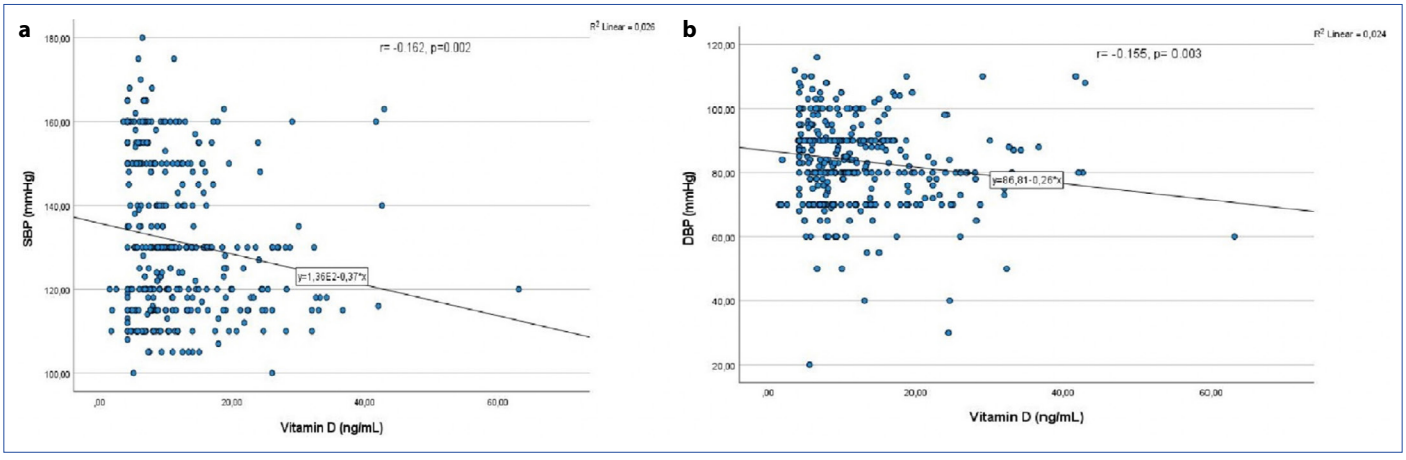

Figure 2. Scatter plots show the relationships between vitamin D levels and systolic (a) and diastolic (b) blood pressure Linear regression lines with 95% confidence intervals are displayed.

SBP: Systolic blood pressure; DBP: Diastolic blood pressure.

Table 1. Comparison of demographic and blood pressure characteristics among the study groups

	Group 1 (Grade 0; n=190) Mean±SD	Group 2 (Grade 1; n=139) Mean±SD	Group 3 (Grades 2–3; n=40) Mean±SD	p
Age (years)	47.0±13.62	48.35±12.52	50.75±12.51	0.230[a]
SBP (mmHg)	129.30±17.79	133.0±18.67	135.85±16.78	0.057[a]
DBP (mmHg)	82.47±12.56	83.96±14.99	88.05±10.27	0.053[a]

Data are presented as mean±standard deviation [a]: p<005 was considered statistically significant using one-way ANOVA. SD: Standard deviation; SBP: Systolic blood pressure; DBP: Diastolic blood pressure.

Table 2. Comparison of biochemical and hormonal parameters among the study groups

	Group 1 (Grade 0; n=190) Mean±SD	Group 2 (Grade 1; n=139) Mean±SD	Group 3 (Grades 2–3; n=40) Mean±SD	p
AST (U/L)	2138±1111	2321±1394	2270±856	0.387[a]
ALT (U/L)	2635±2201	3136±2203	3275±1744	0.059[a]
GGT (U/L)	3332±421	3732±4239	3472±2262	0.678[a]
ALP (U/L)	7689±3101	7932±2591	7652±2339	0.717[a]
LDH (U/L)	161±33	170±41	184±47	**0.001[a]**
Group 1 vs 2				0.069[b]
Group 1 vs 3				**0.020[b]**
Group 2 vs 3				0.128[b]
CRP (mg/L)	493±622	576±805	641±567	0.352[a]
TG (mg/dL)	142±63	146±85	177±103	**0.032[a]**
Group 1 vs 2				0.840[b]
Group 1 vs 3				**0.024[b]**
Group 2 vs 3				0.073[b]
Total Cholesterol (mg/dL)	202±38	205±40	206±42	0.761[a]
LDL (mg/dL)	111±26	112±26	112±31	0.837[a]
HDL (mg/dL)	5583± 1189	5346± 1120	5187± 1311	0.066[a]
Free T3 (fT3) (pg/mL)	333±040	326±045	331±047	0.335[a]
Free T4 (fT4) (ng/dL)	137± 10	128± 030	128± 016	0.449[a]
Ferritin (ng/mL)	5122±6121	6437±6520	5894±5103	0.161[a]
Vitamin B12 (pg/mL)	350 ±126	331 ±101	349 ±102	0.323[a]
Insulin (µIU/mL)	1571 ±1281	1911 ±1910	2190 ±1454	0.052[a]
Vitamin D (ng/mL)	1140±838	1234±787	1094±622	0.469[a]
TSH (µIU/mL)	222±359	233±230	319±548	0.272[a]
C-peptide (ng/mL)	354±307	405±263	443±227	0.101[a]
Uric acid (mg/dL)	471±132	518±132	515±146	**0.004[a]**
Group 1 vs 2				**0.005[b]**
Group 1 vs 3				0.143[b]
Group 2 vs 3				0.992[b]

Data are presented as mean±standard deviation [a]: p<005 was considered statistically significant using one-way ANOVA; [b]: p<005 was considered statistically significant using post-hoc Tukey test. SD: Standard deviation; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase; ALP: Alkaline phosphatase; LDH: Lactate dehydrogenase; CRP: C-reactive protein; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; WBC: White blood cell count; PLT: Platelets; TSH: Thyroid-stimulating hormone.

tory indices, including NLR, PLR, MLR, and PNI (all p>0.05). Although minor numerical variations were present between groups, these differences did not reach statistical significance.

Discussion

The present study demonstrates that lower serum vitamin D levels are significantly associated with higher SBP and DBP in patients with ultrasonographically confirmed hepatosteatosis. This finding may indicate a potential association between vitamin D deficiency and adverse hemodynamic profiles, suggesting that vitamin D status may be linked to an increased cardiovascular risk profile in this population. Furthermore, key hematological and inflammation-based indices, including LYM count, MONO count, PLR, LDH, triglycerides, and uric acid, showed significant variation across hepatosteatosis grades. These findings suggest that increasing hepatosteatosis severity is accompanied by progressive metabolic and systemic inflammatory alterations. While vitamin D levels remained

Table 3. Comparison of hematological indices and inflammation-based ratios among the study groups

	Group 1 (Grade 0; n=190) Mean±SD	Group 2 (Grade 1; n=139) Mean±SD	Group 3 (Grades 2–3; n=40) Mean±SD	p
WBC (×10³/μL)	741±225	761±171	758±173	0.658[a]
Platelet (×10³/μL)	286±69	275 ±65	291±64	0.229[a]
MCV (fL)	8634±586	8602±605	8604±570	0.879[a]
RDW-C (%)	1368±150	1378±160	1368±099	0.804[a]
MPV (fL)	1021±111	1012±099	1030±108	0.607[a]
Neutrophil (×10³/μL)	431±141	441±137	444±146	0.764[a]
LYM (×10³/μL)	239±069	260±079	248±050	**0.031**[a]
Group 1 vs 2				**0.023**[b]
Group 1 vs 3				0.753[b]
Group 2 vs 3				0.608[b]
MONO (×10³/μL)	042±013	046±013	047±014	**0.014**[a]
Group 1 vs 2				**0.029**[b]
Group 1 vs 3				0.094[b]
Group 2 vs 3				0.906[b]
NLR	194±089	181±071	183±069	0.349[a]
PLR	129±46	112±34	121±35	**0.001**[a]
Group 1 vs 2				**0.001**[b]
Group 1 vs 3				0.489[b]
Group 2 vs 3				0.479[b]
MLR	019±0067	019±006	020±008	0.677[a]
PNI	4766±270	4790±274	4759±243	0.671[a]

Data are presented as mean±standard deviation [a]: p<005 was considered statistically significant using one-way ANOVA; [b]: p<005 was considered statistically significant using post-hoc Tukey test. SD: Standard deviation; MCV: Mean corpuscular volume; RDW-C: Red cell distribution width; MPV: Mean platelet volume; LYM: Lymphocyte; MONO: monocytes; NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio; MLR: Monocyte-to-lymphocyte ratio; PNI: Prognostic nutritional index.

comparable across all steatosis severities, their negative association with BP underscores a systemic cardiovascular effect that appears to be independent of the histological or sonographic degree of liver fat accumulation.

Although serum vitamin D levels did not differ significantly across hepatosteatosis grades, significant inverse correlations with SBP and DBP were still observed. Similarly, blood pressure parameters did not show significant differences between hepatosteatosis groups despite the observed weak inverse correlations with vitamin D levels. PLR levels differed significantly according to hepatosteatosis severity. This finding may suggest that the vascular and hemodynamic effects of vitamin D deficiency occur independently of the ultrasonographic severity of hepatosteatosis. In addition, the generally low vitamin D levels observed throughout the cohort may have reduced the ability to detect significant differences between steatosis grades. The multifactorial pathophysiology of MASLD, involving metabolic, inflammatory, and cardiovascular mechanisms, may also contribute to this dissociation. However, statistical significance was not consistently maintained across all subgroup comparisons, particularly between more advanced hepatosteatosis grades, which may reflect biological heterogeneity and the multifactorial inflammatory nature of MASLD.

Routine CBC offers a practical and cost-effective means of evaluating systemic immune dynamics. Parameters such as NLR, MLR, PLR, and the systemic immune-inflammation index (SII) have emerged as accessible biomarkers that capture the balance between distinct leukocyte subpopulations [18–20]. A major finding of our study is the grade-dependent alteration in PLR, which reflects both platelet activation and relative lymphopenia. PLR emerged as a significant marker associated with hepatosteatosis severity, supporting its role as an indicator of subclinical inflammation and prothrombotic tendency. As hepatosteatosis progresses, lipid-laden hepatocytes trigger evolving inflammatory and fibrotic cascades. Previous research, such as the study by Demiröz et al. [21], demonstrated that systemic inflammatory markers like the Pan-Immune-Inflammation Value (PIV) positively correlate with hepatosteatosis severity; our findings extend this observation by identifying PLR as a particularly sensitive marker in a graded clinical setting. Although significant inverse correlations were observed between serum vitamin D levels and blood pressure parameters, no statistically significant differences in vitamin D levels or blood pressure measurements were detected across hepatosteatosis grades. In addition, while certain inflammatory indices demonstrated significant variation between some subgroups, these differences were not consistently maintained across all hepatosteatosis stages, particularly in more advanced grades. These findings suggest that the observed associations should be interpreted cautiously given the multifactorial and heterogeneous nature of MASLD.

Table 4. Comparison of hemodynamic, biochemical, hormonal, and metabolic parameters according to vitamin D status groups

	Group 1 (<20 ng/mL, n=322) Mean±SD	Group 2 (20–29 ng/mL, n=33) Mean±SD	Group 3 (≥30 ng/mL, n=14) Mean±SD	p
SBP (mmHg)	132.45±18.43	132.45±12.75	126.64±17	**0.016**[a]
Group 1 vs 2				**0.020**[b]
Group 1 vs 3				0.463[b]
Group 2 vs 3				0.854[b]
DBP (mmHg)	84.66±12.73	74.63±15.53	82.57±15.91	**0.001**[a]
Group 1 vs 2				**0.001**[b]
Group 1 vs 3				0.829[b]
Group 2 vs 3				0.141[b]
AST (U/L)	22.29±8.60	21.90±6.55	21.14±7.20	0.929[a]
ALT (U/L)	29.01±7.40	29.39±6.07	25.78±5.55	0.855[a]
GGT (U/L)	35.32±11.78	35.63±12.39	24.92±12.60	0.640[a]
ALP (U/L)	77.29±25.28	83.39±18.68	75.71±11.76	0.482[a]
LDH (U/L)	166.86±38.50	169.03±38.82	163.35±42.20	0.897[a]
CRP (mg/L)	5.56±3.21	4.93±2.05	3.42±1.85	0.483[a]
TG (mg/dL)	149.91±79.95	123.18±41.49	138.28±63.98	0.150[a]
Total cholesterol (mg/dL)	204.05±39.19	199.48±36.30	197.28±44.53	0.684[a]
LDL (mg/dL)	111.80±27.22	108.42±22.05	105.57±27.27	0.569[a]
HDL (mg/dL)	54.32± 11.85	56.74± 11.64	52.80± 11.97	0.463[a]
Free T3 (fT3) (pg/mL)	3.31±0.41	3.31±0.31	3.02±0.82	0.064[a]
Free T4 (fT4) (ng/dL)	1.33± 0.79	1.25± 0.17	1.40± 0.20	0.769[a]
Ferritin (ng/mL)	57.0±21.55	56.49±21.26	56.47±16.47	0.999[a]
Vitamin B12 (pg/mL)	340.67 ±113.81	366.96 ±125.18	336.57 ±106.30	0.455[a]
Insulin (µIU/mL)	17.19±6.88	21.38±5.44	21.94±4.85	0.209[a]
TSH (µIU/mL)	2.41±1.64	2.06±1.30	2.09±1.14	**0.015**[a]
C-peptide (ng/mL)	3.69±2.27	5.20±3.19	3.82±1.78	**0.015**[a]
Group 1 vs 2				**0.010**[b]
Group 1 vs 3				0.984[b]
Group 2 vs 3				0.280[b]
Uric acid (mg/dL)	4.92±1.34	5.10±1.36	5.13±1.71	0.730[a]

Data are presented as mean±standard deviation. [a]: p<0.05 was considered statistically significant using one-way ANOVA. [b]: p<0.05 was considered statistically significant using post-hoc Tukey test. SD: Standard deviation; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase; ALP: Alkaline phosphatase; LDH: Lactate dehydrogenase; CRP: C-reactive protein; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; WBC: White blood cell count; PLT: Platelets; TSH: Thyroid-stimulating hormone.

Table 5. Comparison of hematological and inflammatory indices according to vitamin D status groups

	Group 1 (<20 ng/mL, n=322) Mean±SD	Group 2 (20–29 ng/mL, n=33) Mean±SD	Group 3 (≥30 ng/mL, n=14) Mean±SD	p
WBC ($\times10^3$/µL)	7.49±1.77	7.68±3.79	7.80±1.76	0.773[a]
Platelet ($\times0^3$/µL)	283.68±66.55	274.78±70.80	280.92±64.15	0.271[a]
MCV (fL)	86.28±5.92	85.09±6.11	86.92±4.66	0.879[a]
RDW-C (%)	13.71±1.45	13.81 ±1.93	13.69±1.26	0.713[a]
MPV (fL)	10.16±1.06	10.26±0.99	10.4±1.08	0.409[a]
Neutrophil ($\times10^3$/µL)	4.40±1.43	4.0±1.18	4.6±1.41	0.431[a]
LYM ($\times10^3$/µL)	2.48±0.69	2.53±0.92	2.46±0.82	0.925[a]
MONO ($\times10^3$/µL)	0.44±0.13	0.44± 0.14	0.43±0.11	0.903[a]
NLR	1.89±0.82	1.72±0.64	2.03±0.88	0.431[a]
PLR	122±41	118±42	122±38	0.879[a]
MLR	0.189±0.066	0.186±0.0561	0.189±0.0805	0.971[a]
PNI	47.78±2.66	47.70±3.19	41.17±1.76	0.710[a]

Data are presented as mean±standard deviation. [a]: p<0.05 was considered statistically significant using one-way ANOVA. WBC: White blood cell count; MCV: Mean corpuscular volume; MPV: Mean platelet volume; LYM: Lymphocyte count; NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio; MLR: Monocyte-to-lymphocyte ratio; PNI: Prognostic nutritional index.

The pleiotropic effects of vitamin D on immune regulation, insulin sensitivity, and inflammatory pathways provide a biological basis for these observations. The proposed link between vitamin D status and MASLD involves attenuation of adipose tissue-derived inflammation and suppression of hepatic fibrotic processes through vitamin D receptor-dependent pathways [22]. According to the "two-hit" hypothesis, the initial "hit" of insulin resistance drives triglyceride accumulation, whereas the "second hit," characterized by oxidative stress and proinflammatory cytokines, initiates local and systemic inflammation [23]. Our observed negative correlation between PLR and ALT further suggests that inflammatory dysregulation may not always parallel conventional liver enzyme elevations, reinforcing the concept that systemic inflammation can occur independently of overt hepatocellular injury.

While many studies have associated MASLD with lower vitamin D levels due to impaired mobilization from adipose tissue in obese individuals [24–28], some population-based reports have failed to identify such differences [29, 30]. These discrepancies may arise from heterogeneity in diagnostic criteria or genetic polymorphisms in the vitamin D receptor [31, 32]. Although previous studies consistently linked low vitamin D levels to metabolic syndrome [33, 34], data examining hemodynamic parameters across graded hepatosteatosis remain limited. Our study addresses this gap by demonstrating that reduced vitamin D levels correlate with elevated blood pressure independently of hepatosteatosis severity, indicating a predominantly vascular influence rather than one confined to liver pathology. Vitamin D deficiency has been associated with endothelial dysfunction, increased activation of the renin–angiotensin–aldosterone system (RAAS), impaired vascular compliance, and increased arterial stiffness, all of which may contribute to elevated blood pressure in patients with MASLD [35, 36]. In addition, chronic low-grade inflammation and oxidative stress accompanying metabolic liver disease may further exacerbate vascular dysfunction and hemodynamic impairment.

Regarding inflammatory indices, our finding that PLR, rather than NLR or MLR, was significantly associated with disease severity is noteworthy. While some investigations have reported elevated NLR in advanced MASLD [19, 34, 37–42], the lack of significance in our cohort may be explained by the predominance of early-stage hepatosteatosis. In these early stages, systemic inflammatory activation may be sufficient to alter PLR before inducing the marked neutrophil-to-lymphocyte imbalance typically observed in advanced fibrosis or cirrhosis [43–45].

Given the central role of the liver in nutritional metabolism, nutritional impairment is a common feature across chronic liver disease. In this context, PNI, as a composite marker reflecting serum albumin levels and lymphocyte count, provides a cost-effective and readily accessible measure of nutritional and immunological status [46, 47]. Higher PNI values have been shown to correlate with an increased likelihood of MASLD while exhibiting an inverse association with the risk of atrial fibrillation [47]. However, additional well-designed studies are necessary to confirm and validate these findings. Although PNI did not differ significantly across hepatosteatosis grades in our cohort, this finding may reflect the predominance of early-stage disease, in which overt malnutrition has not yet developed. PNI may therefore have greater clinical relevance in advanced stages of liver disease or in longitudinal assessments rather than in cross-sectional evaluations of early hepatosteatosis. Taken together, these findings suggest that lymphocyte dynamics, as a key component of the PNI calculation, vary across different stages of MASLD and may account for the divergent patterns observed in PNI throughout disease progression [48].

Clinical implications and limitations

The observed inverse association between serum vitamin D levels and blood pressure parameters may suggest a potential relationship between vitamin D deficiency and cardiovascular risk in patients with hepatosteatosis. However, given the weak correlation strength, these findings should be interpreted cautiously and may have limited clinical predictive value. In addition, readily accessible hematological indices such as PLR may provide supportive information regarding systemic inflammatory status in clinical practice.

Several limitations should be acknowledged. First, due to the cross-sectional and retrospective design of the study, the observed associations should not be interpreted as causal relationships. Second, although ultrasonography is widely used in routine clinical practice for the assessment of hepatosteatosis, it has lower sensitivity than histological evaluation or advanced imaging modalities such as MRI-PDFF. Although ultrasonographic grading was performed by multiple experienced radiologists who were blinded to clinical and laboratory findings, formal interobserver variability analysis could not be performed because of the retrospective study design. Furthermore, information regarding antihypertensive treatment was not consistently available and therefore could not be comprehensively assessed. Additionally, several important metabolic and cardiovascular confounding factors, including waist circumference, smoking status, and insulin resistance indices such as HOMA-IR, were not consistently available, which may have limited comprehensive risk adjustment. Fibrosis severity was also not systematically evaluated. Seasonal variation in serum vitamin D levels could not be comprehensively assessed due to the retrospective design. In addition, the relatively small sample size in the advanced hepatosteatosis group may have limited statistical power for certain subgroup comparisons. Finally, the relatively weak correlations observed in this study may limit the generalizability and clinical applicability of the findings. Further large-scale prospective and interventional studies are needed to clarify the clinical significance of these associations in patients with MASLD.

Conclusion

In summary, variations in readily accessible hematological inflammatory indices, particularly PLR, across different stages of hepatosteatosis may support the presence of low-grade system-

ic inflammation accompanying the progression of metabolic liver disease. In addition, serum vitamin D levels were significantly associated with blood pressure parameters in patients with hepatosteatosis; however, the strength of these associations was weak and should therefore be interpreted cautiously. These findings suggest that vitamin D status and hematological inflammatory markers may provide supportive clinical information in patients with MASLD. Nevertheless, given the cross-sectional design and modest correlation strengths, further large-scale prospective studies are required to clarify the clinical significance and potential causal relationships of these associations. Future prospective multicenter studies incorporating fibrosis assessment and longitudinal evaluation of inflammatory markers are needed to further elucidate the clinical utility of vitamin D status and inflammatory indices in patients with MASLD.

Disclosures

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Research Article

Application of data mining for monitoring turnaround time in clinical laboratories: A workflow-based model using Orange visual programming

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Abstract

Objectives: Turnaround time (TAT) is a key quality indicator in laboratory medicine, but routine department-level monitoring is limited by fragmented data and scarce analytical resources. We aimed to develop and evaluate a modular, reproducible data analytics workflow—built in the open-source Orange visual programming platform and integrated with hospital information management system (HIMS) data—to monitor laboratory TAT, localize delays, and support quality improvement interventions.

Methods: Approximately 3.75 million timestamped records from a 1,875-bed tertiary care hospital were extracted over three consecutive months. A six-phase Orange pipeline performed data import, concatenation, filtering, unit-level stratification, One-Class Support Vector Machine outlier exclusion, and visualization. Monthly reports were submitted to the laboratory quality committee and prompted targeted interventions. Unit-level monthly means were compared using the Friedman and Wilcoxon signed-rank tests.

Results: Order-to-phlebotomy time decreased significantly across the three months (Friedman p=0.006; −17.5% with Wilcoxon p=0.005 after excluding one administrative unit). Sample reception time varied significantly (p=0.004) owing to a February peak, with no sustained net change by March (p=0.93). Total analytical TAT fell by 19.1% overall (immunoassay: −23.7%; clinical chemistry: −22.3%)—a consistent trend that did not reach statistical significance at the test-group level (p=0.074).

Conclusion: A low-code, visual Orange workflow enabled reproducible, near-real-time monthly TAT monitoring and helped target interventions, offering an accessible alternative to resource-intensive approaches.

Keywords: Clinical laboratory services, data mining, quality improvement, quality indicators, health care, time factors, workflow

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Laboratory testing constitutes a cornerstone of modern clinical decision-making, and the timely delivery of accurate results is essential for optimal patient care. While analytical precision is paramount, clinical laboratories are increasingly required to meet stringent turnaround time (TAT) benchmarks. TAT is broadly defined as the elapsed time from test ordering to result reporting, and it serves as a critical quality performance indicator in laboratory medicine [1].

Timeliness is of particular importance in emergency and inpatient settings, where delayed results can adversely affect diagnostic and therapeutic decision-making [2]. Achieving optimal TAT necessitates continuous monitoring and improvement across all workflow subprocesses, including sample collection, transportation, analysis, and result validation. The influence of TAT on patient satisfaction, clinical efficiency, and institutional accreditation has been extensively documented [3, 4].

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Nevertheless, accurate TAT assessment remains challenging owing to variability in laboratory information system configurations, inconsistent timestamp recording practices, and operational fragmentation across clinical departments. Numerous institutions have reported the benefits of TAT dashboards, Lean Six Sigma methodologies, and root cause analysis frameworks in reducing delays and enhancing process transparency [5–7].

An increasing body of evidence underscores the need for data-driven, visual workflow-based tools to analyze laboratory processes and systematically identify bottlenecks [8, 9]. Platforms such as Orange Data Mining provide a comprehensive range of analytical functionalities, including data merging, manipulation, feature engineering, cleaning, pivoting, outlier exclusion, descriptive statistics, and visualization, that facilitate efficient processing of datasets retrieved from the HIMS, transforming raw time-stamped records into actionable analytical outputs for continuous quality improvement.

This study aimed to develop and evaluate a modular data analytics workflow constructed using Orange and integrated with HIMS data, with the objectives of tracking and analyzing laboratory TATs, generating visual and stratified assessments of process delays, and supporting continuous quality improvement in laboratory operations.

Materials and Methods

Data collection

The study was conducted at a tertiary care hospital with a bed capacity of 1,875. The central laboratory processes approximately 50,000 tests daily, and the TAT analysis encompassed a dataset of approximately 3,750,000 test records. Retrospective laboratory data were extracted from the Hospital Information Management System (HIMS). The dataset comprised test requests, sample types, and timestamps for each workflow stage, from physician order entry to result release, enabling detailed TAT analysis across a three-month observational period.

Although the validation dataset was retrospective, the workflow was designed for recurring, near-real-time use. Because all timestamps are captured automatically by the HIMS and the saved Orange pipeline can be re-executed on each new monthly export without manual re-entry, the system supports prospective monthly (rather than continuous live) evaluation of TAT.

Data preprocessing and preparation

The raw dataset was cleaned and transformed for compatibility with the Orange Data Mining environment. Missing values were addressed through imputation, and categorical variables (e.g., sample type, error category) were encoded appropriately. Duplicate records were identified and removed using Orange's preprocessing widgets. Timestamp intervals were stratified into standard laboratory workflow phases: order to phlebotomy (pre-laboratory TAT); phlebotomy to sample reception (transport time); sample reception to start of analysis (laboratory TAT, including centrifugation and preparation); start of analysis to result in analyzer (analytical TAT); result in analyzer to result release (post-analytical TAT); and result release to physician access (clinician access time). Data on sample rejections, rejection reasons, and their distribution by clinical location were additionally incorporated. All timestamps were digitally extracted from the HIMS, enabling recurring monthly evaluations without manual data entry.

Monitoring-driven interventions

The Orange workflow was not used solely as a passive monitoring instrument; the monthly stratified outputs were fed back into the laboratory's quality improvement cycle and triggered targeted operational interventions. On the basis of each monthly report, the quality management committee reviewed the units and workflow phases with the longest or most variable TATs and implemented corrective actions, including: (i) structured feedback meetings with units showing the highest order-to-phlebotomy times; (ii) optimization of inter-tower sample transport scheduling and porter routes; (iii) reinforcement and retraining of phlebotomy and sample-handling procedures in units with recurrent pre-analytical delays; and (iv) adjustment of staffing allocation during identified peak-demand intervals. The observed TAT changes should therefore be interpreted as the combined effect of continuous monitoring and these monitoring-driven interventions.

Data analysis using orange

Orange Data Mining is an open-source visual analytics platform designed for both novice and expert users [10]. It provides a drag-and-drop interface in which workflows are constructed from modular visual components (widgets) supporting data preprocessing, statistical analysis, machine learning, and interactive visualization without programming. A visual workflow was constructed spanning monthly data import, statistical presentation, and visualization (Fig. 1). Process data files were concatenated vertically using the Concatenate widget, followed by targeted row and column filtering and stratification across towers, floors, and clinical units. New monthly data can be incorporated into the saved workflow without rebuilding the pipeline; additional analyses or visualizations require only connecting the corresponding widgets to the pre-filtered data stream. The monthly report was formally recognized by the hospital's quality management department as a new institutional quality indicator. TAT metrics were visualized using box plots and complementary tools (Fig. 2), enabling rapid identification of outliers and process delays.

Outlier detection and handling

The dataset was first stratified by tower and clinical unit to enable location-specific analysis (Fig. 3). Considerable variability was observed in the order-to-phlebotomy interval, attributable in part to patients providing blood samples on a subsequent date, for example, on the second day of the menstrual cycle, as requested by endocrinology units to eval-

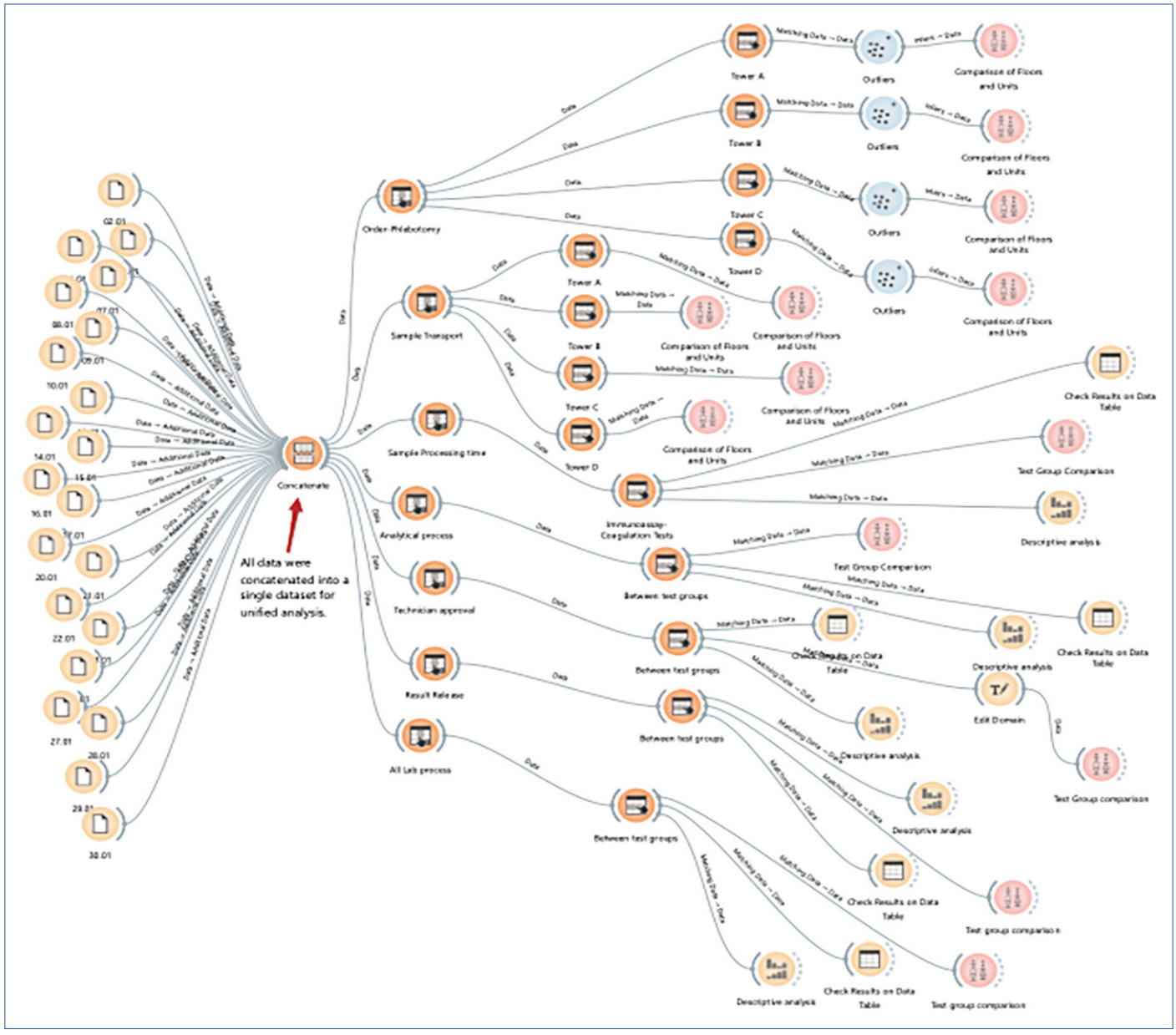

Figure 1. Visual workflow created in Orange Data Mining software. The cluster of File widgets represents daily data exports retrieved from the Hospital Information Management System (HIMS) over the course of a month. Each File widget corresponds to a specific day (e.g., 01.01, 02.01, etc.) and is merged using the Concatenate widget. This is followed by filtering steps and stratification based on location, enabling tailored analysis of relevant subsets. Subsequent modules include statistical analysis and data visualization components. The modular structure of the workflow allows for flexible expansion, integration of new analyses, or the construction of predictive models.

Overview of the modular Orange visual-programming workflow, spanning monthly HIMS data import and concatenation, row/column filtering, stratification by tower and clinical unit, outlier detection, descriptive statistics, and visualization.

uate the early follicular phase. To account for such clinically motivated timing discrepancies, anomalous observations were detected and excluded using a One-Class Support Vector Machine (One-Class SVM), implemented through the Orange Outliers widget. The One-Class SVM is an unsupervised, kernel-based algorithm that learns a boundary enclosing the bulk of the observations in feature space and labels points outside this boundary as outliers; it requires neither labelled data nor an assumption of normality. This method was preferred over simpler univariate rules (e.g., interquartile range [IQR] or fixed-percentile thresholds) because the data were strongly right-skewed and contained clinically legitimate but extreme deferred-collection values; a single global cut-off across heterogeneous units would misclassify routine values in slow units while missing context-specific anomalies. A radial basis function (Gaussian) kernel was used with the contamination (ν) parameter set to 0.10, consistent with the Orange default; flagged observations were excluded only from

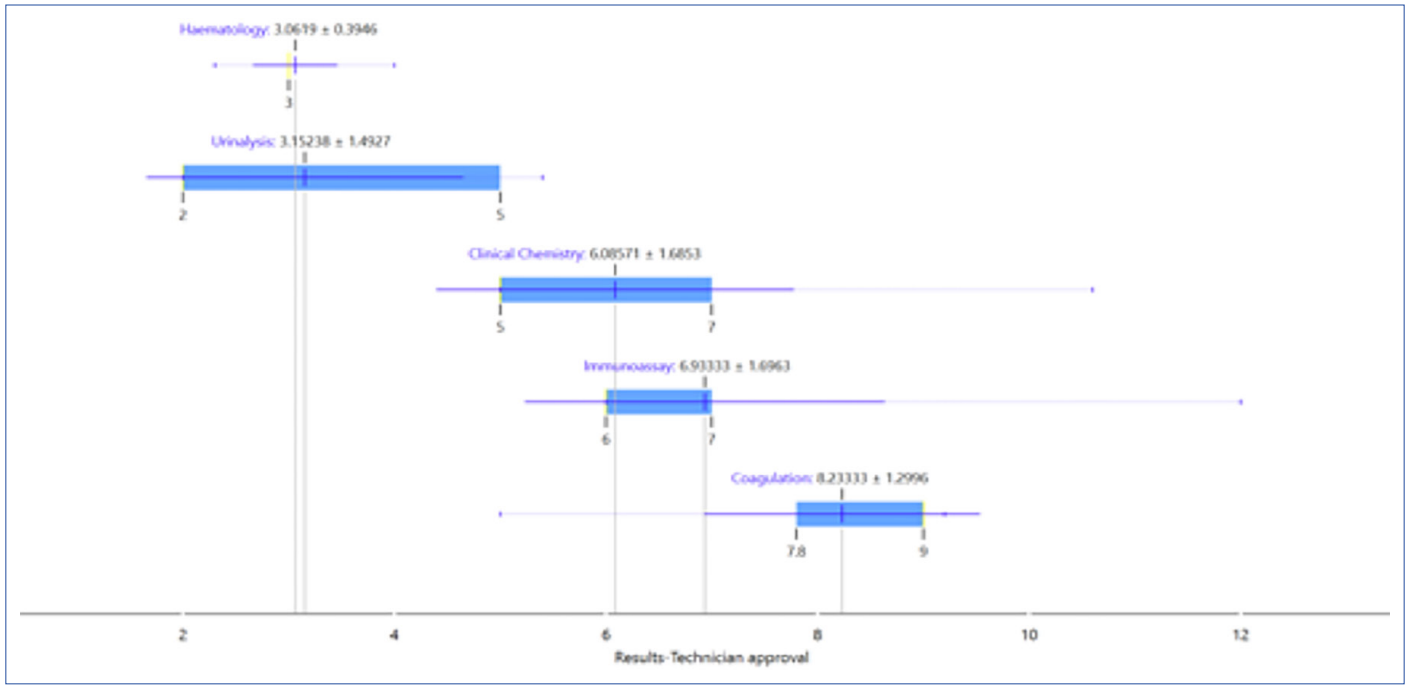

Figure 2. Time from result to technician approval (in minutes) across different test groups. Mean ± standard deviation values are shown for each group, illustrating variation in post-analytic TATs. Coagulation and immunoassay test groups had the longest average approval times, while hematology showed the shortest.

Box-plot visualization of turnaround-time metrics generated within Orange, enabling rapid identification of outliers and process delays across workflow phases.

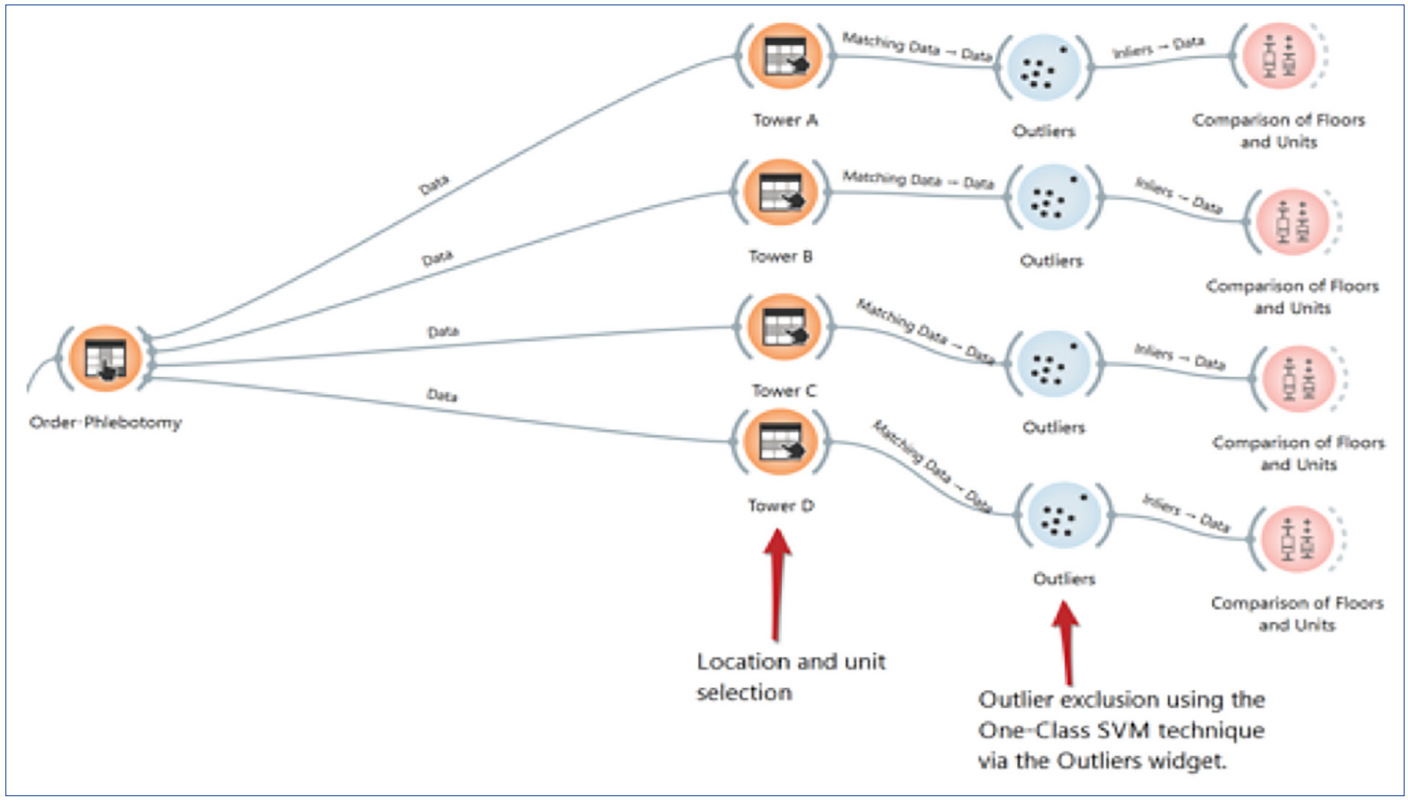

Figure 3. Location-based workflow for evaluating the time interval between order entry and phlebotomy across different towers. Data from Tower A, B, C, and D were filtered and processed separately to allow floor- and unit-specific comparisons. Each subset was analyzed using matching and visualization widgets to assess intra-location variability and highlight structural workflow differences between departments.

Tower- and unit-level stratification of order-to-phlebotomy times, illustrating location-specific variability used to target process-improvement efforts.

Table 1. Widgets used in the construction of the data analysis model

Widget	Purpose of Use
File	Used to import the dataset into the Orange environment.
Concatenate	Used to merge HIMS datasets collected on different dates.
Select Rows	Used to filter samples based on specific criteria (e.g., adult patients).
Select Columns	Used to select variables for analysis or remove unnecessary columns.
Outliers	Used to detect and exclude outliers using the One-Class Support Vector Machine (One-Class SVM) technique.
Data Table	Used to display the final version of the data in a tabular format.
Box Plot	Used to visualize the distribution of variables and identify potential outliers.
Feature Statistics	Used to obtain basic statistical summaries (mean, median, SD) of selected variables.
Edit Domain	Used to rename variables or edit labels of categorical data.

the corresponding TAT comparison and were not deleted from the source dataset. Excluded observations corresponded predominantly to (a) next-day or later deferred sample collection, (b) physiologically scheduled draws, and (c) timestamp-entry errors producing implausible or negative intervals. [Authors' note: Please insert the exact number and type of excluded observations per table (n excluded / N total).] This preprocessing step improved the accuracy and interpretability of TAT comparisons across floors and clinical units. The custom widget pipeline (Table 1) supported data import, preprocessing, One-Class SVM outlier detection, and basic statistical evaluation within an interactive visual environment. Validation of the developed tool was performed using virtual timestamp data.

Ethical considerations

This study analyzed fully anonymized, aggregated operational data routinely generated by the HIMS for laboratory quality-indicator monitoring; no patient-identifying information, clinical outcomes, or individually identifiable records were accessed. Because the analysis was limited to de-identified operational and quality-management data, it was deemed exempt from formal institutional review board approval in accordance with institutional policy and applicable national regulations governing the secondary use of non-identifiable operational data. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Statistical analysis

Because the data available for these comparisons consisted of de-identified, unit-level monthly summaries (mean±standard deviation) rather than individual sample-level records, comparisons were performed at the level of clinical units (order-to-phlebotomy and sample-reception times) or test groups (total laboratory TAT). For each table, differences across the three consecutive months (January, February, and March) were assessed with the non-parametric Friedman test for related samples, treating clinical units (or test groups) as the repeated subjects. Where the Friedman test indicated a significant overall effect, the January-versus-March contrast was examined post hoc with the Wilcoxon signed-rank test. Non-parametric tests were chosen because TAT distributions are right-skewed and the number of units per comparison was small. A two-sided p-value<0.05 was considered statistically significant. Analyses were performed in Python (version 3) using NumPy. Accordingly, the terms "significant" and "marked" are used in the Results only where supported by these tests; numerically large but non-significant changes are described as trends.

Results

Following the implementation of process monitoring and the associated interventions, most clinical units across all towers showed a progressive reduction in order-to-phlebotomy times over the three-month period. The shortest March durations were observed in Cardiology (Tower A) and Internal Medicine (Tower C), both reaching 7.65 min. Pediatrics (Tower D) peaked in February (17.79 min) and improved in March (13.41 min). In contrast, the Medical Board unit in Tower A, which recorded the highest TATs throughout, increased from February (33.70 min) to March (54.46 min), indicating persistent variability in workflow adherence. Across all eleven units, the change over the three months was statistically significant (Friedman χ^2=10.36, p=0.006); the January-to-March reduction approached significance with all units included (Wilcoxon p=0.051) but became clearly significant after excluding the Medical Board administrative unit, whose times rose against the general trend (10 clinical units: −17.5%; Friedman χ^2=12.80, p=0.002; Wilcoxon p=0.005). Thus, data-driven monitoring combined with targeted interventions was associated with measurable reductions in order-to-phlebotomy time across most departments—particularly in general internal medicine and surgical specialties—while a subset of administrative units showed inconsistent progress (Table 2).

Sample-reception times revealed substantial inter-departmental and temporal variation. In Tower A, the Medical Board unit recorded the longest times, notably in January (50.80 min), with a gradual decline thereafter; Cardiology and Oncology were shorter and more stable. In Tower B, General Surgery peaked in February (44.50 min) before falling in March, while Urology and Ophthalmology remained elevated. In Tower C, Nephrology and Internal Medicine were higher and more stable than Endocrinology, which declined to 32.0 min by March. In Tower D, Pediatrics and Gy-

Table 2. Three-month order-to-phlebotomy and sample-reception times across hospital towers and units (minutes, mean±SD)

Section / unit	Order-to-phlebotomy			Sample-reception		
	Jan	Feb	Mar	Jan	Feb	Mar
Tower A						
Cardiology	9.95±6.09	9.21±2.40	7.65±1.94	30.95±2.39	38.93±8.14	34.12±5.04
Oncology	11.85±3.92	7.71±2.55	10.12±5.52	32.13±3.14	36.36±8.90	30.47±5.28
Medical board	40.40±20.11	33.70±24.88	54.46±31.46	50.80±17.60	50.50±15.81	47.46±29.47
Tower B						
General surgery	10.28±6.44	9.71±3.73	9.00±3.05	33.09±8.58	44.50±16.58	34.35±7.42
Urology	13.00±4.93	11.14±3.91	11.06±4.49	33.08±3.44	35.93±6.49	37.12±7.87
Ophthalmology	13.99±6.02	11.50±3.33	10.77±4.65	32.61±7.53	42.64±11.56	35.71±6.56
Tower C						
Internal medicine	8.52±1.37	9.07±2.09	7.65±1.57	40.74±6.21	48.36±10.09	40.77±6.12
Endocrinology	13.71±1.72	12.57±2.16	10.71±4.13	36.11±5.65	44.64±9.40	32.00±5.50
Nephrology	14.02±3.59	11.36±3.31	9.18±1.85	46.19±12.13	47.43±8.22	42.17±9.29
Tower D						
Pediatrics	15.10±3.72	17.79±6.10	13.41±2.70	41.23±6.67	51.71±9.23	39.35±6.61
Gynecology-Obstetrics	15.71±4.20	12.29±2.46	14.53±3.62	42.48±6.48	51.79±13.43	49.94±10.70

Order-to-phlebotomy — Friedman across the three months (11 units): χ^2=10.36, p=0.006; excluding the Medical Board unit (10 units): χ^2=12.80, p=0.002; Wilcoxon Jan vs Mar p=0.005 (−17.5%). Sample-reception — Friedman across the three months (11 units): χ^2=11.09, p=0.004 (February peak); Wilcoxon Jan vs Mar p=0.93 (no sustained change). SD: Standard deviation.

Table 3. Three-month total laboratory TATs across different test groups (minutes, mean±SD)

Test group	January	February	March
Urinalysis	56.90±13.36	64.71±22.29	62.18±7.91
Hematology	77.16±11.57	77.79±15.48	59.76±18.72
Coagulation	101.38±18.30	94.07±14.90	80.06±12.40
Immunoassay	148.51±10.97	147.57±24.96	113.35±18.26
Clinical Chemistry	152.72±20.35	158.43±28.99	118.59±19.85

Friedman across the three months (5 test groups): χ^2=5.20, p=0.074; Wilcoxon Jan vs Mar p=0.080. The overall reduction (−19.1%) is a consistent trend that did not reach significance at the test-group level. TAT: Turnaround time; SD: Standard deviation.

necology-Obstetrics showed prolonged times, particularly in February. Across units, sample-reception times differed significantly between months (Friedman χ^2=11.09, p=0.004); however, this reflected a transient February peak rather than a sustained reduction, as values returned to approximately January levels by March (Wilcoxon p=0.93). These findings identify a February bottleneck as a priority target for process improvement (Table 2).

Total analytical TATs declined across most test groups over the three-month period. The most pronounced improvements were in the Immunoassay and Clinical Chemistry groups: mean immunoassay TAT fell from 148.51±10.97 min in January to 113.35±18.26 min in March (−23.7%), and Clinical Chemistry from 152.72±20.35 min to 118.59±19.85 min (−22.3%); Hematology and Coagulation TATs also fell. At the test-group level, the overall three-month change (mean −19.1%) did not reach statistical significance (Friedman χ^2=5.20, p=0.074; Wilcoxon p=0.080), most plausibly reflecting the small number of test groups (n=5); the consistent downward trend nonetheless supports improved analytical throughput (Table 3).

Discussion

Since the landmark publication of *To Err is Human*, global awareness of the imperative to improve patient safety has grown substantially over the past two decades [11]. TAT broadly refers to the interval between test requisition and result reporting; its operational definition varies across institutions depending on the designated start point. TAT is commonly stratified into pre-analytical, analytical, and post-analytical phases or classified by request urgency (STAT, urgent, routine) [12]. As a critical quality indicator, TAT reflects the commitment to timely result delivery, and delays at any phase can adversely affect patient outcomes [12].

In the present study, a modular Orange-based analytics workflow was integrated with HIMS data to track TAT, visualize delays by test type and patient cohort, and generate actionable insights. Over 3.7 million timestamped records were processed through a six-phase modular pipeline incorporating widgets for import, concatenation, filtering, outlier detection, and visualization, supporting reproducible monthly reporting without rebuilding the workflow.

The operational effectiveness of this workflow is reflected in consistent reductions in key indicators: immunoassay TAT declined by 23.7% and clinical chemistry TAT by 22.3%, while the Endocrinology unit's sample-reception time declined to 32.0 min by March. All of these findings highlight the workflow's capacity to generate stratified, actionable insights. However, although these reductions were numerically substantial, the three-month change at the aggregate test-group level did not reach statistical significance (Friedman p=0.074); the results are therefore best interpreted as a favorable operational trend pending confirmation in a sample-level analysis.

Cassim et al. [13] reported a substantial improvement in TAT performance, with results delivered within target rising from below 10% to over 90% and the 75th percentile TAT falling from 10 hours to under 3 hours, underscoring that TAT monitoring combining central tendency and distributional tails—coupled with quality management interventions—can enhance efficiency. The authors caution that root cause analysis and multidisciplinary management are essential given the multifactorial determinants of TAT. In the present study, particular attention was directed to processes susceptible to extreme values, most notably the requisition-to-collection interval; deferred-collection observations were systematically detected and excluded using the One-Class SVM, improving the reliability of TAT estimates.

Gupta and colleagues applied Lean methodology—value stream mapping, Gemba walks, Pareto analysis, and root cause analysis—reducing TAT from approximately 180 to 95 min in hematology and 268 to 208 min in biochemistry; however, confinement to two departments in a single institution limits generalizability [14]. Large-scale Lean application across all units of a 1,875-bed hospital presents practical and logistical challenges, whereas the present approach offers a more feasible, resource-efficient alternative for routine multi-departmental TAT monitoring with minimal workflow disruption.

An alternative approach using statistical process control (SPC) charts enables dynamic TAT monitoring by identifying trends and deviations relative to control limits adapted to skewed distributions; however, implementation is resource-intensive and presupposes advanced statistical expertise, rendering it less practicable in large, multi-unit settings [15]. By comparison, the Orange-based workflow is accessible, transparent, and adaptable, enabling visual data tracking without specialized statistical proficiency while prioritizing practicality and integration into routine operations.

Limitations

This study has several limitations. The analysis covered a three-month period at a single institution, constraining generalizability and precluding assessment of long-term sustainability. Statistical comparisons used de-identified, unit-level monthly summaries rather than sample-level records, so the aggregate tests are conservative and may underestimate effects detectable at the sample level. Finally, because monitoring and interventions were implemented concurrently within a real-world quality improvement program, the design is observational and the contribution of each component cannot be fully disentangled.

Conclusion

A modular, open-source Orange workflow provided an accessible and reproducible means of monitoring laboratory TAT, localizing pre-analytical and analytical delays, and guiding targeted quality improvement interventions. Formal significance was reached for the reduction in order-to-phlebotomy time, whereas the aggregate analytical TAT reduction represented a favorable but non-significant trend. Sample-level analyses and longer follow-up are warranted to confirm the magnitude and sustainability of these improvements..

Disclosures

Ethics Committee Approval: This study analyzed fully anonymized, aggregated operational data routinely generated by the HIMS for laboratory quality-indicator monitoring; no patient-identifying information, clinical outcomes, or individually identifiable records were accessed.

Informed Consent: Informed consent was obtained from all participants.

Conflict of Interest Statement: None declared.

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Research Article

Evaluation of HbA1c analytical performance using sigma metrics and total allowable error models

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Abstract

Objectives: Accurate measurement of glycosylated hemoglobin (HbA1c) is essential for the correct diagnosis and treatment of diabetes. The Implementation Task Force of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC-TF) recommends the standardization of HbA1c testing and its monitoring using the sigma metric model. The aim of this study was to evaluate the analytical performance of the HbA1c test using sigma metrics according to different quality specifications and to examine its impact on quality control planning.

Methods: Coefficients of variation were evaluated using IQC materials at two levels. Bias values were calculated from 12 months of PT results. Sigma metrics were calculated according to the Clinical Laboratory Improvement Amendments (CLIA) 2024, National Glycohemoglobin Standardization Program (NGSP), and IFCC total allowable error (TEa) criteria. Total analytical error (TAE) was also calculated and compared with TEa limits. The Quality Goal Index (QGI) was calculated from sigma values to identify the primary source of analytical error.

Results: All CV values were below 3%, except for the 12-month pooled CV1 value. The weighted mean signed bias was −0.98%, while the weighted mean absolute bias was 1.99%. Sigma metrics for the 12-month period at IQC-1 were 2.18 (IFCC), 1.64 (CLIA), and 1.09 (NGSP), indicating acceptable performance only under the IFCC criteria. For IQC-2, sigma values were 4.05 (IFCC), 3.04 (CLIA), and 2.03 (NGSP), indicating acceptable performance under all three specifications.

Conclusion: The performance of the same analytical system was classified differently depending on the selected quality specification. Long-term sigma results at low concentration levels near clinical decision limits were borderline according to the IFCC criteria and unacceptable according to the CLIA and NGSP criteria, suggesting that stricter quality control strategies may be required to ensure the reliability of patient results.

Keywords: Analytical performance, HbA1c standardization, sigma-metric

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Diabetes is a chronic disease that affects the whole world. When the International Diabetes Federation (IDF) 2021 data were examined, it was reported that 10.5% of the population had diabetes at that time, and a 46% increase is expected by 2045 [1].

The glycosylated hemoglobin (HbA1c) test, which is used to monitor average blood glucose levels over a 3-month period, has also been used as a diagnostic test for a long time [2–4]. Therefore, laboratories have important responsibilities in the early and accurate diagnosis of diabetes.

In order to determine whether HbA1c standardization has been achieved, the Implementation Task Force of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC-TF) developed a model containing quantitative data. It has been reported that sigma metric and biological variation (BV) models can be used [5]. However, for the purpose of international consensus, the Sigma metric model is recommended [6]. In this context, the IFCC-TF recommends minimum and optimal sigma performance targets for HbA1c testing depending on the type of laboratory. A sigma level of

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≥2 is considered acceptable for routine laboratories, while ≥4 is expected for high-accuracy or reference laboratories.

The Six Sigma methodology enables the identification and measurement of process defects and is used as a quality indicator according to ISO 15189:2022 Medical laboratories—Requirements for quality and competence, meaning that it can measure the quality of all operational processes within an organization [7]. After the defects observed in the process are identified, the number of defects is converted to defects per million (DPM). DPM is then converted to a sigma value using standard conversion tables. Six Sigma indicates that there are 3.4 defects per million opportunities, that is, at most four defective results out of one million test results [8]. Six Sigma was first implemented in industry [9]. Its use in medical laboratories began in 2000 [10]. It is now applied in almost every area related to laboratories, including External Quality Assessment (EQA) programs and software manufacturers [8]. The Six Sigma method helps laboratories determine and monitor their own quality performance.

When calculating the sigma metric, which indicates the analytical performance of laboratory tests, reliable data on Total Allowable Error (TEa), bias, and coefficient of variation (CV) are required. Target values obtained from proficiency testing (PT)/EQA programs or certified reference materials and methods can be used to determine bias. Internal quality control (IQC) data can be used to calculate CV [5, 8]. TEa values differ between countries and EQA programs [11].

According to the IFCC criterion, the TEa is 5 mmol/mol at an HbA1c concentration of 50 mmol/mol with a risk level of 2 sigma, corresponding to approximately 10% TEa [6, 12]. According to the updated 2024 acceptable performance criteria, the allowable limit is ±8% in the Clinical Laboratory Improvement Amendments (CLIA) and the College of American Pathologists (CAP). However, for CAP-accredited laboratories, it is ±6%. CAP targets CV values of ≤3.0% for all methods [13]. While the acceptable performance criterion in CLIA in 2019 was ±10%, the targets have become increasingly stringent over the years [14]. Furthermore, while National Glycohemoglobin Standardization Program (NGSP)-aligned PT programs commonly apply a ±6% TEa limit for routine laboratory performance, NGSP method certification employs even stricter criteria of ±5%, reflecting its focus on method validation rather than routine laboratory assessment [13, 14].

The primary aim of this study is to evaluate how different total allowable error (TEa) models affect the sigma metric-based classification of HbA1c assay performance. The secondary aim is to assess the implications of these differences for internal quality control (QC) strategy and planning in routine laboratory practice. In addition, an exploratory objective of the study is to investigate potential sources of analytical error during periods of reduced performance using the Quality Goal Index (QGI), particularly in cases where sigma values indicate suboptimal analytical quality. QGI helps determine whether the problem originates from imprecision, bias, or both [15].

Materials and Methods

Materials

The study was conducted retrospectively in the Biochemistry Laboratory of Eskişehir City Hospital using an HbA1c analyzer (Adams Brand HA-8180V, Arkray KDK, Kyoto, Japan). Traceable, NGSP-certified calibrators supplied by Arkray were used. HbA1c measurements were based on HPLC methodology. The study was approved by the Eskişehir City Hospital Ethics Committee (Decision date: 08/03/2024, Decision No: ESH/BAEK 2024/7) and complies with the Declaration of Helsinki. Internal quality control materials from the extendSURE brand were also used throughout the study. The lot numbers of the controls used during the year were 7125, 7132, 7137, and 7140. No patient data were used; only IQC and PT data were analyzed.

Methods

The analyzer processed two levels of IQC samples each day, one containing low values and the other containing high values. IQC data were obtained from the Laboratory Module Quality Control page, which received data transferred from the analyzer to the Hospital Information Management System between January 1, 2023, and December 31, 2023. Internal quality control was performed using the 13s/R4s/41s rules, and inappropriate control results were excluded.

Between-day CV% values (CVbetween) were calculated separately for each month for low-level IQC (CV1%) and high-level IQC (CV2%). During periods in which different IQC lots were used, the pooled CV approach was applied to avoid overestimation of analytical imprecision resulting from lot-to-lot variations in control means. This approach minimizes the artificial inflation of CV values caused by such shifts and provides a more accurate estimation of analytical performance. Pooling was performed using standard deviation (SD) values in the original measurement units. First, the pooled standard deviation (SDpooled) was calculated by combining the standard deviations of individual lots weighted according to their respective numbers of measurements. The pooled mean (Meanpooled) was calculated as the weighted average of the means of individual lots according to their sample sizes. Subsequently, the pooled coefficient of variation (CVpooled%) was calculated using SDpooled and Meanpooled. Additionally, to reflect long-term performance, 12-month CV1pooled and 12-month CV2pooled values were calculated using the same formulas.

The PT study was performed once a month at a single level. PT data for 2023 (12 samples) were obtained from the QualiCont program, and bias% values were calculated for each month using peer-group means as target values. Both signed bias and absolute bias values were evaluated. Signed bias values were used to assess the direction of systematic error and for clinical interpretation, whereas absolute bias values were used in sigma metric and total analytical error (TAE) calculations to represent the magnitude of analytical error. To obtain a more robust estimate of long-term bias, a weighted mean signed bias was calculated by considering the peer-group size for each PT cy-

Table 1. Monthly bias, imprecision (CV%), and total analytical error (TAE) values for HbA1c assay

Month	Bias%		CV1 %	TAE 1	CV2%	TAE 2
	n	Bias %				
January	4	-1.64	2.40*	5.60	1.99	4.92
February	5	-1.79	2.04	5.16	1.55	4.35
March	9	-0.63	2.06	4.03	2.74	5.15
April	4	-6.84	2.05*	10.22▪	2.37*	10.75▪
May	5	-1.25	1.96	4.48	2.07	4.66
June	6	2.58	1.06	4.33	1.21	4.58
July	5	-6.07	1.35	8.30•	1.38*	8.35•
August	4	-2.02	1.60	4.66	1.56	4.60
September	7	2.00	1.68*	4.77	2.19	5.62
October	7	0.69	1.62	3.36	1.73	3.54
November	5	-1.45	2.42	5.44	2.51	5.59
December	7	-0.11	1.39	2.40	1.64	2.81
12-month	Weighted Absolute Bias	1.99	3.67*	8.05*	1.98*	5.26
	Weighted Signed Bias	-0.98				

Bias% values were derived from proficiency testing (PT) results using peer-group means as target values. CV1% and CV2% represent between-day imprecision for low- and high-level IQC materials, respectively. TAE was calculated for both control levels. n indicates the number of laboratories in the peer group using the same method, instrument, and reagent combination. Pooled CV values (CVpooled) marked with * were calculated during lot transition periods. ▪: Indicates unacceptable performance according to all quality specifications. •: Indicates unacceptable performance according to CLIA and NGSP criteria. IQC: Internal quality control; IFCC: International Federation of Clinical Chemistry; CLIA: Clinical Laboratory Improvement Amendments; TAE: Total analytical error; PT: Proficiency testing.

cle. In addition, a weighted mean absolute bias was calculated and used for the 12-month sigma metric and TAE calculations. This approach minimizes the influence of PT cycles with smaller peer-group sizes and ensures consistency between bias estimation and sigma-based performance evaluation. Bias estimation may be unstable because of small peer-group sizes, which directly affect sigma metrics. Sensitivity analysis comparing weighted and unweighted bias values was not performed.

Sigma metrics and total analytical error (TAE) of the test were calculated using the obtained CV% and bias% values with the relevant formulas.

The following TEa limits were applied: 8% for CLIA 2024, 6% for NGSP, and 10% for IFCC.

QGI results were interpreted descriptively to support error characterization and were not used as a primary decision criterion for analytical performance classification or sigma metric calculation.

Statistical analysis

Calculations were performed using Microsoft Excel 2010 based on the formulas presented below, using data obtained from the Hospital Information Management System and the QualiCont website.

Equation 1. CV% = (Standard deviation / Mean)×100

Equation 2. $SD_{pooled} = \sqrt{\frac{\sum(ni-1) \times (SDi)^2}{\sum(ni-1)}}$

$Mean_{pooled} = \frac{\sum(ni \times Meani)}{\sum ni}$

$CV_{pooled}\% = (SD_{pooled} / Mean_{pooled}) \times 100$

ni: represents the number of measurements for the corresponding IQC lot.

SD i: represents the standard deviations of the corresponding IQC lot.

Mean i: represents the mean value of the corresponding IQC lot.

The index i refers to different IQC lots.

Equation 3. Bias% = [(Laboratory result – Peer group mean) / Peer group mean] × 100

Equation 4. 12-month Weighted Absolute Bias%= $\sum (w_i \times |Bias_i|) / \sum w_i$

$Bias_i$: Absolute Bias value (%) for each month.

w_i: Monthly PT participant number.

Equation 5. 12-month Weighted Signed Bias%= $\sum (w_i \times Bias_i) / \sum w_i$

$Bias_i$: Signed Bias value (%) for each month.

w_i: Monthly PT participant number.

Equation 6. Sigma value = (TEa% – |Bias%|) / CV%

Equation 7. TAE = |Bias%| + (1.65 × CV%)

Equation 8. QGI = |Bias|/ (1.5 × CV)

According to this classification, QGI values <0.8 indicate imprecision, values between 0.8 and 1.2 reflect a combination of imprecision and inaccuracy, and values >1.2 indicate inaccuracy as the predominant source of error.

Results

CV% values obtained from both levels of HbA1c internal quality control (IQC) measurements are presented in Table 1. All monthly CV1 and CV2 values remained below 3%. In contrast,

Table 2. Monthly sigma performance and analytical error classification according to IFCC, CLIA, and NGSP criteria

Month	IQC-1 σ (IFCC / CLIA / NGSP)	IQC-2 σ (IFCC / CLIA / NGSP)	QGI (IQC-1 / IQC-2)	Error type
January	3.49/2.65/1.82	4.20/3.20/2.19	0.5 / —	Imprecision
February	4.02/3.04/2.06	5.30/4.01/2.72		
March	4.55/3.58/2.61	3.42/2.69/1.96	— / 0.2	Imprecision
April	1.54/0.57/0.41	1.33/0.49/-0.35	2.2 / 1.9	Inaccuracy
May	4.47/3.45/2.43	4.23/3.26/2.30		
June	7.00/5.11/3.23	6.13/4.48/2.83		
July	2.91/1.43/-0.05	2.85/1.34/-0.05	3.0 / 2.9	Inaccuracy
August	4.99/3.74/2.49	5.11/3.83/2.55		
September	4.76/3.57/2.38	3.65/2.74/1.83	— / 0.6	Imprecision
October	5.75/4.51/3.28	5.38/4.23/3.07		
November	3.53/2.71/1.88	3.41/2.61/1.81	0.4/0.4	Imprecision
December	7.12/5.68/4.24	6.03/4.81/3.59		
long term	2.18/1.64/1.09	4.05/3.04/2.03	0.4/ —	Imprecision

Sigma values were calculated using IFCC, CLIA, and NGSP total allowable error (TEa) specifications. QGI (Quality Goal Index) was applied for identification of dominant analytical error sources. Negative sigma values indicate extremely poor analytical performance. IQC: Internal quality control; IFCC: International Federation of Clinical Chemistry; CLIA: Clinical Laboratory Improvement Amendments.

Table 3. Comparative classification of analytical performance according to TEa models

Month	IFCC (σ≥2)	CLIA (σ≥2)	NGSP (σ≥2)
January	√	√	×
February	√	√	√
March	√	√	×
April	×	×	×
May	√	√	√
June	√	√	√
July	√	×	×
August	√	√	√
September	√	√	×
October	√	√	√
November	√	√	×
December	√	√	√

√: Acceptable performance (σ ≥2), ×: Unacceptable performance (σ <2). IFCC: International Federation of Clinical Chemistry; CLIA: Clinical Laboratory Improvement Amendments; TEa: Total allowable error.

Table 4. Proportion of acceptable analytical performance across TEa models

TEa Model	IQC-1 acceptable months (σ≥2)	IQC-2 acceptable months (σ≥2)
IFCC	11/12 (91.7%)	11/12 (91.7%)
CLIA	10/12 (83.3%)	10/12 (83.3%)
NGSP	8/12 (66.7%)	9/12 (75.0%)

The proportion of months meeting acceptable performance (σ ≥ 2) was highest for IFCC, followed by CLIA, and lowest for NGSP for both IQC levels. IQC: Internal quality control; TEa: total allowable error.

the 12-month CV1pooled value was 3.67%, which exceeded the acceptable limit and indicated poor overall precision. During the study period, shifts in control means were observed between different IQC lots (for IQC Level 1: means of 5.5%, 5.09%, 5.5%, and 5.4%; for IQC Level 2: means of 10.4%, 10.19%, and 11.4%). Due to differences in mean values during lot transition periods, CVpooled% was calculated for certain months. The marked symbols represent the CVpooled% values.

The bias% values obtained from the 12-month PT program data for the HbA1c test are presented in Table 1. Monthly bias values ranged between −6.84% and +2.58%. The weighted mean signed bias was −0.98%, indicating a slight systematic underestimation of HbA1c results. In contrast, the weighted mean absolute bias was 1.99%, which was used for sigma metric calculations to represent the magnitude of total analytical error. Most bias results were negative, except for Months 6, 9, and 10 of the QualiCont program, which showed positive bias values. Two PT cycles (Months 4 and 7) demonstrated marked negative bias (−6.84% and −6.07%, respectively).

The TAE values are also presented in Table 1. In April, unacceptable performance was observed for both TAE at the low level of internal quality control (10.22) and at the high level (10.75) according to all criteria (IFCC, CLIA, and NGSP). In July, TAE at the low (8.30) and high (8.35) IQC levels was considered acceptable according to IFCC criteria but was deemed unacceptable according to CLIA and NGSP criteria. TAE1 was 8.05% for the 12-month period (acceptable only according to IFCC), whereas 12-month TAE2 was 5.26% (acceptable according to IFCC, CLIA, and NGSP).

Monthly sigma values calculated using IFCC, CLIA, and NGSP TEa specifications are presented in Table 2. April was the only month with unacceptable performance across all models. July showed acceptable performance only under IFCC criteria but not under CLIA or NGSP criteria, as shown in Table 3. Analytical performance varied substantially depending on the selected TEa model. According to IFCC criteria, performance was acceptable (σ≥2) in 11 out of 12 months (91.7%) for both IQC levels. Under CLIA criteria, acceptable performance decreased to 10 out of 12 months (83.3%). Using NGSP criteria, acceptable performance further declined to 8/12 months (66.7%) for IQC-1 and 9/12 months (75.0%) for IQC-2 (Table 4).

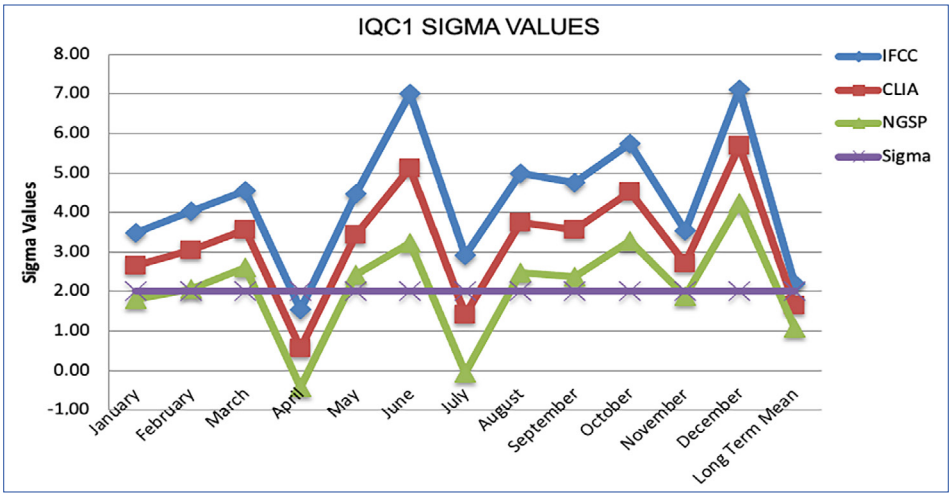

Figure 1. Sigma metrics for HbA1c at IQC-1, which is closer to clinical decision limits, were evaluated according to IFCC, CLIA, and NGSP quality specifications.

A horizontal reference line at 2 sigma indicates the minimum acceptable performance threshold. Performance deterioration is evident in April, July and long term, particularly under stricter quality criteria. IFCC: International Federation of Clinical Chemistry; CLIA: Clinical Laboratory Improvement Amendments; TEa: Total allowable error.

Sigma metrics for the 12-month period at the IQC-1 level were 2.18 (IFCC), 1.64 (CLIA), and 1.09 (NGSP), indicating acceptable performance only under IFCC criteria, while performance under CLIA and NGSP criteria was unacceptable. For IQC-2, sigma values were 4.05 (IFCC), 3.04 (CLIA), and 2.03 (NGSP), indicating acceptable performance under all three quality specifications (Table 2).

The IQC-1 level, being closer to clinical decision limits, is additionally presented in Figure 1 to more clearly demonstrate its variation across months and under different quality specifications. Overall, performance varied markedly by month and quality specification, with the highest sigma values observed under IFCC criteria and the lowest under NGSP criteria.

QGI analysis identified the dominant source of analytical error (Table 2). Inaccuracy was identified in April and July (QGI>1.2), whereas imprecision was observed in several months, including January, March, September, and November (QGI<0.8). The long-term QGI value (0.4) indicated that imprecision was the dominant contributor to overall performance limitations at the low IQC level.

The distribution of sigma values revealed a substantial operational impact, as shown in Table 5. Under IFCC criteria, 33.3% (IQC-1) and 41.7% (IQC-2) of results fell below 4 sigma (moderate QC burden). Under CLIA criteria, this increased to 75.0% and 66.7%, respectively (high QC burden). Under NGSP criteria, 91.7% (IQC-1) and 100% (IQC-2) of results were below 4 sigma, indicating a substantial increase in QC burden. These findings demonstrate that stricter TEa models significantly increase the required QC intensity.

In terms of quality control (QC) requirements, stricter TEa models led to a substantial increase in QC stringency. When NGSP criteria were applied, multirule QC strategies with increased control frequency (n=4, R=45) were required in the majority of months, whereas IFCC criteria allowed less stringent QC procedures during several periods. This corresponds to a relative increase in QC requirements when transitioning from IFCC- to NGSP-based evaluation.

Discussion

This study evaluated the analytical performance of the HbA1c assay using sigma metrics based on three different TEa models: IFCC, CLIA, and NGSP. The main finding was that the same analytical system can be classified into different performance categories depending on the selected TEa model, leading to corresponding changes in QC requirements.

While the IFCC model demonstrated acceptable performance, the CLIA and, particularly, the NGSP criteria revealed lower sigma values and greater analytical limitations, espe-

Table 5. Observed QC burden based on <4 sigma performance for IQC-1 and IQC-2

Criteria	QC intensity		QC intensity	
	% σ <4	(IQC-1)	% σ <4	(IQC-2)
IFCC	33.3%	Moderate QC Burden	41.7%	Moderate - Increased QC Burden
CLIA	75.0%	Increased QC Burden	66.7%	Increased QC Burden
NGSP	91.7%	Markedly Increased QC Burden	100%	Markedly Increased QC Burden

Percentages were calculated based on monthly sigma values. QC burden classification was derived from sigma thresholds <4. IQC-1 and IQC-2 datasets were evaluated independently. IQC: Internal quality control.

cially for the clinically significant IQC-1 (decision level) in long-term sigma evaluations.

April and July were identified as the most critical periods. April showed failure across all TEa models, while July failed under CLIA and NGSP criteria. QGI analysis indicated that most errors during these periods were due to inaccuracy, supporting the likelihood of systematic error. Possible causes may include calibration issues and lot-to-lot variations; however, a root-cause analysis was not performed in this study.

These three TEa models were compared because IFCC is accepted as the reference framework for international standardization [16], while NGSP links HbA1c results to clinical interpretation by associating them with diagnostic thresholds and treatment targets [17]. The TEa limits defined by CLIA are based on a combination of biological variation, clinical risk, and analytical performance [18]. There is currently no consensus in the literature regarding the selection of a TEa model [6, 9, 19–23], and a resolution is not expected in the near future [24].

According to sigma metric-based QC planning, the design of statistical quality control procedures was stratified according to analytical performance levels. For assays with sigma values greater than 6, a simple 13s rule with two control levels and a large run size (R≈1000) was considered sufficient. For sigma values between 5 and 6, a multirule strategy (13s/22s/R4s) with two control levels and a reduced run size (R≈450) was applied. As performance decreased further (sigma between 4 and 5), additional rules (41s) and increased control levels (n=4) were required, with a run size of approximately 200. For assays with sigma values below 4, the most stringent QC design (13s/22s/R4s/41s/6x) with four control levels and a significantly reduced run size (R≈45) was implemented, reflecting the increased need for error detection in low-performance assays [25–27]. In this study, the QC burden increased from IFCC to CLIA and was highest under NGSP criteria.

QGI was used solely to identify the source of errors. It showed that most errors were due to imprecision in the majority of months, whereas inaccuracy was dominant in April and July. Therefore, a targeted QC approach, rather than a fixed QC strategy, is required.

Since processed samples are used in the PT program, peer-group means were used for bias calculation [13]. The use of weighted bias estimation increased reliability [28]. A negative weighted signed bias may lead to underreporting of HbA1c, which is particularly critical at clinical decision limits [29]. IFCC has also announced that, starting in 2025, the direction of bias will be incorporated into quality targets.

From a clinical perspective, higher sigma values indicate lower analytical error and reduce the risk of misdiagnosis or inappropriate treatment [30]. Importantly, differences in sigma classification across TEa models may lead to different clinical interpretations of the same analytical performance.

This study has some limitations. The sigma calculations were based on a single center and a defined monitoring period. The study did not include correlations with patient outcomes; therefore, clinical impact was inferred indirectly from analytical performance metrics. Future studies involving different instruments, longer monitoring durations, and clinical outcomes will help provide a more comprehensive understanding of the relationship between analytical quality and clinical impact. Additionally, the relatively low number of participants in the peer group of the PT program represents a significant limitation. This may affect the reliability of bias estimation and consequently influence sigma metrics, particularly in months with smaller peer-group sizes.

These findings highlight the importance of continuous monitoring in laboratory quality management. However, no root-cause analysis or corrective and preventive actions were performed within the scope of this study; therefore, the results should be interpreted as performance indicators rather than confirmed analytical errors. Nevertheless, regardless of the selected quality specification, borderline sigma performance at low concentration levels indicates the need for ongoing efforts to improve analytical performance.

The analytical findings of this study suggest that performance differences observed across TEa models may have clinical relevance when HbA1c results are interpreted near diagnostic decision limits. In particular, the presence of a slight negative bias and reduced sigma performance at low concentration levels indicate that analytical variability may become more critical in borderline patient results. These observations do not introduce new findings beyond the analytical results but rather translate them into a clinical context, highlighting the importance of selecting appropriate quality specifications for reliable result interpretation.

Conclusion

The analytical performance of HbA1c varied significantly depending on the selected total allowable error (TEa) model, resulting in different sigma-based classifications for the same analytical system. This demonstrates that TEa selection has a direct impact on performance evaluation and quality control strategy in routine laboratory practice.

Overall, while acceptable performance was observed under less stringent criteria, reduced sigma performance at clinically relevant concentration levels highlighted potential limitations when stricter quality specifications were applied. These findings emphasize the importance of selecting appropriate quality goals to ensure consistent and reliable analytical performance in laboratory medicine.

Disclosures

Ethics Committee Approval: The study was approved by the Eskişehir City Hospital Ethics Committee (no: ESH/BAEK 2024/7, date: 08/03/2024).

Informed Consent: Written informed consent was obtained.

Conflict of Interest Statement: None declared.

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Research Article

Comparison of Vacusera blood collection tubes with BD and Greiner tubes for routine biochemical parameters

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Abstract

Objectives: Preanalytical factors, including the choice of blood collection tubes, are a major source of laboratory errors and can affect patient care. Therefore, local verification of newly introduced tubes is essential for quality assurance and accreditation. This study aimed to evaluate the analytical comparability and clinical acceptability of Vacusera CAT Serum tubes versus established BD Vacutainer SST and Greiner Vacuette serum tubes for routine biochemistry.

Methods: In March 2025, venous blood was collected by a single phlebotomist from 42 adults. Fifteen analytes [glucose, urea, total protein, albumin, alanine aminotransferase, aspartate aminotransferase (AST), creatine kinase (CK), C-reactive protein, calcium (Ca), phosphorus, magnesium (Mg), and uric acid by photometry; sodium (Na), potassium (K), and chloride (Cl) by indirect ion-selective electrode] were measured within the same analytical run on a Mindray BS2000M.

Results: Compared with BD tubes, Vacusera showed statistically significant differences for AST (p=0.046), CK (p=0.001), Na (p<0.001), Cl (p<0.001), Ca (p=0.024), and Mg (p<0.001); compared with Greiner tubes, differences were observed for K (p=0.019) and Mg (p=0.002). Nevertheless, all analytes, except Na and Cl, fulfilled the biological variation-derived total allowable error specifications. Deming regression indicated no statistically significant constant or proportional systematic error [intercept confidence intervals (CIs) including 0; slope CIs including 1]. At predefined medical decision limits, differences between Vacusera and BD or Greiner tubes were within the reference change value limits, supporting clinically acceptable agreement.

Conclusion: Despite statistically significant differences in some analytes, Vacusera CAT Serum tubes provided clinically acceptable results comparable to those obtained with BD and Greiner tubes for the tested routine biochemical parameters.

Keywords: Biochemical analytes, blood collection, clinical significance, preanalytical errors, serum separator tube, total analytical error

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The total testing process is commonly evaluated in three phases, namely the preanalytical, analytical, and postanalytical phases, according to the concept of the "brain-to-brain turnaround time cycle." It has been shown that most errors in this cycle occur during the preanalytical phase [1, 2]. Preanalytical errors account for up to 70% of the total errors encountered in laboratory diagnostic processes, and factors such as improper sample collection, incorrect labeling, insufficient sample volume, inappropriate sample transport, and the use of poor-quality blood collection tubes may lead to erroneous laboratory results and adversely affect patient care [3–7]. Despite increasing evidence regarding the importance of preanalytical quality assurance, the selection and procurement of blood collection devices in healthcare facilities often remain overlooked [8]. Therefore, the International Federation of Clinical Chemistry and Laboratory Medicine Working Group on Laboratory Errors and Patient Safety (IFCC WG-LEPS) developed a globally applicable panel

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of six essential quality indicators aimed at minimizing laboratory errors and improving patient safety. These essential indicators cover the total testing process and include critical preanalytical measures necessary to ensure the reliability of laboratory results, such as the rates of misidentified requests and samples, sample rejections, and hemolysis [4].

For clinical laboratory accreditation, it is essential that both manufacturers and local users assess the suitability and performance of new laboratory equipment, instruments, methods, and *in vitro* diagnostic devices before routine implementation. Failure to comply with appropriate manufacturing or laboratory practices may negatively affect the safety of both operators and patients. Increasing evidence indicates that the introduction of new blood collection devices, such as blood collection tubes, may alter local procedures and influence measured analyte concentrations. Although serum separator tubes are designed for similar purposes, tubes produced by different manufacturers may differ in terms of tube wall material, clot activator composition, separator gel characteristics, surfactants, lubricants, additives, and vacuum properties. These differences may influence clot formation, serum separation, analyte adsorption, contamination risk, and the duration of contact between serum and blood cells. They may also affect analytical accuracy and sample stability during clotting, transport, short-term storage, and other preanalytical handling steps, thereby leading to measurable differences in analyte concentrations. Therefore, the selection and procurement of blood collection systems should be regarded as a vital component in ensuring the quality, safety, and effectiveness of the preanalytical phase in laboratory diagnostics and, consequently, of the entire testing process [7–9].

BD Vacutainer SST and Greiner Vacuette tubes are widely used in routine laboratory practice, whereas Vacusera CAT Serum tubes have been introduced more recently. Recent comparative studies have shown that although many analytes may remain within clinically acceptable limits, certain parameters may still demonstrate statistically or clinically relevant discrepancies between tube brands, emphasizing the need for local tube-specific verification and caution against assuming interchangeability between manufacturers [9–11]. Because the implementation of a new tube system may affect analytical comparability under local laboratory conditions, local verification is necessary before routine use. Our laboratory is currently using Vacusera CAT Serum Gel Q Clot Activator tubes. Therefore, this study aimed to compare Vacusera CAT Serum tubes with BD Vacutainer SST and Greiner Vacuette tubes and to determine whether Vacusera CAT Serum tubes provide analytically comparable and clinically acceptable results for routine biochemical testing.

Materials and Methods

Ethical considerations

The study was approved by the Istanbul Medipol University Non-interventional Clinical Research Ethics Committee (approval no: 268; date: 06.03.2025). Informed consent was obtained from all participants, and the study was conducted in accordance with the principles of the Declaration of Helsinki.

Study design and participants

This single-center comparative study was conducted as a local verification study to assess the analytical comparability of different serum tube systems under routine laboratory conditions. As an additive-free glass reference tube was not included, the study should be interpreted as an inter-tube comparison rather than a formal validation study. In March 2025, 42 adults were enrolled, including inpatients from the Department of Internal Medicine at Bagcilar Training and Research Hospital and apparently healthy volunteers. Healthy individuals and participants with diverse biochemical values were included to provide a broader distribution across the analytical measurement range. Eligible participants were aged between 18 and 65 years.

Pregnancy, lactation, analytical measurement errors, results below the analytical limit of detection, and samples affected by hemolysis, icterus, or lipemia were defined as the exclusion criteria. To avoid potential analytical interference, hemolysis, icterus, and lipemia were assessed using automated serum index analysis on the Mindray BS2000M analyzer (Mindray, Shenzhen, China). Samples exceeding the lowest manufacturer-declared interference thresholds among the evaluated analytes were excluded from the analysis. In addition, participants in whom an appropriate vein could not be identified, those with deterioration of vascular integrity during phlebotomy, those with evidence of intravenous contamination, and those in whom tourniquet application exceeded 1 minute were excluded.

Blood collection and preanalytical handling

After 8–12 hours of overnight fasting, venous blood samples were collected between 08:00 and 10:00 a.m. from the antecubital vein by a single experienced phlebotomist using an evacuated blood collection system and a BD Vacutainer Eclipse blood collection needle (21G×1–1/4", 0.8×32 mm; catalog no: 368609; Becton Dickinson, NJ, USA). A 21G needle was preferred because it is standard for routine adult venipuncture, provides adequate blood flow, and helps minimize mechanically induced hemolysis during sample collection. Blood was collected into three serum tube types: Vacusera® CAT serum tubes (13×100 mm, 5.0 mL; catalog no: 635305; Disera, Türkiye), BD Vacutainer® SST™ II Advance tubes (13×100 mm, 5.0 mL; catalog no: 367955; Becton Dickinson, NJ, USA), and VACUETTE® Serum Separator Clot Activator tubes (13×100 mm, 5.0 mL; catalog no: 456073; Greiner Bio-One, Austria). Approximately 15 mL of blood was collected from each participant.

All samples were obtained during the same venipuncture session under the same fasting and morning collection conditions and handled according to routine standardized laboratory procedures. To reduce posture-related variation, all

participants remained seated for 15 minutes before venipuncture. The order of drawing among the comparative (BD and Greiner) and control (Vacusera) tubes was randomized for each participant, in accordance with the CLSI GP34-A guideline [12]. Immediately after collection, each tube was gently inverted five times to ensure adequate mixing of blood with the clot activator. Following blood collection, all tubes were maintained in an upright position at room temperature (25°C) and transported to the laboratory without delay. To ensure complete clot formation, the samples were allowed to clot for 45 minutes at room temperature prior to centrifugation. Subsequently, the samples were centrifuged using an NF 800 R centrifuge (Nüve, Ankara, Türkiye) at 2000×g for 10 minutes at 25°C. All serum analytes were measured within 2 hours of blood collection. Throughout the preanalytical phase, standardized procedures were applied for sample handling and processing to minimize variability.

Analytical methods

Fifteen routine biochemical parameters were assessed, including glucose, total protein (TP), albumin, urea, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), C-reactive protein (CRP), calcium, phosphorus, magnesium (Mg), and uric acid (UA), using photometric methods. Additionally, sodium, potassium, and chloride were measured using an indirect ion-selective electrode module on a Mindray BS2000M autoanalyzer (Mindray, Shenzhen, China). Instrument performance remained stable throughout the study. Routine maintenance of the analyzer was conducted according to the manufacturer's guidelines, and analytical stability was regularly assessed. Internal quality control and external quality assessment results remained within the analytical performance criteria.

Statistical analysis

The appropriateness of the test results for a normal distribution was assessed using the Shapiro-Wilk test. A paired-sample t-test or Wilcoxon test was used to assess statistically significant differences between the two dependent groups. Correlation coefficients between the variables were determined using Spearman correlation analysis. Passing-Bablok and Deming regression analyses were conducted to assess proportional and constant systematic errors, including the intercept and slope values, along with their 95% confidence intervals (CIs). Additionally, Bland-Altman analysis was used to evaluate both the absolute and percentage mean differences between the two methods, providing a visual assessment of agreement. Each analysis was conducted in duplicate, and the mean values of the results were evaluated. The percentage coefficient of variation (CV%) for the Vacusera CAT serum tubes was computed based on the results of the duplicate runs. The mean percentage difference (bias%) was determined by averaging all calculated differences between the control and comparative tubes for each analyte. The bias percentage was calculated as follows: Bias%=[Control tube (Vacusera)–Comparative tube (BD or Greiner)]×100/Comparative tube (BD or Greiner), where the Vacusera SST tube served as the control (new) tube and the BD or Greiner tube was the comparative tube. The percentage of total analytical error (TAE) was calculated as the sum of bias% and 1.65 times CV%. The assessment of TAE was based on total allowable error (TEa) limits obtained from the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) biological variation database [13], Clinical Laboratory Improvement Amendments (CLIA) guidelines, and the Reference Interval Limits for Biological Analytes in Clinical Chemistry (RILIBAK) database [14]. Reference change value (RCV) values were calculated using analytical imprecision (CV_a) derived from duplicate measurements performed with Vacusera tubes. The within-subject biological variation (CV_i) values were obtained from the EFLM Biological Variation Database [13] and incorporated into the RCV calculation: $RCV=\sqrt{2}\times Z\times\sqrt{(CV_a^2+CV_i^2)}$. Asymmetric RCV values were considered to allow separate evaluation of increases and decreases. For each predefined medical decision limit (MDL), the corresponding Vacusera values were estimated using Deming regression equations derived from the comparative tubes (BD or Greiner). Subsequently, the differences between the two tube types were calculated and evaluated against the RCV limits. Statistical analyses were performed using MedCalc v. 23.4.5 (MedCalc Software Ltd, Ostend, Belgium). A p-value below 0.05 was considered indicative of statistical significance.

Sample size and power

The sample size of 42 participants was determined based on the CLSI EP09-C (3rd Edition) guideline [15], which recommends a minimum of 40 samples for the verification of measurement procedures and bias estimation using patient samples. To further assess the adequacy of this cohort, a post hoc power analysis was conducted using G*Power software (version 3.1.9.7). Given the high correlation coefficients ($r\geq0.95$) observed in our results, the statistical power (1-β) for a bivariate normal model with $\alpha=0.05$ and $n=42$ was calculated to be >0.99, confirming that the study was sufficiently powered to detect the analytical associations between the tube brands.

Results

Table 1 presents the descriptive statistics and statistical and clinical comparisons of the 15 routine biochemical parameters analyzed using Vacusera and BD serum tubes. Among these parameters, AST ($p=0.046$), CK ($p=0.001$), sodium ($p<0.001$), chloride ($p<0.001$), calcium ($p=0.024$), and Mg ($p<0.001$) levels exhibited statistically significant differences (Table 1). Table 2 presents the descriptive statistics and statistical and clinical comparisons of the 15 routine biochemical parameters analyzed using Vacusera and Greiner serum tubes. Among these parameters, potassium ($p=0.019$) and Mg ($p=0.002$) showed statistically significant differences (Table 2). Tables 1 and 2 present the CV% values for the variables examined in duplicate within the Vacusera CAT serum tubes, as well as the mean

Table 1. Descriptive statistics and comparison result for Vacusera CAT and BD Vacutainer SST tubes

Parameter	Vacusera CAT	BD SST	p	CV%	Bias%	TAE%	TEa%
Glucose (mg/dL)	91.9 (85.6–102)	92.3 (85.3–101)	0.769	1.05	0.24	1.97	9.30
TP (g/dL)	6.90 (5.75–7.35)	6.80 (5.80–7.30)	0.426	1.15	0.21	2.11	5.10
Albumin (g/dL)	4.17 (3.09–4.70)	4.17 (3.13–4.67)	0.388	1.17	0.22	2.15	5.00
Urea (mg/dL)	32.3 (25.0–55.5)	32.0 (25.5–57.0)	0.572	1.32	-0.36	2.54	25.3
ALT (IU/L)	15.3 (10.5–25.5)	15.5 (10.0–26.0)	0.082	3.01	1.66	6.63	27.8
AST (IU/L)	18.3 (15.5–24.3)	18.8 (15.8–24.8)	**0.046**	2.50	-1.13	5.26	18.7
CK (IU/L)	57.3 (37.0–104)	58.0 (36.5–105)	**0.001**	2.63	-1.19	5.53	30.9
CRP (mg/L)	6.10 (2.00–26.1)	6.23 (2.00–26.5)	0.914	1.52	-0.16	2.67	78.1
Sodium (mmol/L)	138 (136–140)	138 (135–139)	**<0.001**	1.22	0.36	**2.37**	1.00
Potassium (mmol/L)	4.24 (4.06–4.60)	4.26 (4.06–4.55)	0.333	1.29	-0.37	2.50	7.40
Chloride (mmol/L)	105 (100–106)	105 (100–106)	**<0.001**	0.99	0.35	**1.98**	1.90
Calcium (mg/dL)	9.38 (8.65–9.75)	9.28 (8.55–9.75)	**0.024**	1.51	0.47	2.96	3.30
Phosphorus (mg/dL)	3.43 (3.05–4.15)	3.48 (3.00–4.25)	0.388	2.62	0.18	4.50	14.5
Mg (mg/dL)	1.96 (1.76–2.09)	1.93 (1.74–2.09)	**<0.001**	2.20	0.94	4.57	5.80
UA (mg/dL)	4.65 (3.70–5.60)	4.68 (3.70–5.50)	0.218	1.27	0.88	2.98	18.7

Data are presented as the median (25^{th}–75^{th} percentile). Parameters showing statistically significant differences according to the Wilcoxon signed-rank test are indicated in bold ($p<0.05$). TEa targets were obtained from the EFLM biological variation database. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CK: Creatine kinase; CRP: C-reactive protein; CV: Coefficient of variation; Mg: Magnesium; TAE: Total analytical error; TEa: Total allowable error; TP: Total protein; UA: Uric acid.

Table 2. Descriptive statistics and comparison result for Vacusera SST and Greiner Vacuette tubes

Parameter	Vacusera CAT	Greiner Vacuette	p	CV%	Bias%	TAE%	TEa%
Glucose (mg/dL)	91.9 (85.6–102)	91.2 (85.9–103)	0.053	1.05	-0.23	1.96	9.30
TP (g/dL)	6.90 (5.75–7.35)	6.90 (5.75–7.30)	0.179	1.15	-0.30	2.20	5.10
Albumin (g/dL)	4.17 (3.09–4.70)	4.17 (3.13–4.68)	0.816	1.17	-0.07	2.00	5.00
Urea (mg/dL)	32.3 (25.0–55.5)	32.0 (25.0–54.5)	0.597	1.32	0.03	2.21	25.3
ALT (IU/L)	15.3 (10.5–25.5)	16.0 (10.0–24.0)	0.269	3.01	0.05	5.02	27.8
AST (IU/L)	18.3 (15.5–24.3)	18.5 (15.5–25.3)	0.177	2.50	-1.27	5.40	18.7
CK (IU/L)	57.3 (37.0–104)	56.8 (38.0–103)	0.604	2.63	-1.43	5.77	30.9
CRP (mg/L)	6.10 (2.00–26.1)	6.18 (2.00–25.8)	0.801	1.52	0.06	2.57	78.1
Sodium (mmol/L)	138 (136–140)	138 (136–140)	0.128	1.22	-0.08	**2.09**	1.00
Potassium (mmol/L)	4.24 (4.06–4.60)	4.32 (4.13–4.57)	**0.019**	1.29	-0.93	3.06	7.40
Chloride (mmol/L)	105 (100–106)	105 (100–107)	0.697	0.99	-0.05	1.68	1.90
Calcium (mg/dL)	9.38 (8.65–9.75)	9.30 (8.50–9.75)	0.087	1.51	0.31	2.80	3.30
Phosphorus (mg/dL)	3.43 (3.05–4.15)	3.43 (3.00–4.15)	0.479	2.62	-0.61	4.93	14.5
Mg (mg/dL)	1.96 (1.76–2.09)	1.94 (1.75–2.09)	**0.002**	2.20	0.66	4.29	5.80
UA (mg/dL)	4.65 (3.70–5.60)	4.68 (3.80–5.55)	0.657	1.27	-0.19	2.29	18.7

Data are presented as median (25^{th}–75^{th} percentile). Parameters showing statistically significant differences according to the Wilcoxon signed-rank test are indicated in bold ($p<0.05$). TEa targets were obtained from the EFLM biological variation database. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CK: Creatine kinase; CRP: C-reactive protein; CV: Coefficient of variation; Mg: Magnesium; TAE: Total analytical error; TEa: Total allowable error; TP: Total protein; UA: Uric acid.

bias percentages. As the calculated TAE values for the sodium and potassium tests did not meet the minimum performance goals established by the EFLM Biological Variation Database [13], a comparison was conducted between sodium and chloride with the current RILIBAK and CLIA values [14], respectively. All parameters met these targets (Tables 1, 2).

Bland-Altman analysis demonstrated that the mean differences between the two tube types were close to zero across all evaluated analytes, indicating minimal systematic bias. The majority of the data points were distributed symmetrically around the zero line and remained within the 95% limits of agreement. No clear trend toward increasing or decreasing differences across the analytical measurement range was observed, suggesting the absence of significant proportional bias (Appendix 1, 2).

The Deming regression parameters for the Vacusera and BD serum tubes are summarized in Table 3. For all biochemical analytes, the 95% CIs of the slope included 1, and the 95% CIs of the intercept included 0, indicating no evidence of significant proportional or constant bias across the measured concentration range. Similarly, the Deming regression results for the Vacusera and Greiner serum tubes are presented in Table

Table 3. Deming regression analysis for Vacusera CAT and BD Vacutainer SST tubes

Parameter	Regression Equation	Slope (95% CI)	Intercept (95% CI)
Glucose (mg/dL)	y= 2.9415 + 0.9727 x	0.8979 to 1.0476	-4.3256 to 10.209
TP (g/dL)	y= 0.0369 + 0.9964 x	0.9723 to 1.0205	-0.1115 to 0.1853
Albumin (g/dL)	y= -0.0403 + 1.0132 x	0.9886 to 1.0377	-0.1320 to 0.0515
Urea (mg/dL)	y= -0.1170 + 1.0009 x	0.9974 to 1.0044	-0.3689 to 0.1350
ALT (IU/L)	y= 0.0022 + 1.0102 x	0.9900 to 1.0304	-0.3685 to 0.3730
AST (IU/L)	y= -0.1407 + 0.9950 x	0.9890 to 1.0010	-0.4512 to 0.1699
CK (IU/L)	y= -0.4032 + 0.9945 x	0.9848 to 1.0042	-1.1502 to 0.3438
CRP (mg/L)	y= -0.1220 + 1.0052 x	0.9987 to 1.0117	-0.2533 to 0.0093
Sodium (mmol/L)	y= 4.2990 + 0.9722 x	0.9214 to 1.0230	-2.6521 to 11.250
Potassium (mmol/L)	y= -0.0951 + 1.0189 x	0.9672 to 1.0706	-0.3117 to 0.1215
Chloride (mmol/L)	y= 1.3126 + 0.9907 x	0.9670 to 1.0144	-1.0908 to 3.7160
Calcium (mg/dL)	y= -0.0515 + 1.0103 x	0.9683 to 1.0522	-0.4152 to 0.3122
Phosphorus (mg/dL)	y= -0.0892 + 1.0239 x	0.9586 to 1.0891	-0.3159 to 0.1375
Mg (mg/dL)	y= 0.0159 + 1.0006 x	0.9695 to 1.0316	-0.0426 to 0.0745
UA (mg/dL)	y= 0.0375 + 0.9989 x	0.9779 to 1.0200	-0.0814 to 0.1563

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CI: Confidence interval; CK: Creatine kinase; CRP: C-reactive protein; Mg: Magnesium; TP: Total protein; UA: Uric acid.

Table 4. Deming regression analysis for Vacusera CAT and Greiner Vacuette tubes

Parameter	Regression Equation	Slope (95% CI)	Intercept (95% CI)
Glucose (mg/dL)	y= 0.3292 + 0.9946 x	0.9588 to 1.0305	-3.2251 to 3.8835
TP (g/dL)	y= 0.0594 + 0.9877 x	0.9579 to 1.0175	-0.1327 to 0.2515
Albumin (g/dL)	y= -0.0439 + 1.0112 x	0.9899 to 1.0324	-0.1213 to 0.0334
Urea (mg/dL)	y= 0.0161 + 1.0006 x	0.9943 to 1.0070	-0.2646 to 0.2967
ALT (IU/L)	y= 0.0329 + 0.9936 x	0.9844 to 1.0027	-0.2112 to 0.2771
AST (IU/L)	y= 0.0119 + 0.9738 x	0.7782 to 1.1693	-4.0165 to 4.0403
CK (IU/L)	y= -0.7141 + 1.0038 x	0.9885 to 1.0191	-1.9943 to 0.5661
CRP (mg/L)	y= -0.0387 + 1.0008 x	0.9898 to 1.0118	-0.2162 to 0.1388
Sodium (mmol/L)	y= 3.1793 + 0.9760 x	0.9098 to 1.0423	-5.9645 to 12.323
Potassium (mmol/L)	y= 0.0507 + 0.9781 x	0.9249 to 1.0313	-0.1547 to 0.2562
Chloride (mmol/L)	y= 1.1956 + 0.9878 x	0.9628 to 1.0128	-1.3440 to 3.7352
Calcium (mg/dL)	y= -0.0082 + 1.0040 x	0.9647 to 1.0432	-0.3649 to 0.3485
Phosphorus (mg/dL)	y= -0.1569 + 1.0386 x	0.9922 to 1.0850	-0.3301 to 0.0163
Mg (mg/dL)	y= -0.0031 + 1.0079 x	0.9682 to 1.0477	-0.0789 to 0.0728
UA (mg/dL)	y= -0.0013 + 0.9977 x	0.9821 to 1.0133	-0.0664 to 0.0638

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CI: Confidence interval; CK: Creatine kinase; CRP: C-reactive protein; Mg: Magnesium; TP: Total protein; UA: Uric acid.

4. The 95% CIs for the slope encompassed 1, and those for the intercept encompassed 0 for all analytes, suggesting the absence of significant proportional or constant bias. Appendix 3 presents the results of the Passing-Bablok regression analysis and the correlation between Vacusera and BD serum tubes. The correlation analysis conducted between the test results of Vacusera and BD serum tubes revealed that all correlation coefficients exceeded 0.97 (Appendix 3). In the Passing-Bablok regression analysis, the presence of a value of 1 within the slope or its 95% CI suggested the absence of proportional bias. Similarly, if the intercept or its 95% CI includes the value of 0, constant bias is not present. Appendix 4 presents the results of the Passing-Bablok regression analysis and the correlation for the Vacusera and Greiner serum tubes. The correlation analysis conducted between the test results of Vacusera and Greiner serum tubes revealed that all correlation coefficients exceeded 0.95 (Appendix 4). The 95% CI for the slope encompassed 1, and the 95% CI for the intercept encompassed 0 across all analytes, suggesting that there was no significant proportional or constant bias (Appendix 3, 4).

The percentage difference values calculated at predefined MDLs are presented in Table 5. Asymmetric RCVs, determined based on biological variation for clinical significance, were calculated. At the MDL levels, the deviations calculated for both the Vacusera vs. BD and Vacusera vs. Greiner comparisons remained within a clinically acceptable range when compared with the RCV limits for all analytes (Table 5).

Table 5. Estimated percentage bias of Vacusera tubes at MDLs compared with BD and Greiner tubes

Parameter	MDL1	Bias % (vs BD)	Bias % (vs Greiner)	MDL2	Bias % (vs BD)	Bias % (vs Greiner)	*RCV% increase	*RCV% decrease
Glucose (mg/dL)	126	-0.40	-0.28	400	-2.00	-0.46	11.8	-10.5
TP (g/dL)	6.40	0.22	-0.30	8.30	0.08	-0.51	6.80	-6.40
Albumin (g/dL)	3.50	0.17	-0.14	5.20	0.54	0.27	6.60	-6.20
Urea (mg/dL)	20	-0.50	0.14	100	-0.03	0.08	35.8	-26.4
ALT (IU/L)	40	1.03	-0.55	120	1.02	-0.62	35.2	-26.0
AST (IU/L)	40	-0.85	-2.60	120	-0.62	-2.61	23.1	-18.7
CK (IU/L)	200	-0.75	0.02	1000	-0.59	0.31	39.5	-28.3
CRP (mg/L)	5	-2.00	-0.69	100	0.40	0.04	119.2	-54.4
Sodium (mmol/L)	120	0.80	0.25	160	-0.09	-0.41	3.20	-3.10
Potassium (mmol/L)	2.50	-1.91	-0.16	6.50	0.43	-1.41	10.0	-9.10
Chloride (mmol/L)	80	0.71	0.27	120	0.16	-0.22	3.30	-3.20
Calcium (mg/dL)	6.50	0.24	0.27	13.0	0.63	0.34	5.60	-5.30
Phosphorus (mg/dL)	2.50	-1.18	-2.42	4.50	0.41	0.44	20.7	-17.1
Mg (mg/dL)	1.00	1.65	0.48	3.50	0.51	0.70	8.30	-7.70
UA (mg/dL)	6.80	0.44	-0.25	13.0	0.15	-0.24	21.4	-17.6

*: EFLM Biological variation database. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CK: Creatine kinase; CRP: C-reactive protein; MDL: Medical decision limit; Mg: Magnesium; TP: Total protein; RCV: Reference change value; UA: Uric acid.

Discussion

In recent years, an increasing number of new blood collection tube brands have entered routine laboratory practice, highlighting the importance of continuous local assessment to determine their comparability with established systems and ensure analytical accuracy and patient safety [10]. In the present study, the analytical performance of Vacusera serum tubes, newly introduced for routine laboratory use, was compared with that of Greiner and BD serum tubes using 15 routine biochemical analytes. Although statistically significant differences were observed for certain analytes, evaluation at predefined MDLs demonstrated that the observed differences between Vacusera and comparator tubes remained within RCV limits, indicating clinically acceptable agreement across routine biochemical parameters.

When the Vacusera tubes were compared with the BD tubes, statistically significant differences were observed in AST, CK, sodium, chloride, calcium, and Mg. In comparison with the Greiner tubes, statistically significant differences were found for potassium and Mg. However, when TAE values were evaluated against TEa limits to determine clinical relevance, all analytes, except sodium and chloride, met the EFLM biological variation-based performance specifications [13]. While biological variation is a key model for establishing analytical performance specifications in laboratory medicine, its application to analytes under strict homeostatic control, such as sodium and chloride, may be associated with methodological challenges. The very low CV_i values of these electrolytes can lead to highly stringent biological variation-based targets, which may be difficult to achieve in routine practice and may not always reflect clinical requirements. In accordance with the Milan hierarchy, which allows the use of alternative models when biological variation-based specifications are not considered appropriate for a given measurand [16, 17], we additionally evaluated analytical performance using state-of-the-art criteria based on CLIA and RiliBÄK guidelines [14], and the observed total error for sodium and chloride remained within the allowable limits (CLIA: chloride±5%; RiliBÄK: sodium±5%). Additionally, when assessing the preanalytical phase, the differences observed between different types of collection tubes for sodium and chloride remained within the calculated RCV limits. This indicates that the variation introduced by tube types does not exceed the threshold for a clinically significant change, further supporting the clinical utility of these measurements despite the exceptionally stringent nature of their biological variation-based targets. In addition, Deming regression analysis showed that the 95% CIs for the slope included 1, and those for the intercept included 0 for all analytes, indicating the absence of clinically relevant proportional or constant systematic bias.

These results also emphasize the distinction between statistical and clinical significance. In paired comparison studies performed under controlled preanalytical conditions, small absolute differences may reach statistical significance despite having little or no effect on clinical interpretation. Therefore, statistical significance alone should not be regarded as sufficient evidence of clinically meaningful disagreement. In the present study, the combined interpretation of TAE, RCV, Deming regression, and Bland-Altman agreement indicated that the observed differences were not large enough to compromise clinical usability. Taken together, these findings suggest that the observed differences remained within clinically acceptable limits and that the Va-

cusera serum separator tubes were analytically comparable to the comparator tubes under the conditions of this study.

The statistically significant differences observed, particularly for electrolytes and Mg, may be explained by differences in tube composition and preanalytical behavior. Although serum separator tubes are intended for similar purposes, they may differ in clot activator composition, separator gel characteristics, tube wall material, surfactants, lubricants, and vacuum properties. Such differences may influence clot formation, serum separation efficiency, duration of serum contact with blood cells, and the potential for subtle analyte adsorption or contamination [7]. These effects may be more apparent for electrolytes measured using indirect ion-selective electrode methods, in which even minor matrix-related differences can produce measurable shifts. Likewise, calcium and Mg may be affected by small differences in gel composition or additive-related interactions. The fact that Mg showed significant differences in both comparisons suggests that this analyte may be particularly sensitive to tube-dependent preanalytical variation.

Our findings are in agreement with previous studies reporting that different serum separator tubes may be broadly comparable for routine chemistry testing while still showing analyte-specific differences. Salvatici et al. [10] reported no clinically significant differences between Disera Vacusera and BD tubes for several routine biochemical parameters, including potassium, sodium, chloride, AST, ALT, and lactate dehydrogenase, although some statistically significant differences were observed for selected analytes. Similarly, Lima-Oliveira et al. [18] compared five brands of serum vacuum tubes and found that most routine biochemical parameters were comparable across tube systems, whereas clinically relevant deviations were observed for specific analytes, such as creatinine, amylase, phosphorus, and Mg. The authors emphasized that clot activators and separator gels may influence analytical performance and that particular caution is needed when collection devices are changed.

Recent reports have further supported this interpretation. Bowen and Dasgupta emphasized that components of blood collection devices, including additives, lubricants, stopper materials, and separator gels, may directly or indirectly affect specimen quality and analyte stability, underscoring the importance of careful local assessment when new collection systems are introduced [7]. Balci et al. [9] also reported that tube-related variability may affect the reliability of biochemical measurements, particularly for analytes susceptible to contamination or tube-specific interference, and highlighted Mg among the parameters that may be influenced by inter-tube differences. Likewise, Kang et al. [11] showed that newly introduced alternative tube systems may demonstrate acceptable overall agreement with established tubes while still producing notable discrepancies for selected analytes, indicating that interchangeability between manufacturers should not be assumed automatically.

In addition, recent work by Plebani et al. [19] further emphasized that preanalytical quality should be viewed as a dynamic and continuously monitored component of the total testing process. Their discussion highlighted that specimen collection devices, handling procedures, transport conditions, and other preanalytical factors remain major determinants of result reliability. In this context, our findings support the need for a structured local comparison process before a newly introduced blood collection tube is adopted in routine practice.

Therefore, the effectiveness and reliability of all collection tubes used in the laboratory should be carefully assessed before routine implementation. In clinical laboratories, each component of diagnostic products, including blood collection tubes, should undergo a structured local comparison process before incorporation into routine patient testing [7, 19, 20].

This study had several limitations. First, it was conducted at a single center, which may limit the generalizability of the findings to laboratories operating under different preanalytical and analytical conditions. Second, an additive-free glass serum tube was not included as a reference comparator; therefore, the findings should be interpreted as an inter-tube comparison rather than formal reference-based validation. Third, only a limited panel of routine biochemical parameters was evaluated, and the inclusion of a broader range of analytes, particularly those known to be sensitive to tube additives or separator gels, might have provided a more comprehensive assessment. Additionally, the relatively limited sample size may reduce the statistical power and precision of bias estimates and agreement analyses; therefore, the findings should be interpreted with caution and confirmed in larger cohorts. Another limitation of this study is that the differences in clot activators and gel compositions among the tube brands were not specifically evaluated. Therefore, their potential impact on sample quality could not be fully assessed. Future studies with larger sample series, broader analyte panels, and multicenter designs may provide a more comprehensive understanding of the impact of tube-related factors on routine biochemical testing.

Conclusion

This comparative analytical study evaluated whether newly introduced Vacusera CAT Serum Gel Q Clot Activator tubes yielded clinically acceptable results compared with established BD Vacutainer SST and Greiner Vacuette serum tubes, given that blood collection devices are a critical preanalytical source of error and require careful local assessment for quality assurance and accreditation. Vacusera CAT Serum tubes produced results comparable to those of BD and Greiner tubes for the tested routine biochemical parameters, supporting their suitability for routine use while emphasizing the need for continued local comparison when implementing new blood collection devices.

Disclosures

Ethics Committee Approval: The study was approved by the Istanbul Medipol University Non-interventional Clinical Research Ethics Committee (no: 268, date: 06/03/2025).

Informed Consent: Written informed consent was obtained.

Conflict of Interest Statement: There is no conflict of interest for any author. All authors have read and approved the manuscript.

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Authorship Contributions: Concept – A.K., L.D.; Design – A.K., L.D.; Supervision – A.K., L.D.; Materials – A.K.; Data collection and/or processing – A.K., L.D.; Analysis and/or interpretation – A.K., L.D.; Literature search – A.K., L.D.; Writing – A.K., L.D.; Critical review – A.K., L.D.

Peer-review: Externally peer-reviewed.

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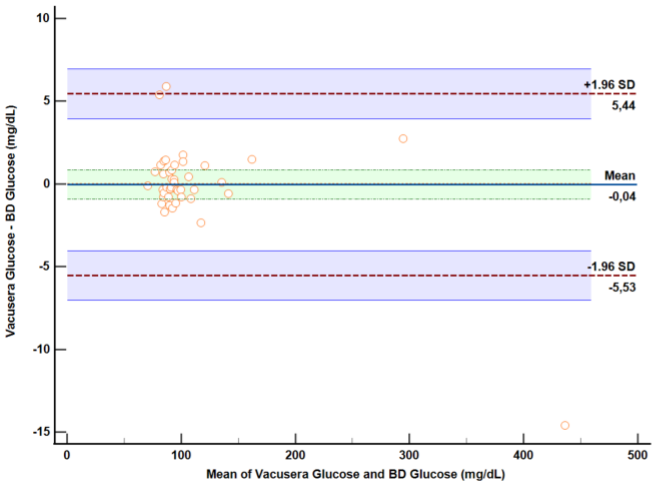
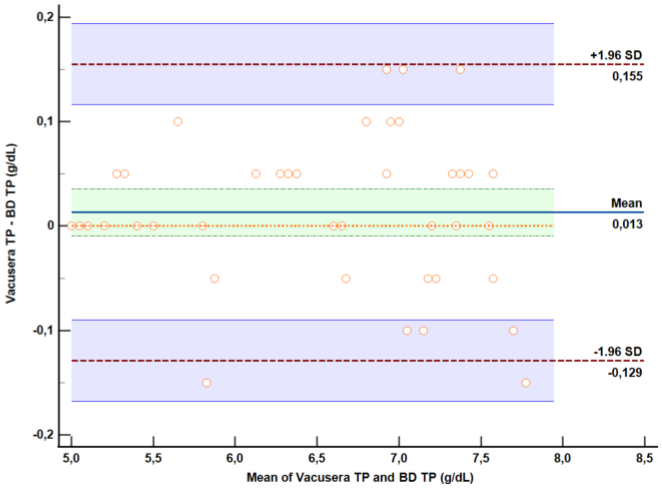
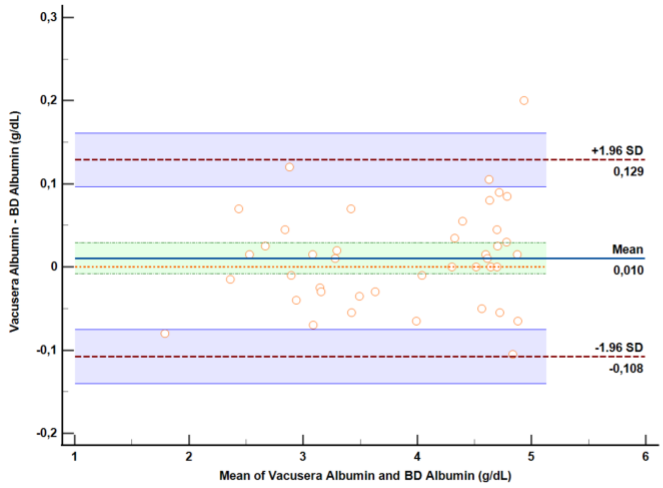
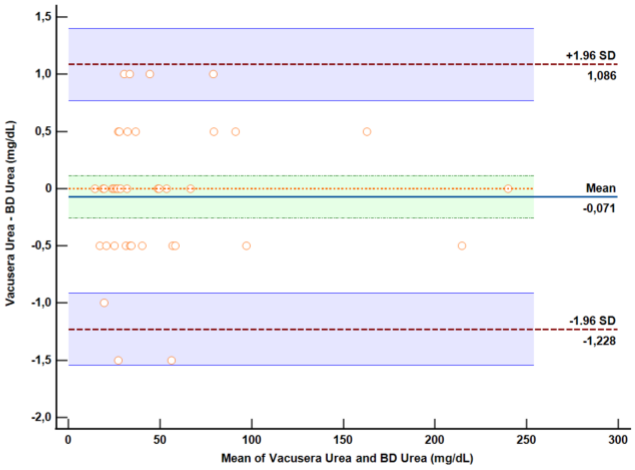
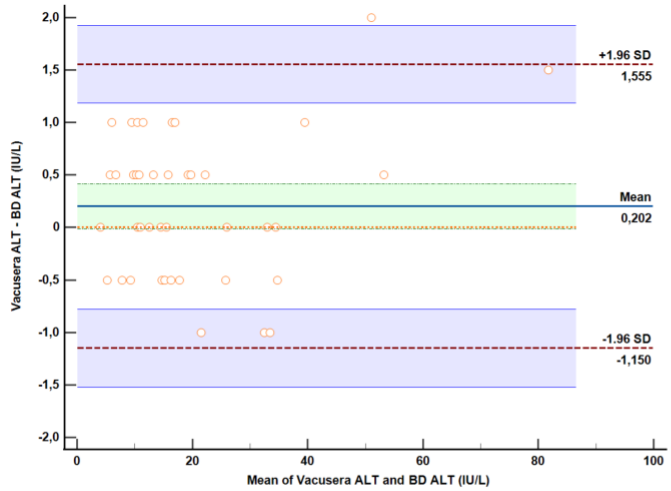
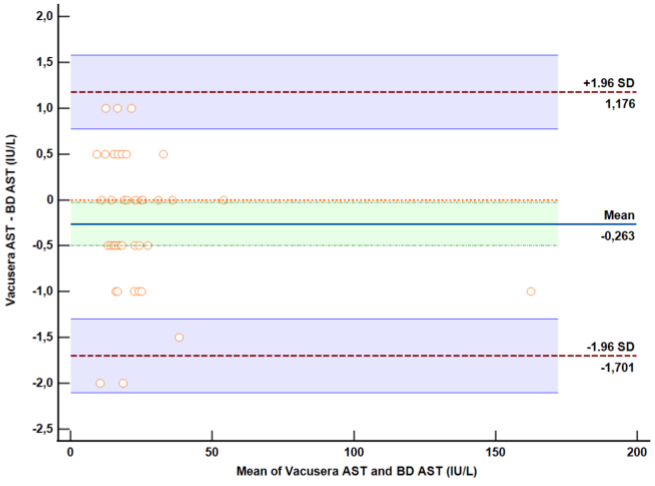

Vacusera Glucose - BD Glucose (mg/dL)
Mean of Vacusera Glucose and BD Glucose (mg/dL)
+1.96 SD
5,44
Mean
-0,04
-1.96 SD
-5,53


Vacusera TP - BD TP (g/dL)
Mean of Vacusera TP and BD TP (g/dL)
+1.96 SD
0,155
Mean
0,013
-1.96 SD
-0,129

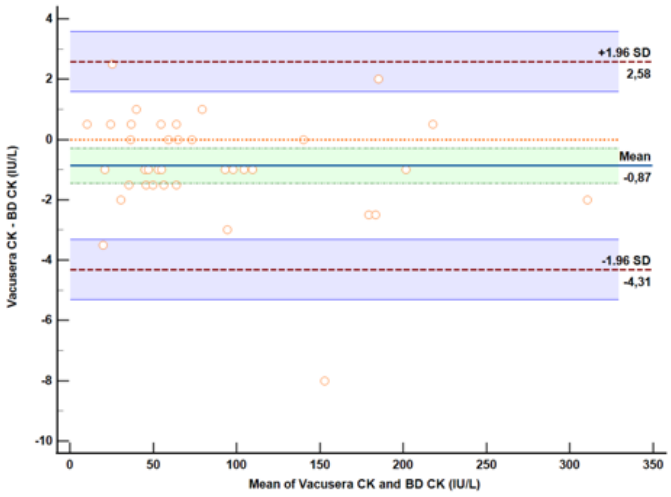

Vacusera Albumin - BD Albumin (g/dL)
Mean of Vacusera Albumin and BD Albumin (g/dL)
+1.96 SD
0,129
Mean
0,010
-1.96 SD
-0,108

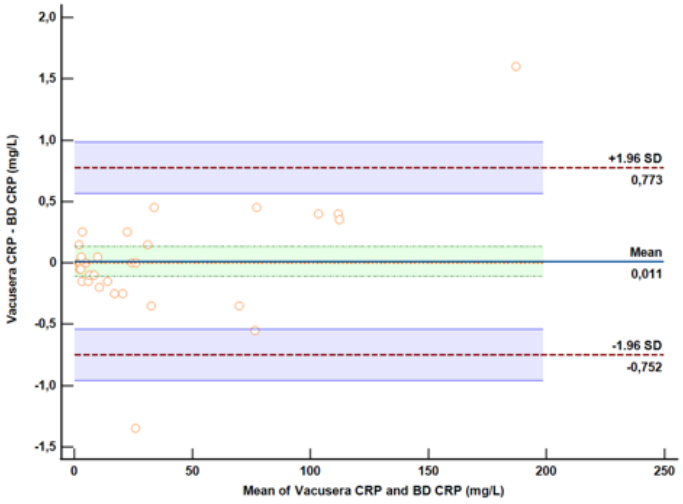

Vacusera Urea - BD Urea (mg/dL)
Mean of Vacusera Urea and BD Urea (mg/dL)
+1.96 SD
1,086
Mean
-0,071
-1.96 SD
-1,228

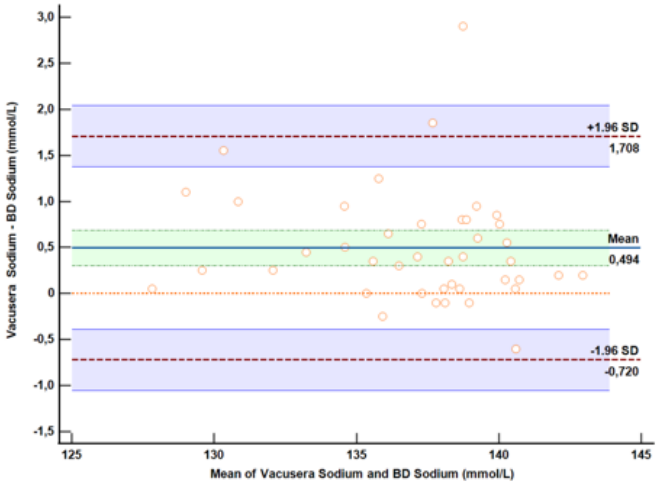

Vacusera ALT - BD ALT (IU/L)
Mean of Vacusera ALT and BD ALT (IU/L)
+1.96 SD
1,555
Mean
0,202
-1.96 SD
-1,150

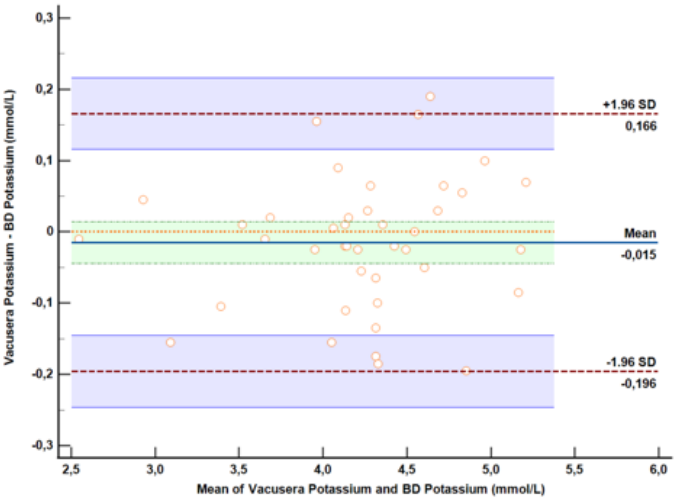

Vacusera AST - BD AST (IU/L)
Mean of Vacusera AST and BD AST (IU/L)
+1.96 SD
1,176
Mean
-0,263
-1.96 SD
-1,701

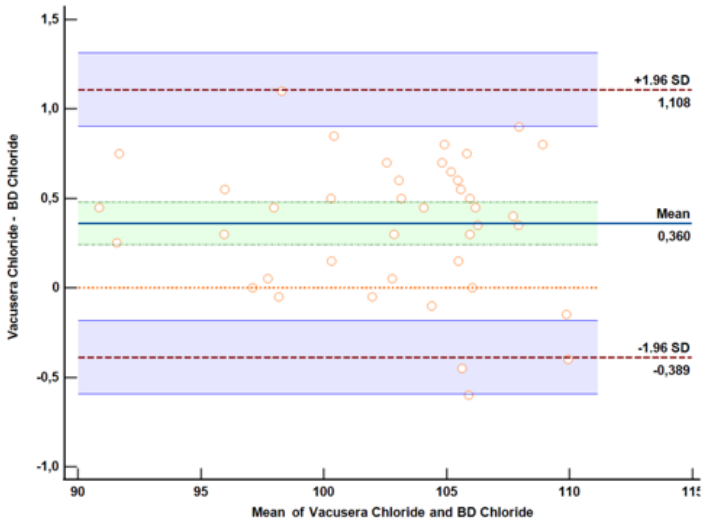
A

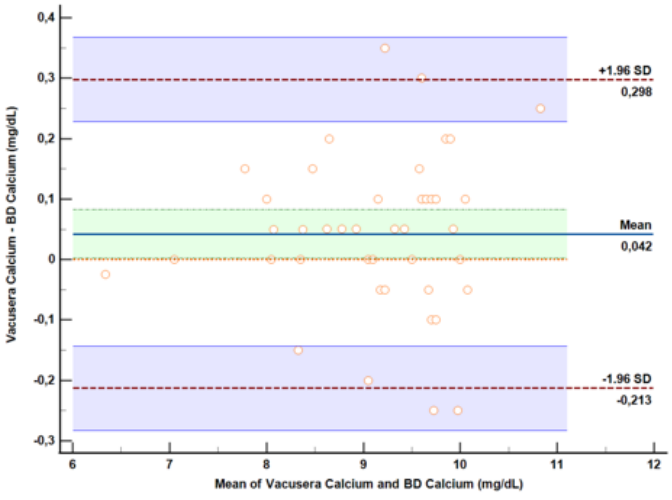

+1.96 SD
2,58
Mean
-0,87
-1.96 SD
-4,31
Vacusera CK - BD CK (IU/L)
Mean of Vacusera CK and BD CK (IU/L)


+1.96 SD
0,773
Mean
0,011
-1.96 SD
-0,752
Vacusera CRP - BD CRP (mg/L)
Mean of Vacusera CRP and BD CRP (mg/L)

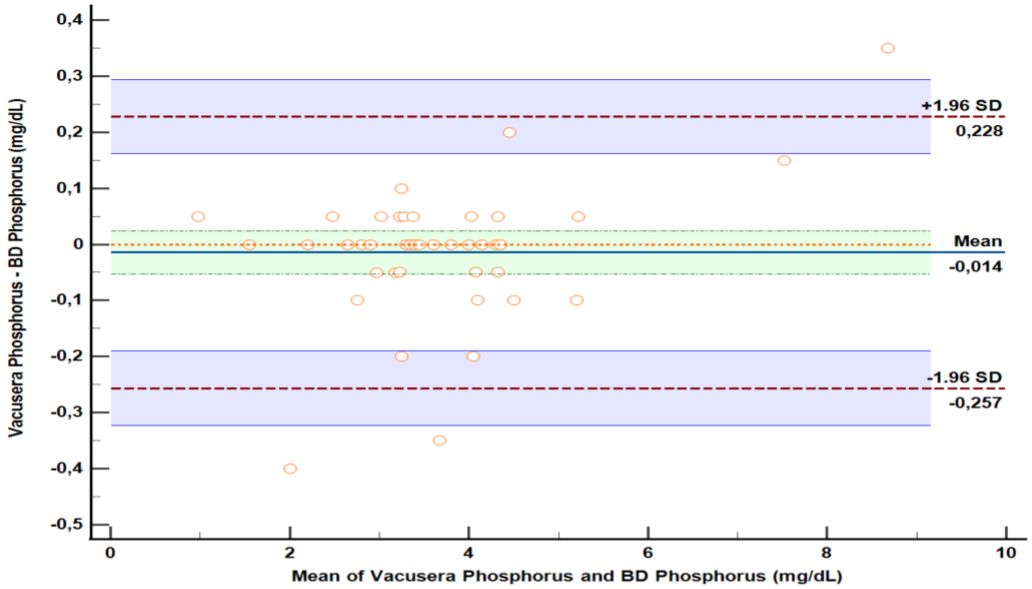

+1.96 SD
1,708
Mean
0,494
-1.96 SD
-0,720
Vacusera Sodium - BD Sodium (mmol/L)
Mean of Vacusera Sodium and BD Sodium (mmol/L)

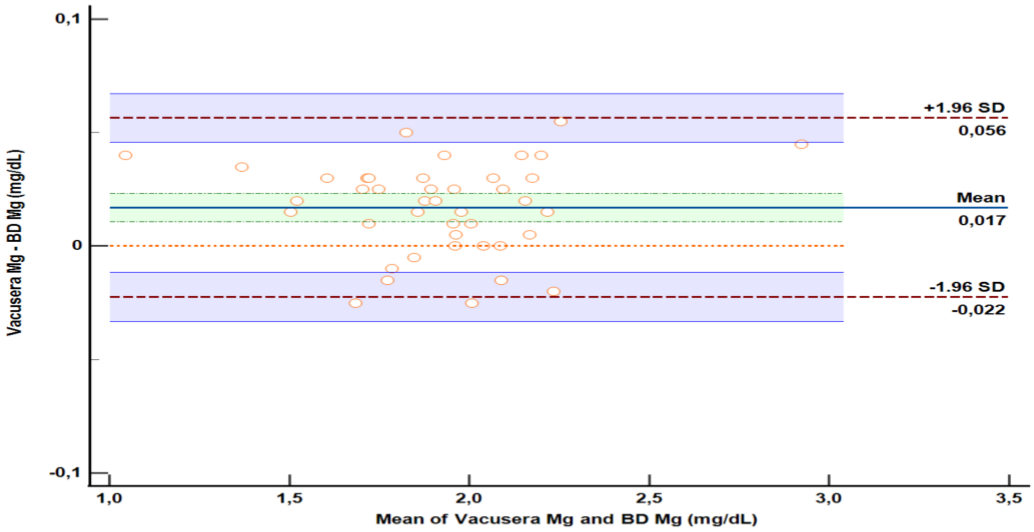

+1.96 SD
0,166
Mean
-0,015
-1.96 SD
-0,196
Vacusera Potassium - BD Potassium (mmol/L)
Mean of Vacusera Potassium and BD Potassium (mmol/L)

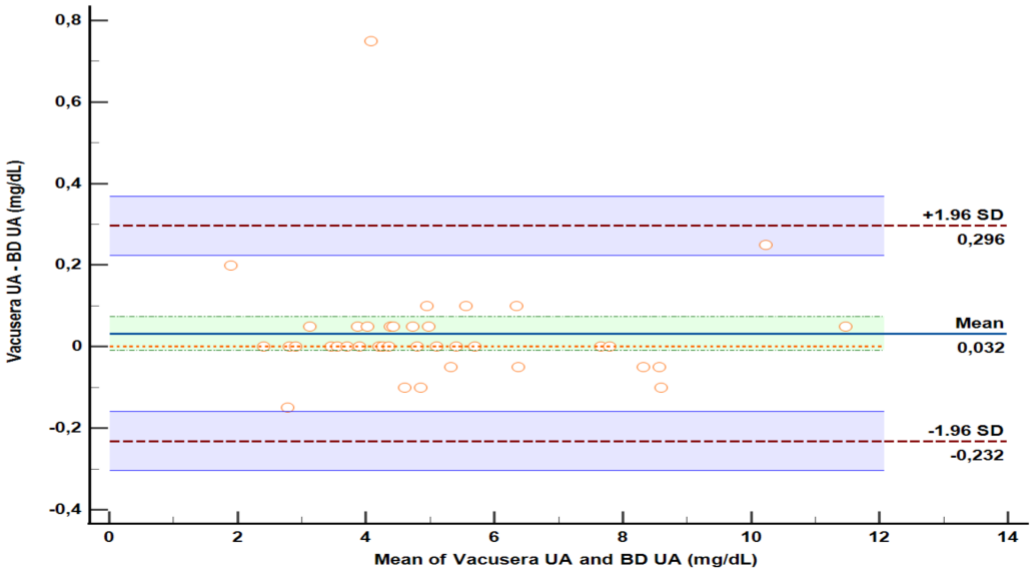

+1.96 SD
1,108
Mean
0,360
-1.96 SD
-0,389
Vacusera Chloride - BD Chloride
Mean of Vacusera Chloride and BD Chloride


+1.96 SD
0,298
Mean
0,042
-1.96 SD
-0,213
Vacusera Calcium - BD Calcium (mg/dL)
Mean of Vacusera Calcium and BD Calcium (mg/dL)

B



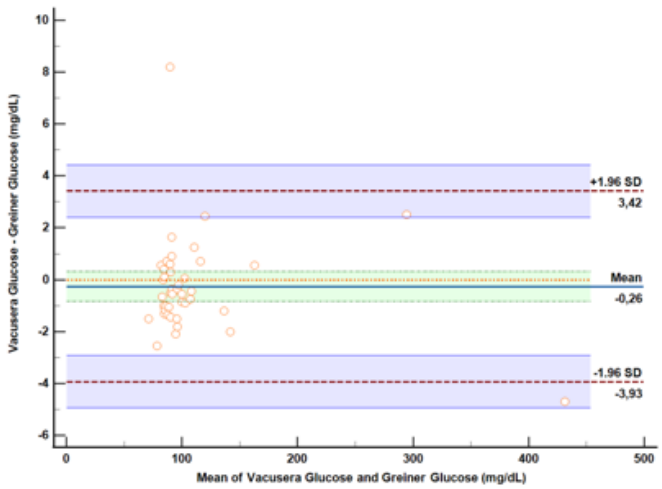


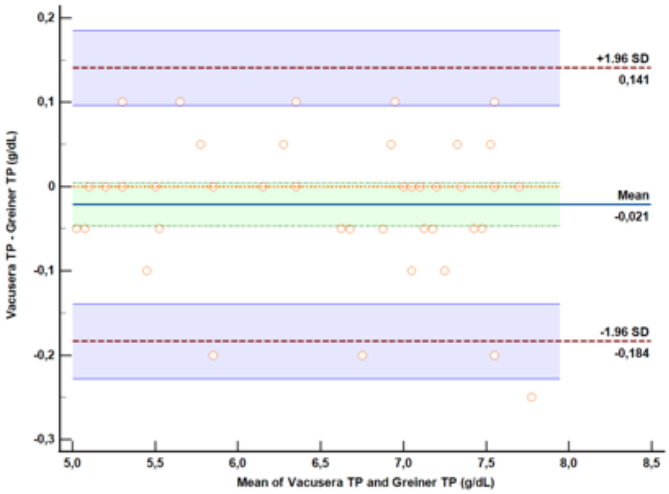


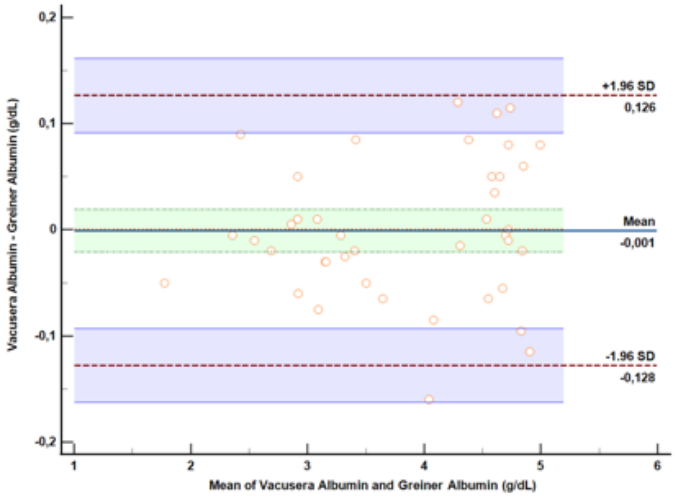
C

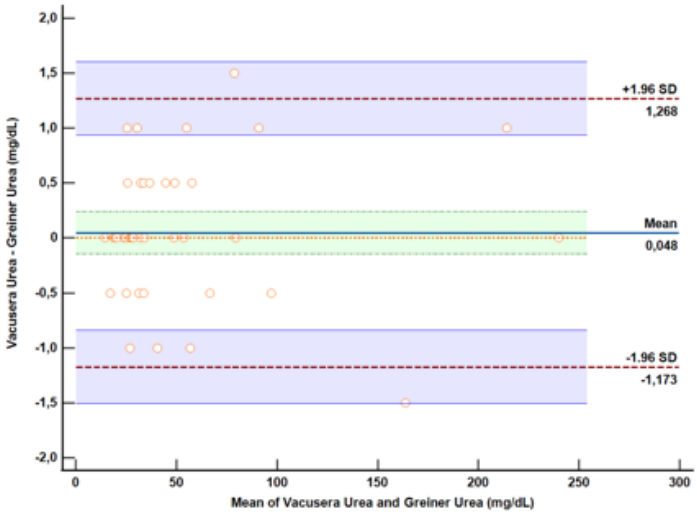
Appendix 1. Bland–Altman plots comparing biochemical analyte results obtained from Vacusera CAT serum tubes and BD Vacutainer SST tubes.

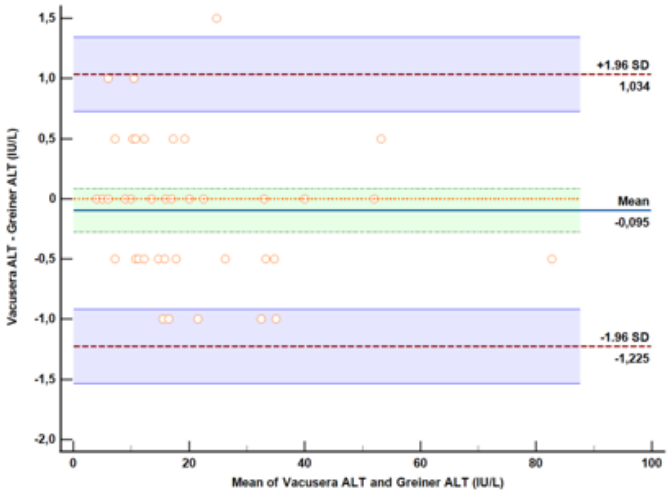
ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CK: Creatine kinase; CRP: C-reactive protein; Mg: Magnesium; TP: Total protein; UA: Uric acid.

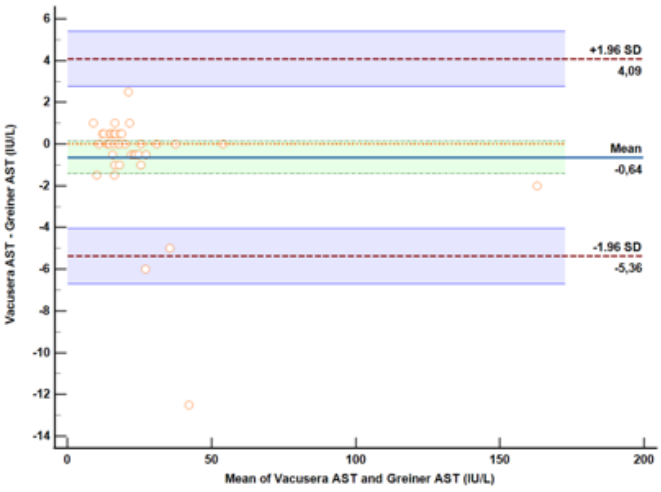

Vacusera Glucose - Greiner Glucose (mg/dL)
+1.96 SD
3,42
Mean
-0,26
-1.96 SD
-3,93
Mean of Vacusera Glucose and Greiner Glucose (mg/dL)


Vacusera TP - Greiner TP (g/dL)
+1.96 SD
0,141
Mean
-0,021
-1.96 SD
-0,184
Mean of Vacusera TP and Greiner TP (g/dL)

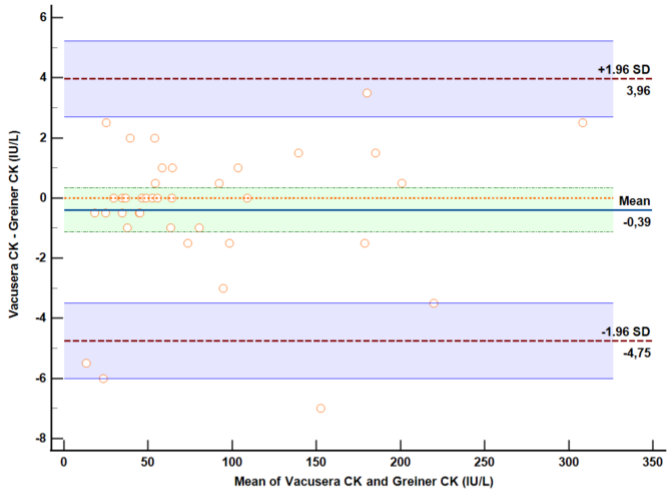

Vacusera Albumin - Greiner Albumin (g/dL)
+1.96 SD
0,126
Mean
-0,001
-1.96 SD
-0,128
Mean of Vacusera Albumin and Greiner Albumin (g/dL)

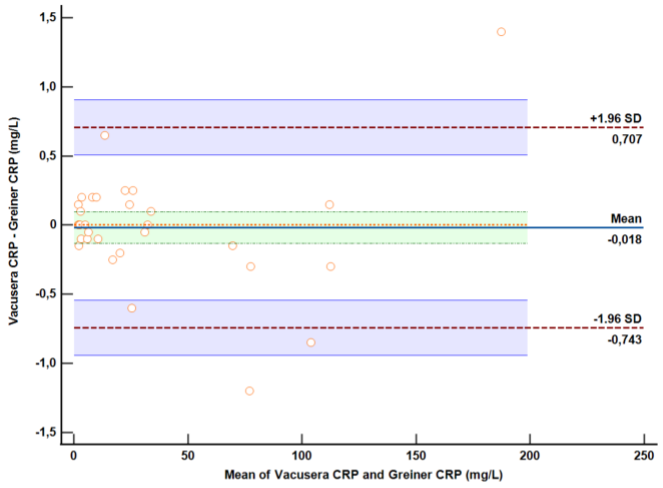

Vacusera Urea - Greiner Urea (mg/dL)
+1.96 SD
1,268
Mean
0,048
-1.96 SD
-1,173
Mean of Vacusera Urea and Greiner Urea (mg/dL)

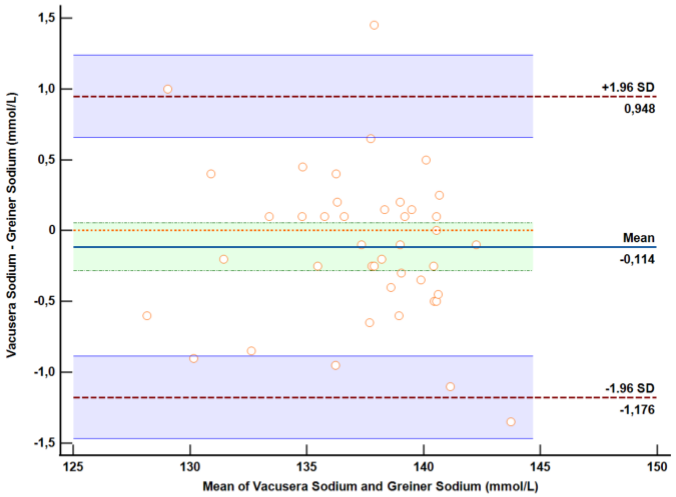

Vacusera ALT - Greiner ALT (IU/L)
+1.96 SD
1,034
Mean
-0,095
-1.96 SD
-1,225
Mean of Vacusera ALT and Greiner ALT (IU/L)

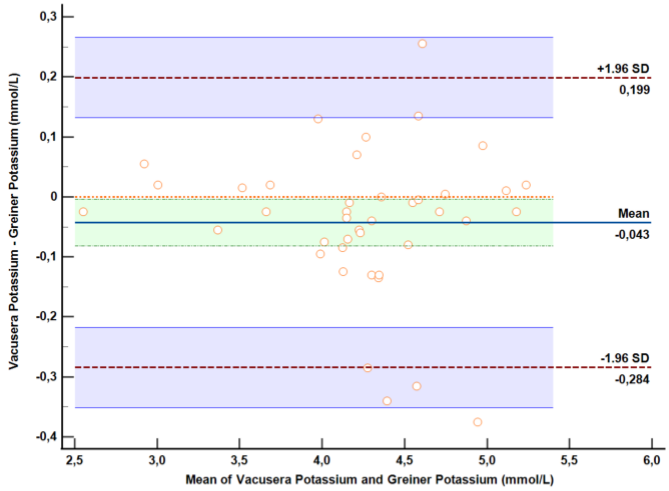

Vacusera AST - Greiner AST (IU/L)
+1.96 SD
4,09
Mean
-0,64
-1.96 SD
-5,36
Mean of Vacusera AST and Greiner AST (IU/L)

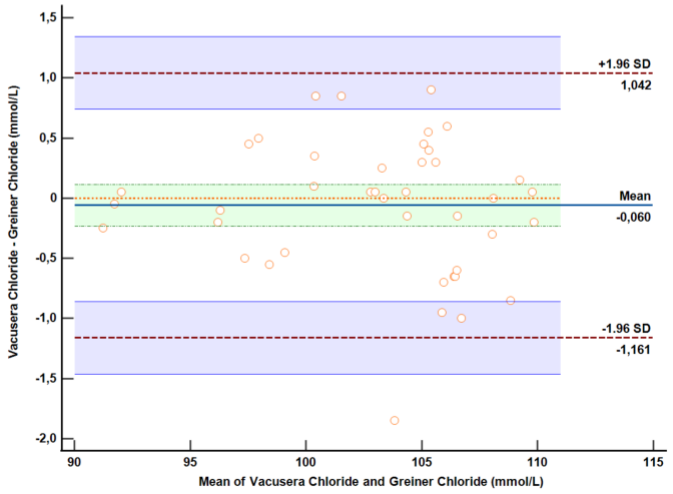
A

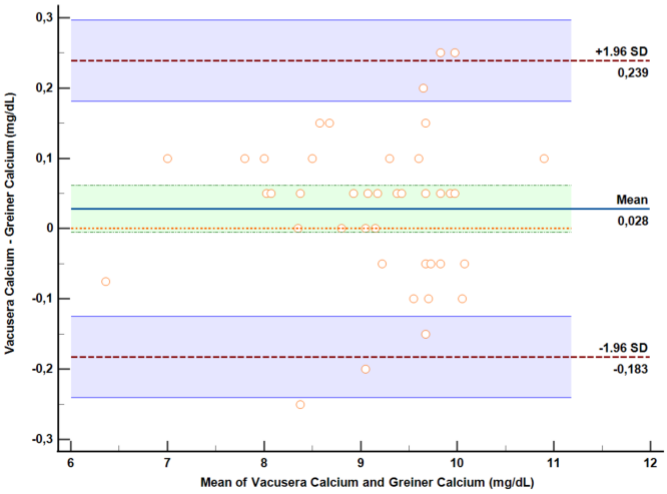

Vacusera CK - Greiner CK (IU/L)
+1.96 SD
3,96
Mean
-0,39
-1.96 SD
-4,75
Mean of Vacusera CK and Greiner CK (IU/L)


Vacusera CRP - Greiner CRP (mg/L)
+1.96 SD
0,707
Mean
-0,018
-1.96 SD
-0,743
Mean of Vacusera CRP and Greiner CRP (mg/L)

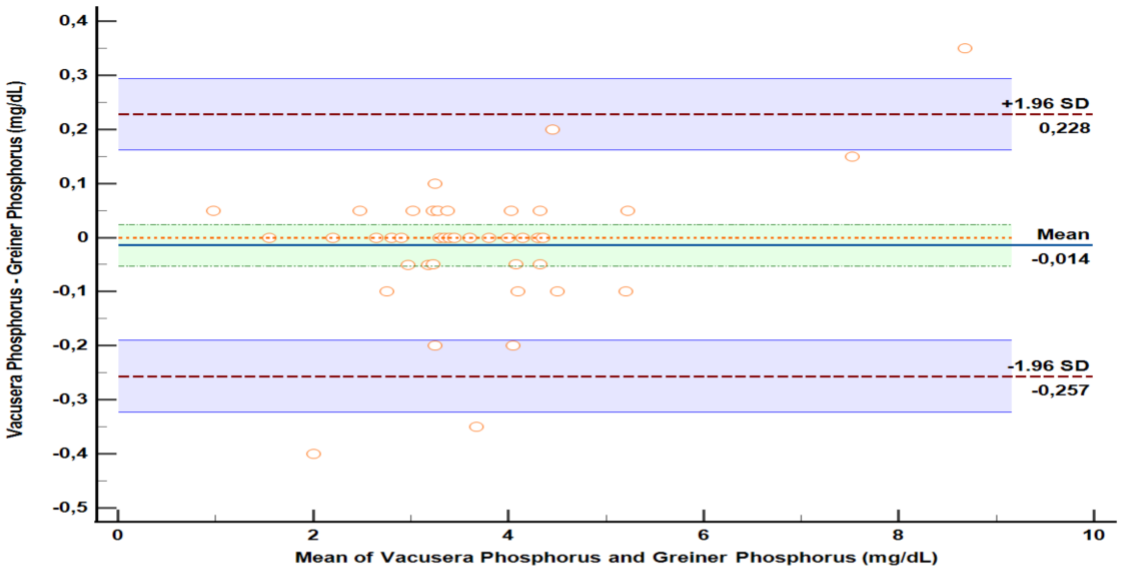

Vacusera Sodium - Greiner Sodium (mmol/L)
+1.96 SD
0,948
Mean
-0,114
-1.96 SD
-1,176
Mean of Vacusera Sodium and Greiner Sodium (mmol/L)

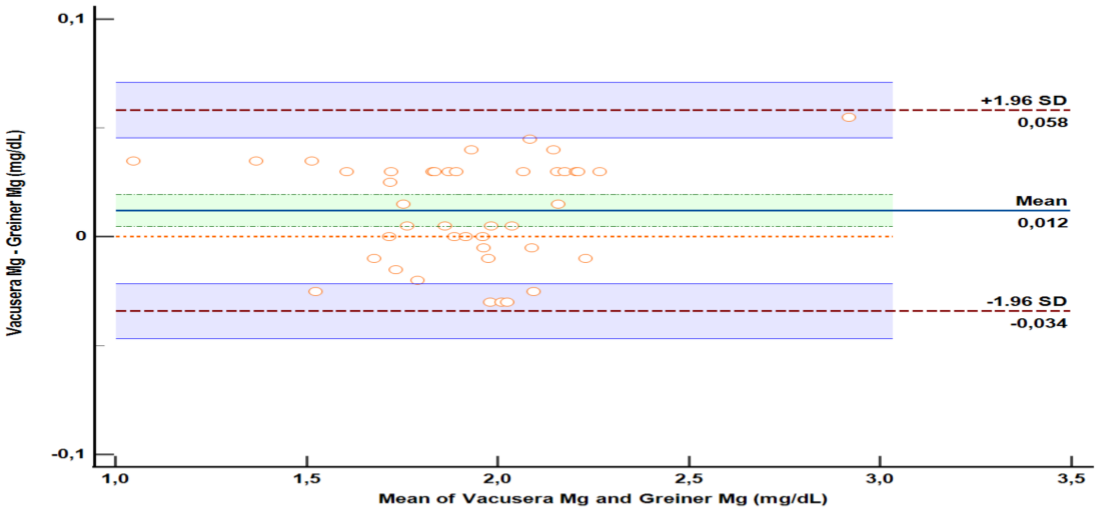

Vacusera Potassium - Greiner Potassium (mmol/L)
+1.96 SD
0,199
Mean
-0,043
-1.96 SD
-0,284
Mean of Vacusera Potassium and Greiner Potassium (mmol/L)

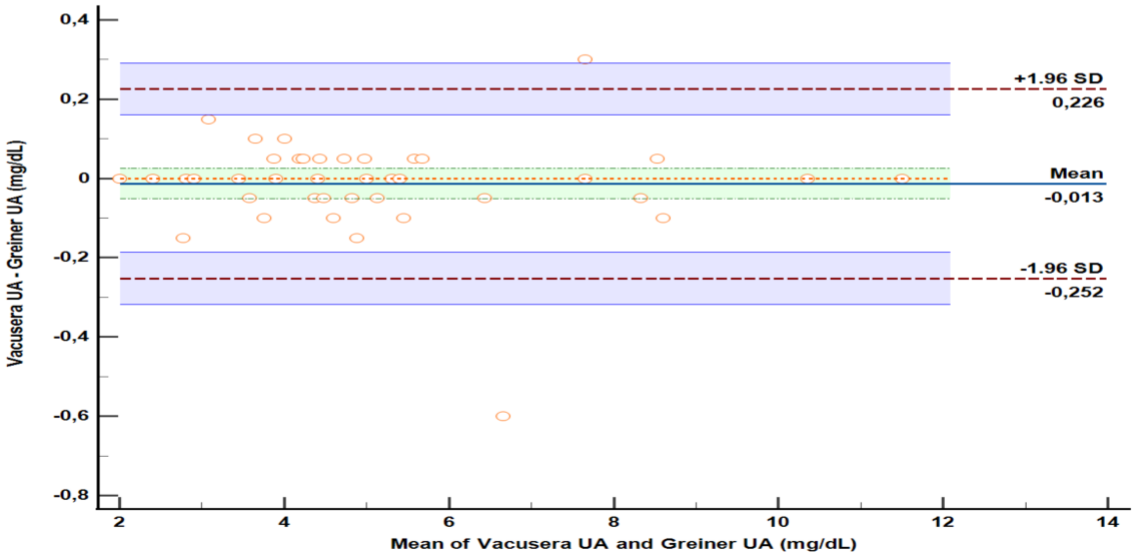

Vacusera Chloride - Greiner Chloride (mmol/L)
+1.96 SD
1,042
Mean
-0,060
-1.96 SD
-1,161
Mean of Vacusera Chloride and Greiner Chloride (mmol/L)


Vacusera Calcium - Greiner Calcium (mg/dL)
+1.96 SD
0,239
Mean
0,028
-1.96 SD
-0,183
Mean of Vacusera Calcium and Greiner Calcium (mg/dL)

B



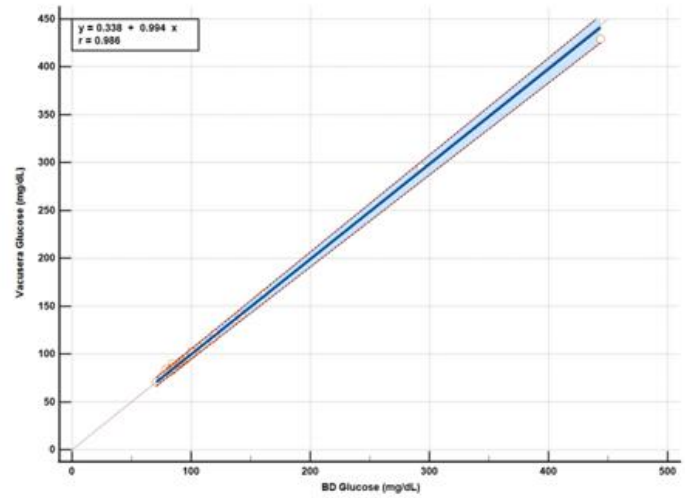


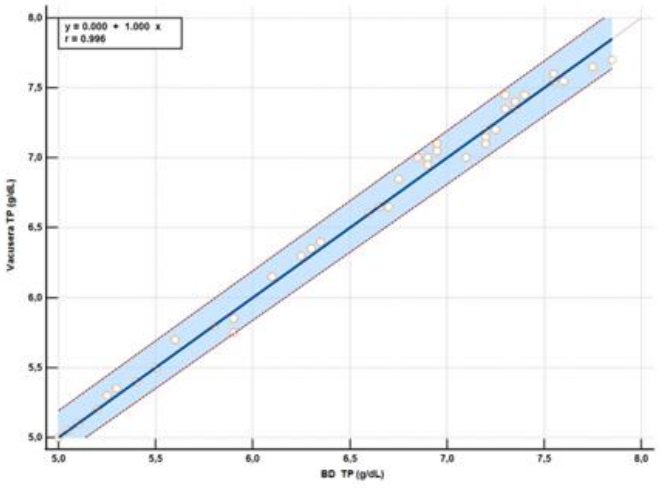


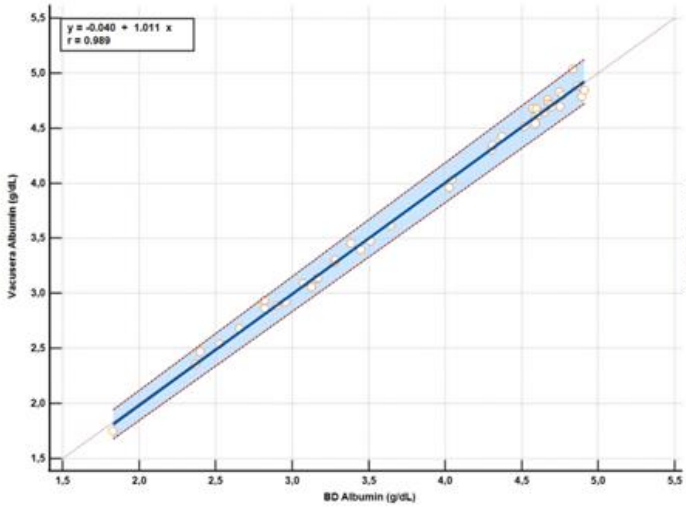
C

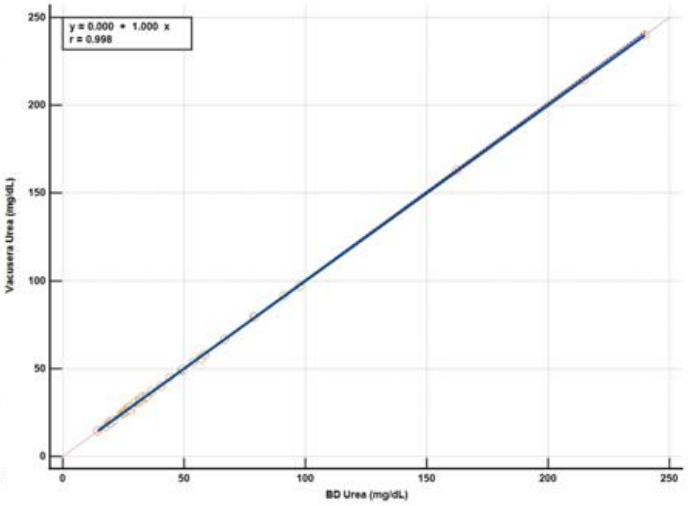
Appendix 2. Bland–Altman plots comparing biochemical analyte results obtained from Vacusera CAT serum tubes and Greiner Vacuette serum tubes.

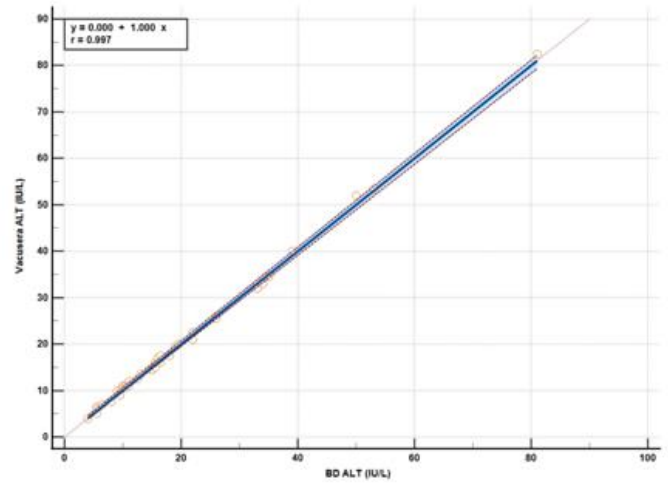
ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CK: Creatine kinase; CRP: C-reactive protein; Mg: Magnesium; TP: Total protein; UA: Uric acid.

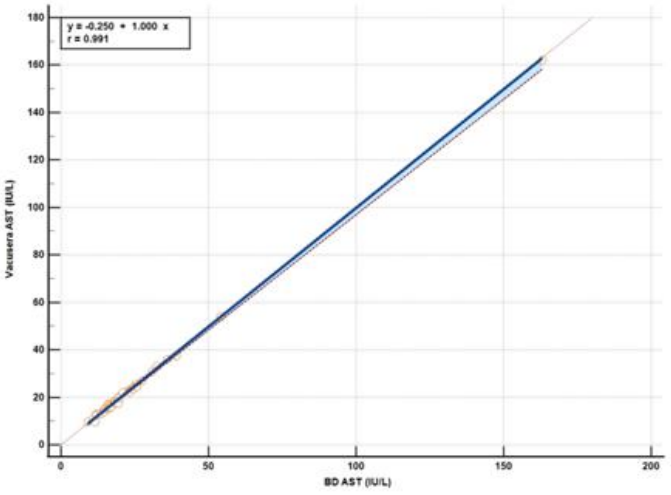

y = 0.338 + 0.994 x
r = 0.986
Vacusera Glucose (mg/dL)
BD Glucose (mg/dL)


y = 0.000 + 1.000 x
r = 0.996
Vacusera TP (g/dL)
BD TP (g/dL)

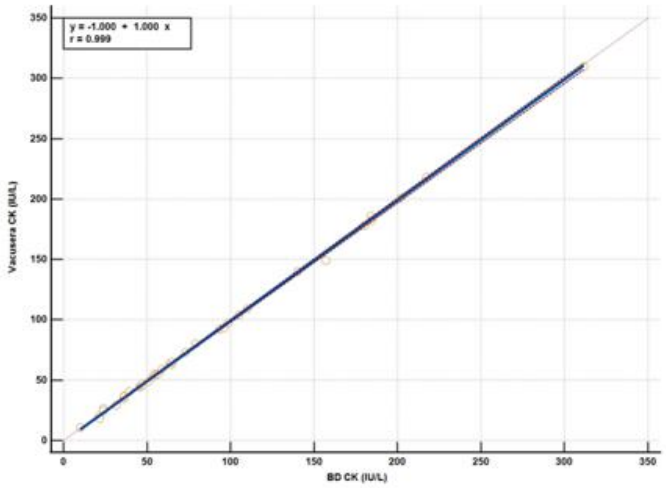

y = -0.040 + 1.011 x
r = 0.989
Vacusera Albumin (g/dL)
BD Albumin (g/dL)

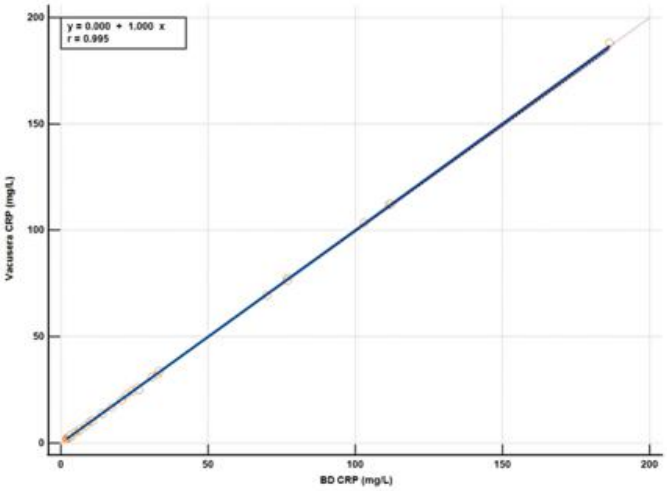

y = 0.000 + 1.000 x
r = 0.998
Vacusera Urea (mg/dL)
BD Urea (mg/dL)

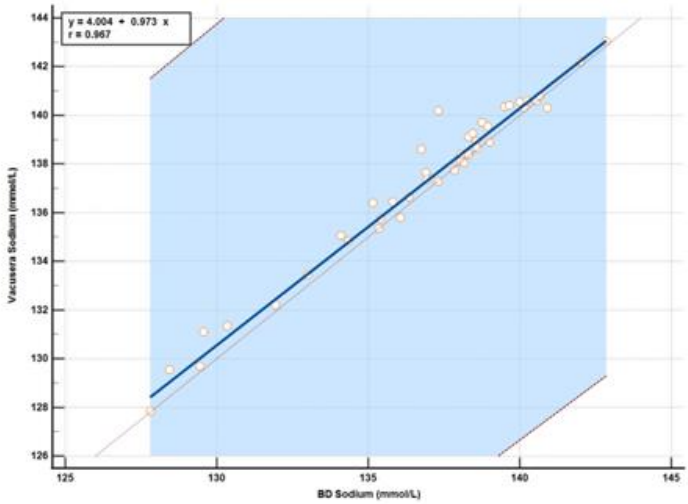

y = 0.000 + 1.000 x
r = 0.997
Vacusera ALT (IU/L)
BD ALT (IU/L)

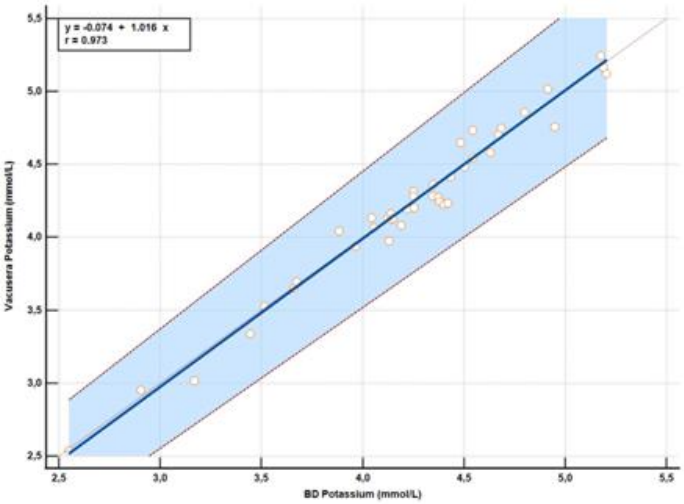

y = -0.250 + 1.000 x
r = 0.991
Vacusera AST (IU/L)
BD AST (IU/L)

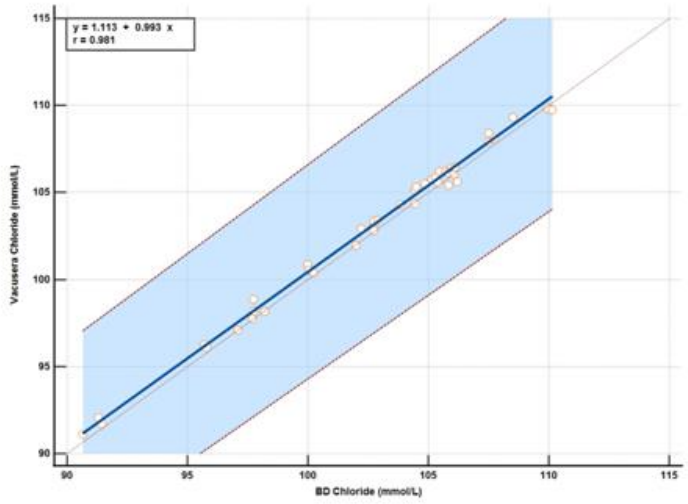
A

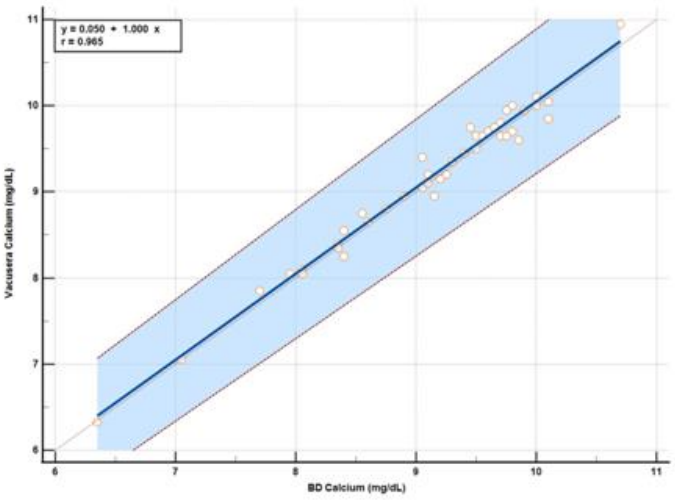

y = -1.000 + 1.000 x
r = 0.999
Vacusera CK (IU/L)
BD CK (IU/L)


y = 0.000 + 1.000 x
r = 0.995
Vacusera CRP (mg/L)
BD CRP (mg/L)

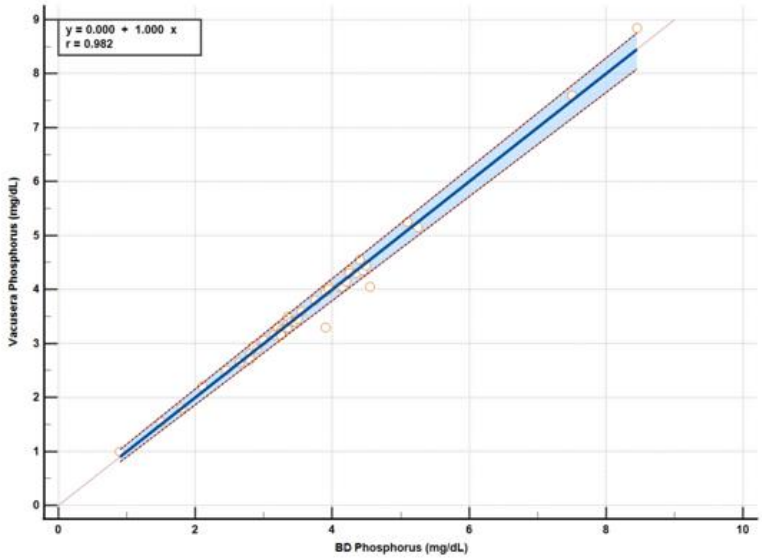

y = 4.004 + 0.973 x
r = 0.967
Vacusera Sodium (mmol/L)
BD Sodium (mmol/L)

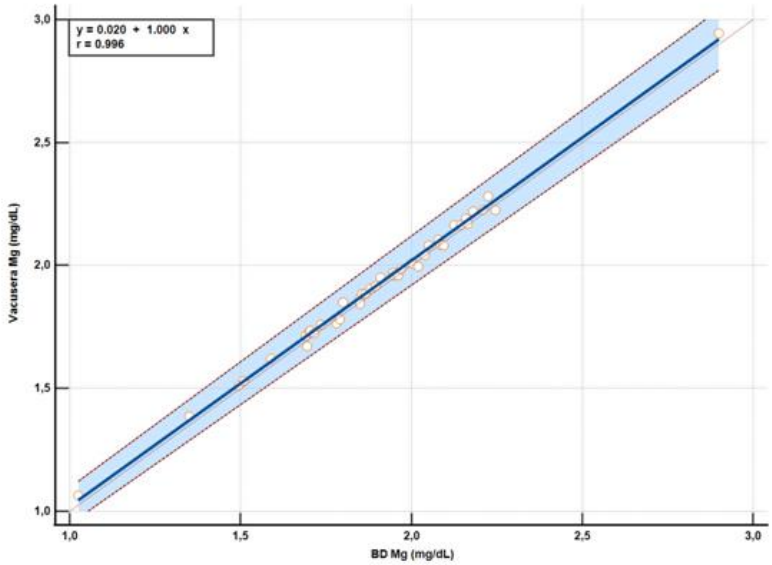

y = -0.074 + 1.016 x
r = 0.973
Vacusera Potassium (mmol/L)
BD Potassium (mmol/L)

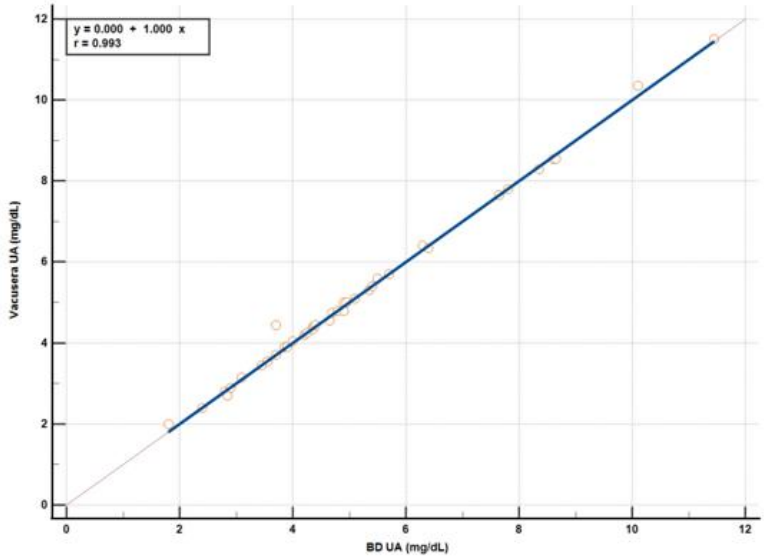

y = 1.113 + 0.993 x
r = 0.981
Vacusera Chloride (mmol/L)
BD Chloride (mmol/L)


y = 0.050 + 1.000 x
r = 0.965
Vacusera Calcium (mg/dL)
BD Calcium (mg/dL)

B



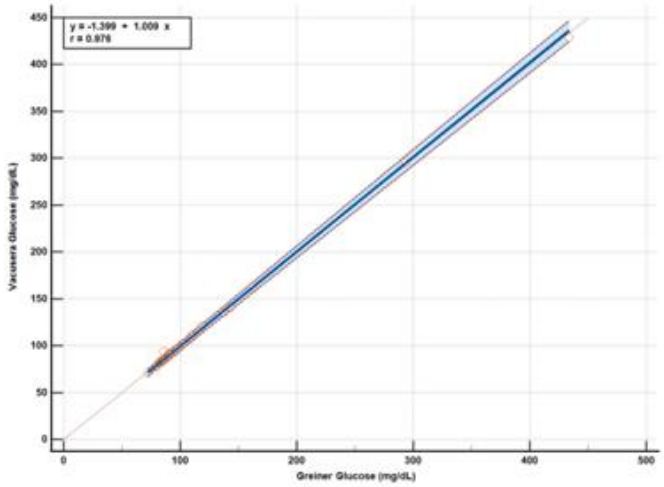


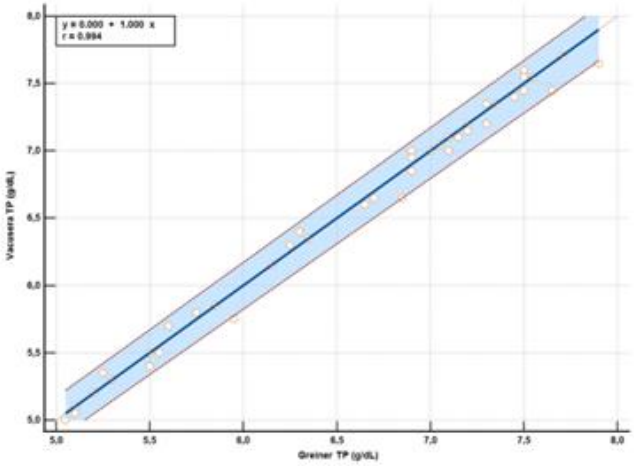


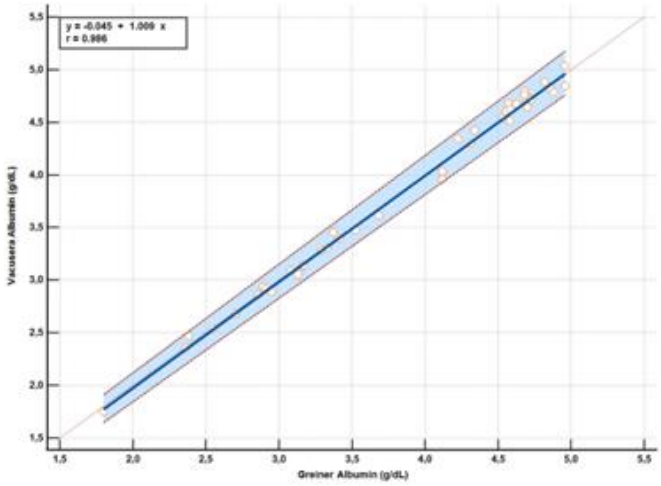
C

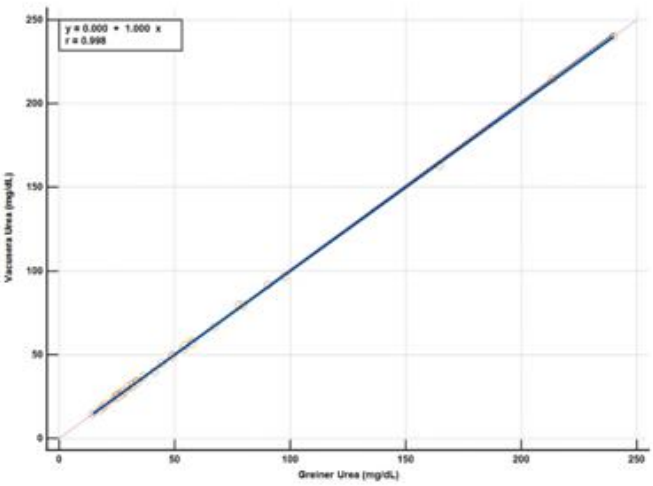
Appendix 3. Passing-Bablok regression analysis for Vacusera CAT and BD Vacutainer SST tubes.

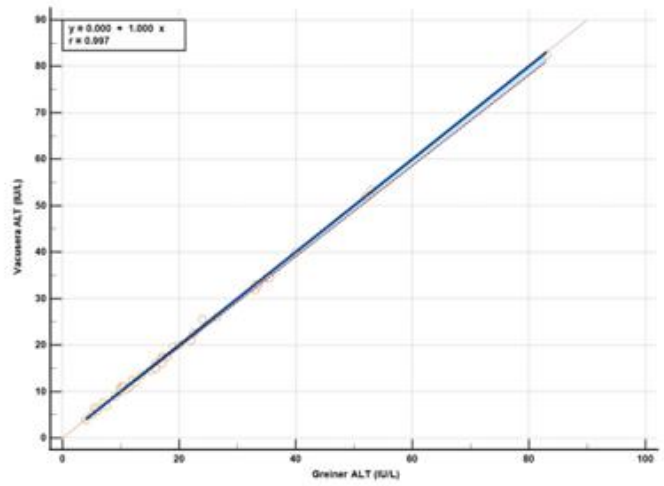
ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CK: Creatine kinase; CRP: C-reactive protein; Mg: Magnesium; TP: Total protein; UA: Uric acid.

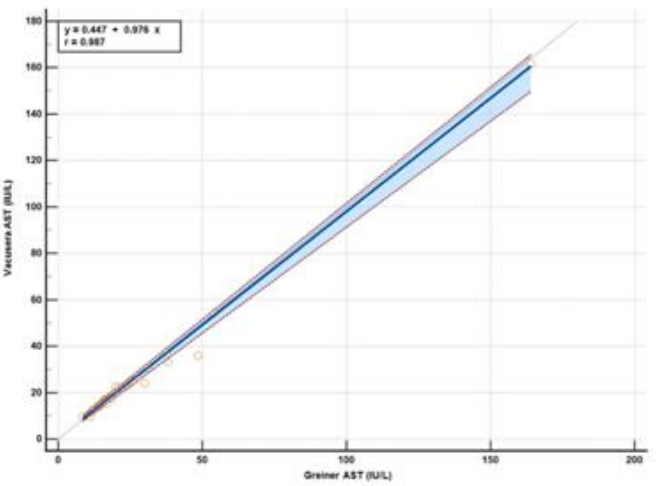

y = -1.399 + 1.009 x
r = 0.978
Vacusera Glucose (mg/dL)
Greiner Glucose (mg/dL)


y = 0.000 + 1.000 x
r = 0.994
Vacusera TP (g/dL)
Greiner TP (g/dL)

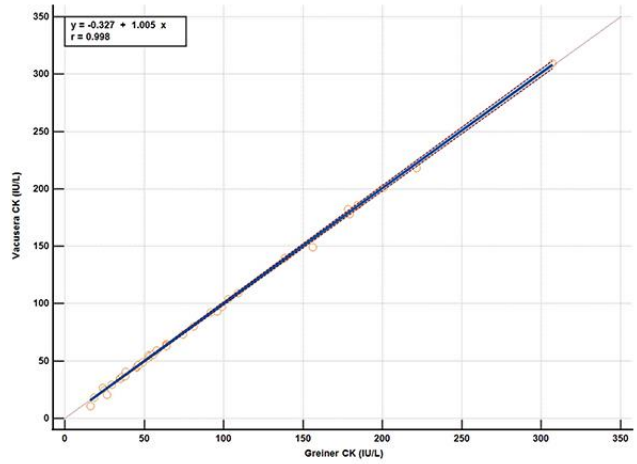

y = -0.045 + 1.009 x
r = 0.986
Vacusera Albumin (g/dL)
Greiner Albumin (g/dL)

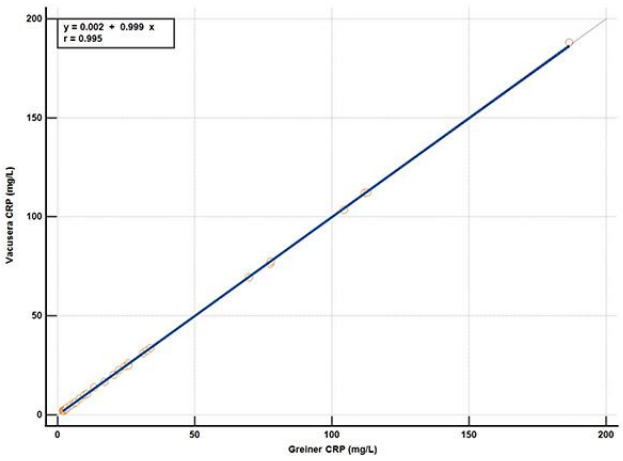

y = 0.000 + 1.000 x
r = 0.998
Vacusera Urea (mg/dL)
Greiner Urea (mg/dL)

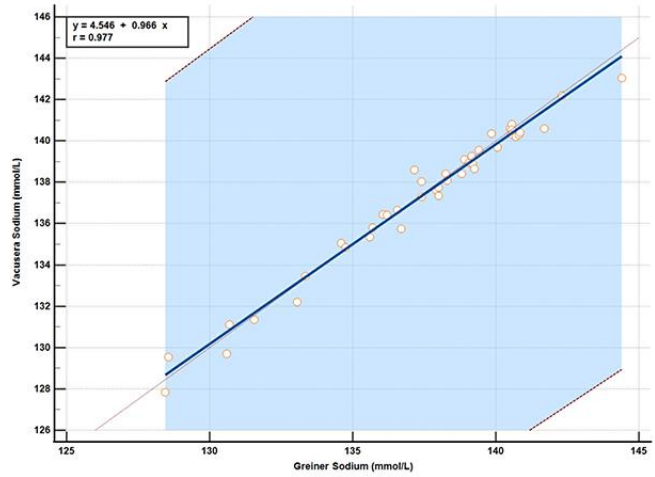

y = 0.000 + 1.000 x
r = 0.997
Vacusera ALT (IU/L)
Greiner ALT (IU/L)

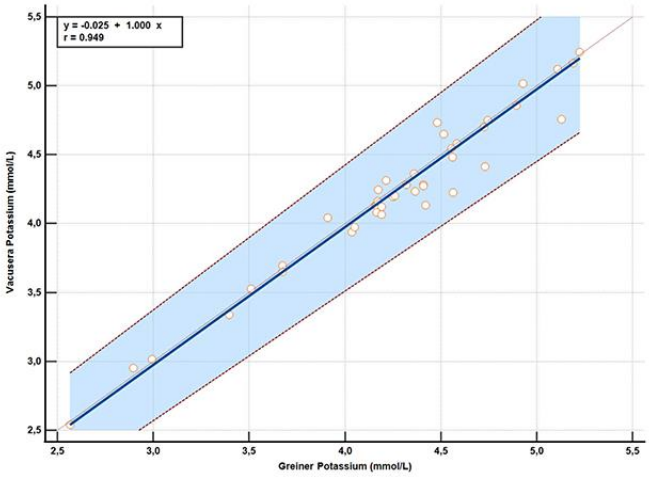

y = 0.447 + 0.976 x
r = 0.987
Vacusera AST (IU/L)
Greiner AST (IU/L)

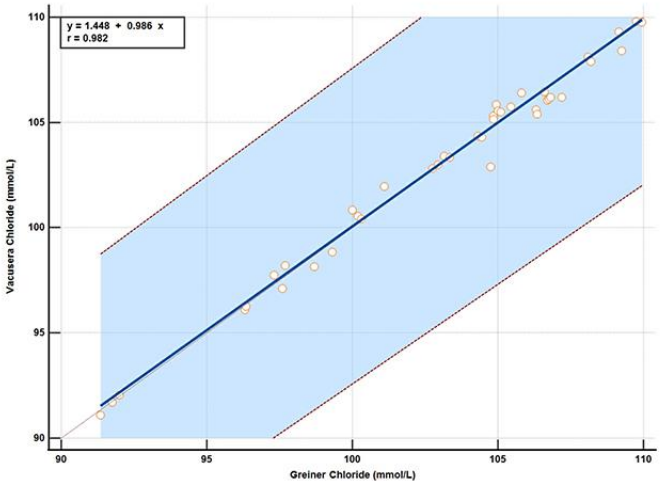
A

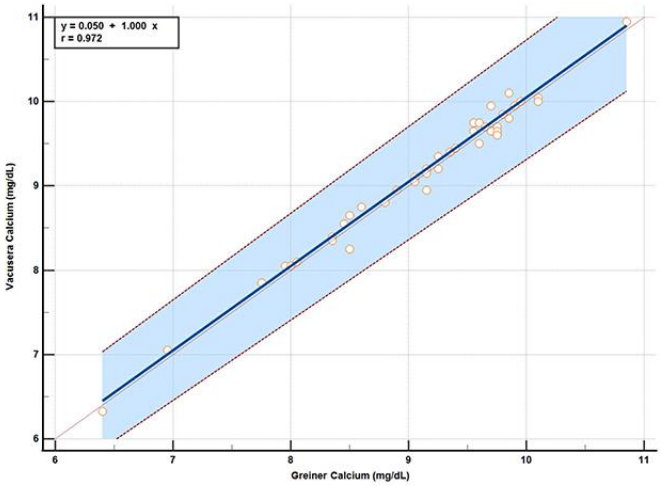

y = -0.327 + 1.005 x
r = 0.998
Vacusera CK (IU/L)
Greiner CK (IU/L)


y = 0.002 + 0.999 x
r = 0.995
Vacusera CRP (mg/L)
Greiner CRP (mg/L)

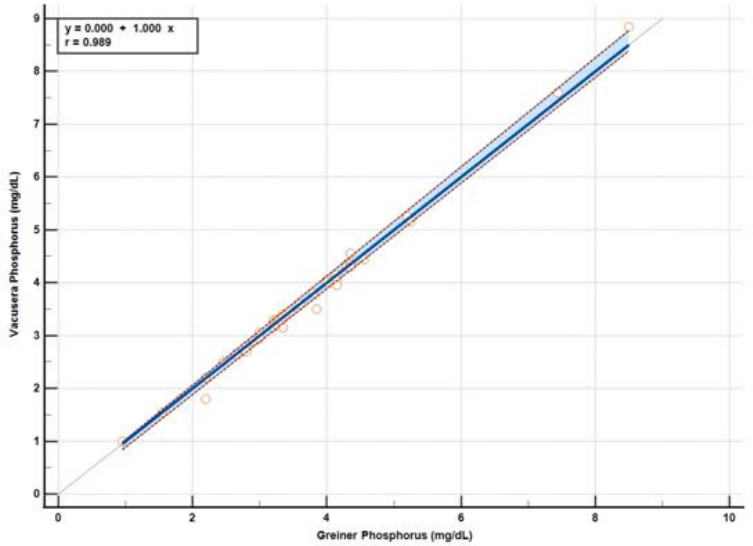

y = 4.546 + 0.966 x
r = 0.977
Vacusera Sodium (mmol/L)
Greiner Sodium (mmol/L)

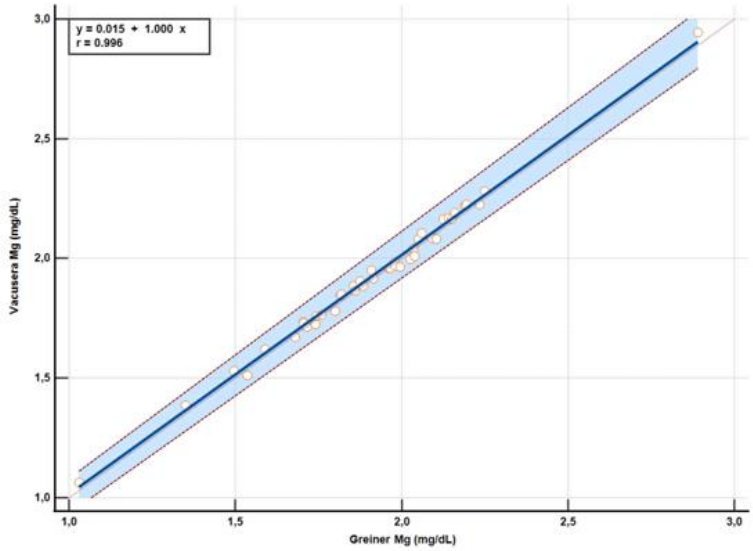

y = -0.025 + 1.000 x
r = 0.949
Vacusera Potassium (mmol/L)
Greiner Potassium (mmol/L)

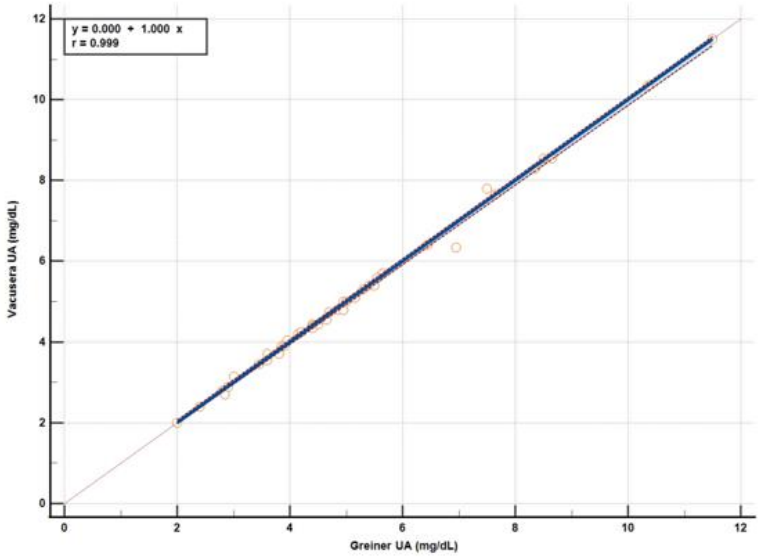

y = 1.448 + 0.986 x
r = 0.982
Vacusera Chloride (mmol/L)
Greiner Chloride (mmol/L)


y = 0.050 + 1.000 x
r = 0.972
Vacusera Calcium (mg/dL)
Greiner Calcium (mg/dL)

B.







C

Appendix 4. Passing-Bablok regression analysis for Vacusera CAT and Greiner Vacuette tubes.

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CK: Creatine kinase; CRP: C-reactive protein; Mg: Magnesium; TP: Total protein; UA: Uric acid.

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Research Article

Evaluation of free light chain measurement in EDTA and Lithium heparin plasma using the Diazyme assay

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Abstract

Objectives: Serum free light chain (FLC) measurement is widely used in the diagnosis and monitoring of monoclonal gammopathies. This study aimed to evaluate the analytical comparability of EDTA and lithium heparin plasma with serum for kappa and lambda FLC measurements using the Diazyme™ assay on the AU5800 analyzer.

Methods: Twenty paired patient samples submitted for routine FLC testing were included. For each case, serum, EDTA-anticoagulated plasma, and lithium heparin plasma (Barricor tube) specimens were collected during the same phlebotomy session and analyzed in parallel. Paired comparisons were performed using the Wilcoxon signed-rank test. Agreement among serum, EDTA plasma, and lithium heparin plasma samples was assessed using Passing–Bablok regression analysis and Bland–Altman plots.

Results: Kappa FLC concentrations measured in EDTA plasma did not differ from serum values ($p>0.05$). Lambda FLC concentrations in EDTA plasma showed a small but statistically significant difference compared with serum ($p=0.037$), while the kappa/lambda ratio was not significantly affected ($p>0.05$). In contrast, lithium heparin plasma demonstrated statistically significant differences from serum for kappa FLC ($p=0.044$), lambda FLC ($p<0.0001$), and the kappa/lambda ratio ($p=0.0004$). Lithium heparin plasma demonstrated broader limits of agreement and a substantially larger positive bias, particularly for lambda FLC.

Conclusion: Compared with lithium heparin plasma, EDTA plasma demonstrated closer agreement with serum for kappa FLC, lambda FLC, and the kappa/lambda ratio. These findings suggest that EDTA plasma may represent a more suitable alternative specimen matrix for FLC measurement using the Diazyme assay on the AU5800 platform, although confirmation in larger studies is warranted.

Keywords: Free light chain, kappa, lambda, plasma, serum

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Monoclonal paraproteins—commonly called M-proteins—are monoclonal immunoglobulins or light chains detected in the blood or urine. The production of excess monoclonal free light chains characterizes neoplastic monoclonal gammopathies. Therefore, the assay of free monoclonal light chains (FLCs) in serum is used as a marker in the diagnosis and monitoring of patients with plasma cell dyscrasias and other monoclonal gammopathies, and as an adjunct to serum protein electrophoresis, serving as a screening method for monoclonal gammopathies [1].

Monoclonal immunoglobulins are known to interfere with various laboratory methods, thereby affecting the reliability of analytical measurements and clinical interpretation. Studies have shown that, among cases with persistently abnormal gel separation, the majority belonged to patients with multiple myeloma [2]. High concentrations of paraproteins may compromise the separation of serum in gel-containing tubes due to increased viscosity or density, which impairs gel barrier formation during centrifugation [3]. Such separation failures may result in inadequate serum recovery or

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contaminated layers, rendering serum samples unsuitable for accurate FLC testing. This vulnerability of serum samples underscores the necessity of exploring alternative specimen types—such as EDTA-anticoagulated plasma or plasma collected in Barricor (mechanical barrier) tubes—that might circumvent these pre-analytical issues. While comparative studies have previously examined other FLC assay methods using alternative matrices (Freelite™ assays) [4], no published data currently exist evaluating the Diazyme™ serum FLC assay on the AU5800 analyzer using EDTA or Barricor plasma. This gap in the literature highlights the originality and importance of our work: assessing whether these alternative sample types can reliably replace serum for FLC quantification on the AU5800 system, potentially improving sample acceptance and laboratory workflow without compromising analytical integrity.

Materials and Methods

This prospective study was conducted over a two-week period in the Zonguldak Bülent Ecevit University Medical Biochemistry Laboratory. Residual patient specimens submitted for routine FLC testing were included in this study. Eligible patients required the availability of matched serum, EDTA plasma, and lithium heparin plasma specimens collected during the same venipuncture procedure. Accordingly, 20 patients were included, providing a total of 60 specimens for analysis. No disease-specific selection criteria were applied, and the study population reflected a routine clinical laboratory population rather than a dedicated hematology cohort.

Blood samples were collected into serum gel tubes (BD Vacutainer SST™ II Advance, 16×100 mm, 8 mL; REF: 367953), EDTA-K2 tubes (Vacusera, 13×100 mm, 2 mL; REF: 234602), and lithium heparin tubes with a mechanical separator (BD Vacutainer Barricor™ LH Plasma, 13×100 mm, 5 mL; REF: 365081). Samples were transported to the laboratory under routine operating conditions. Serum tubes were allowed to clot at room temperature before centrifugation. Subsequently, serum and EDTA plasma tubes were centrifuged at 1500×g for 10 minutes, whereas BD Barricor lithium-heparin plasma tubes were centrifuged at 4000×g for 3 minutes, all at room temperature. Following centrifugation, all specimens were stored at 2–8°C and analyzed within 8 hours of collection. No samples were stored overnight before analysis. Samples were excluded based on predefined pre-analytical quality criteria, including significant hemolysis, icterus, or lipemia as assessed by serum indices, the presence of fibrin, inadequate barrier formation, or insufficient sample volume.

Free light chain analysis was performed on a Beckman Coulter AU5800 analyzer (Beckman Coulter, Brea, CA, USA) in accordance with the manufacturer's instructions. Serum kappa and lambda FLC concentrations were measured using the Diazyme human kappa and lambda FLC assays. These assays are based on a latex-enhanced immunoturbidimetric principle, in which free kappa or lambda light chains in the sample bind to specific anti-kappa or anti-lambda antibodies coated onto latex particles. The resulting agglutination produces turbidity proportional to the concentration of free light chains in the sample. The study protocol was approved by the Zonguldak Bülent Ecevit University Ethics Committee (Approval No: 2025/16; Date: 17/09/2025). All study procedures were conducted in accordance with the ethical principles of the Declaration of Helsinki.

Statistical analysis

Statistical analysis was performed using MedCalc software (version 18.9.0; Ostend, Belgium). All data were checked for normal distribution using the Kolmogorov–Smirnov test. For paired comparisons between plasma and serum specimens, the Wilcoxon signed-rank test was applied. To assess the correlation between the tube types, Passing–Bablok regression and Spearman correlation analyses were performed. Bias was evaluated using Bland–Altman plots. Statistical significance was defined as $p<0.05$.

Results

The clinical and laboratory characteristics of the included samples are summarized in Table 1. None of the included patients had a known diagnosis of monoclonal gammopathy or were under active hematology follow-up at the time of sample inclusion. Among the 20 paired specimens included in the study, a subset of samples demonstrated elevated kappa and/or lambda free light chain concentrations relative to the assay reference intervals. Abnormal serum kappa/lambda ratios were observed in some patients, whereas others showed proportional elevations of both free light chains without alteration of the ratio classification.

Kappa FLC concentrations measured in EDTA plasma did not differ significantly from those obtained in serum ($p=0.430$). In contrast, lithium heparin plasma yielded slightly higher kappa FLC concentrations compared with serum ($p=0.044$). Lambda FLC concentrations measured in EDTA plasma were slightly higher than serum values ($p=0.037$), whereas lithium heparin plasma demonstrated a more pronounced difference ($p<0.0001$). The kappa/lambda ratio calculated from EDTA plasma did not differ significantly from that obtained

Table 1. Clinical and laboratory characteristics of the study population

Patient characteristics	n	%
Number of patients	20	
Elevated κ FLC without abnormal ratio	9	45
Elevated λ FLC without abnormal ratio	7	35
Abnormal serum κ/λ ratio	4	20
Patients with known monoclonal gammopathy	0	
Renal impairment	1	25
Inflammation, liver disease, infections	3	75

FLC: Free light chain.

Table 2. Comparison of kappa FLC, lambda FLC, and kappa/lambda ratio measurements obtained from serum, EDTA plasma, and lithium heparin plasma samples

Analyte	Serum median (IQR)	EDTA median (IQR)	p EDTA vs serum	Lithium heparin median (IQR)
Kappa	30.5 (21.3–73.5)	29.5 (20.8–74.9)	0.430	31.1 (22.1–79.1)
Lambda	21.0 (17.1–35.9)	21.8 (17.0–36.8)	0.037*	23.1 (18.1–39.9)
Kappa/Lambda	1.39 (1.09–1.68)	1.43 (1.04–1.65)	0.097	1.32 (0.99–1.62)

*: p<0.05 was considered statistically significant. Data are presented as median and interquartile range (IQR). Paired comparisons were performed using the Wilcoxon signed-rank test. P values refer to comparisons between each plasma matrix and serum. FLC: Free light chain; EDTA: Ethylenediaminetetraacetic acid; IQR: Interquartile range.

Table 3. Passing–Bablok regression analysis of free light chain measurements obtained from EDTA plasma and lithium heparin plasma compared with serum

Analyte	r	p	Intercept A	95% CI of intercept	Slope B	95% CI of slope
EDTA-Serum Kappa	0.998	<0.001	-0.18	-1.8412 to 0,9196	1.01	0.9800 to 1.0497
Li-Heparin-Serum Kappa	0.988	<0.001	1.15	-0.6783 to 2.1557	1.02	0.9632 to 1.0595
EDTA-Serum Lambda	0.986	<0.001	-0.17	-1.3188 to 1.8239	1.04	0.9588 to 1.0863
Li-Heparin-Serum Lambda	0.986	<0.001	-0.47	-3.0877 to 1.4599	1.11	1.0336 to 1.2216
EDTA-Serum Kappa/ Lambda	0.932	<0.001	0.01	-0.1599 to 0.1783	0.96	0,8582 to 1.0817
Li-Heparin-Serum Kappa/ Lambda	0.922	<0.001	-0.06	-0.1992 to 0.01811	1.02	0,9650 to 1.1152

R: Spearman correlation coefficient; CI: confidence interval. Passing–Bablok regression was used to evaluate agreement between plasma and serum measurements. Intercept A represents constant bias, whereas slope B represents proportional bias. Confidence intervals including 0 for the intercept and 1 for the slope indicate the absence of significant constant and proportional bias, respectively. EDTA: Ethylenediaminetetraacetic acid; CI: Confidence interval.

from serum (p=0.097). However, a significant difference was observed between lithium heparin plasma and serum-derived ratios (p=0.0004) (Table 2).

Passing–Bablok regression analysis showed a high degree of agreement between serum and both EDTA and lithium heparin plasma measurements. The corresponding regression parameters and 95% confidence intervals are summarized in Table 3.

Bland–Altman analysis showed a bias of 1.25% (95% limits of agreement: -1.72 to 4.23) for kappa FLC and 3.95% (95% limits of agreement: -0.06 to 7.96) for lambda FLC in EDTA tubes, and 3.08% (95% limits of agreement: -0.99 to 7.15) for kappa FLC and 12.5% (95% limits of agreement: -6.24 to 18.7) for lambda FLC in lithium heparin tubes (Fig. 1).

Discussion

In this study, we investigated the analytical comparability of EDTA and lithium heparin plasma with serum for the measurement of kappa and lambda FLC using the Diazyme™ immunoturbidimetric assay on the AU5800 analyzer.

Our findings indicate that EDTA-anticoagulated plasma provides FLC measurements that are largely comparable to those obtained from serum when analyzed using the Diazyme assay on the AU5800 platform. Although a small statistically significant difference was observed for lambda FLC, the magnitude of bias remained low, and agreement analyses demonstrated minimal systematic deviation across the evaluated concentration range. Importantly, the kappa/lambda ratio, which frequently guides the clinical interpretation of FLC results, did not differ significantly between EDTA plasma and serum. These findings are consistent with a previous study evaluating alternative specimen types for FLC assays, which reported acceptable analytical agreement between serum and plasma matrices [4].

In contrast, lithium heparin plasma yielded higher lambda FLC concentrations and a corresponding shift in the kappa/lambda ratio compared with serum. The greater magnitude of bias and reduced agreement suggest a matrix-dependent effect associated with lithium heparin. Similar specimen- and anticoagulant-related effects have been reported for immunochemical protein measurements, including nephelometric and latex immunoturbidimetric assays [5, 6]. A matrix effect has also been demonstrated for the Freelite assay, particularly at low analyte concentrations, where differences between serum and saline-based matrices affected both lambda FLC measurements and the derived kappa/lambda ratio [7]. These findings support the concept that FLC assays may be sensitive to changes in sample composition and matrix characteristics. Although the underlying mechanism in the present study may differ, the greater bias observed for lambda FLC in lithium heparin plasma suggests that matrix-dependent effects can influence FLC quantification and should be considered when alternative specimen types are used.

Study limitations

Several limitations of this study should be acknowledged. First, the sample size was relatively small and derived from a routine clinical laboratory population rather than a disease-specific cohort. In addition, extreme free light chain concentrations were underrepresented, which may limit the generalizability of the findings to samples with markedly el-

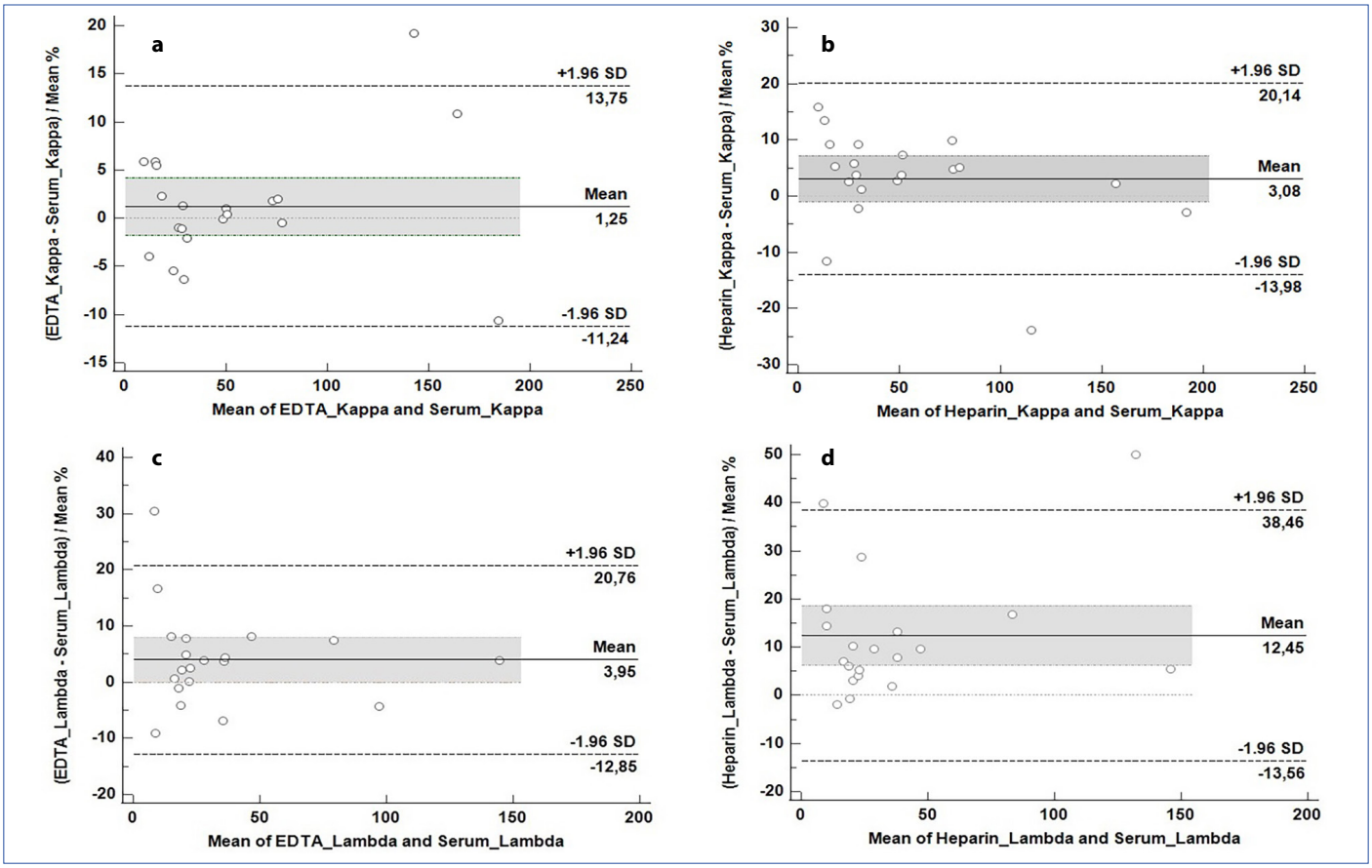

Figure 1. Bland–Altman plots showing agreement between serum and EDTA plasma for kappa FLC (A) and for lambda FLC (C) and between serum and lithium heparin plasma (for kappa FLC (B) and for lambda FLC (D). The solid horizontal line represents the mean percentage bias, and the dashed lines represent the 95% limits of agreement.

evated analyte levels. None of the included patients had a known diagnosis of monoclonal gammopathy or were under active hematological follow-up at the time of sample inclusion. Finally, the study was designed as a sample matrix comparison study rather than a formal analytical performance verification study; therefore, total analytical error calculations based on biological variation-derived performance specifications were not performed. Nevertheless, the paired-sample design, standardized pre-analytical handling, and the use of complementary statistical approaches strengthen the validity of the observed comparisons.

Conclusion

In conclusion, EDTA plasma showed good analytical agreement with serum for the measurement of kappa and lambda free light chains using the Diazyme assay on the AU5800 platform, with no significant difference observed in the kappa/lambda ratio. In contrast, lithium heparin plasma demonstrated greater deviations, particularly for lambda FLC concentrations and the derived kappa/lambda ratio. These findings suggest that EDTA plasma may be a suitable alternative specimen type for Diazyme FLC testing when serum is unavailable, whereas results obtained from lithium heparin plasma should be interpreted with caution. Further studies are needed to confirm these findings in larger and clinically diverse patient populations.

Disclosures

Ethics Committee Approval: The study was approved by the Zonguldak Bülent Ecevit University Clinical Research Ethics Committee (no: 2025/16, date: 17/09/2025).

Informed Consent: Informed consent was not obtained because the study was conducted using residual samples remaining after routine clinical testing.

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Review

Flow cytometry: From cell counting to high-dimensional cytometry

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Abstract

Flow cytometry has evolved from early photoelectric cell-counting methods into a sophisticated, high-dimensional single-cell platform that is indispensable in biomedical research and clinical laboratories. Its historical development reflects the integration of diverse technological modalities, including hydrodynamic focusing, light scattering, fluorescence, monoclonal antibody technology, laser optics, digital electronics, and computational analysis. Conventional flow cytometry remains a cornerstone of immunophenotyping, the diagnosis of hematological malignancies, measurable residual disease assessment, transplantation immunology, selected infectious disease immune monitoring, and translational drug and vaccine development. Technological advances, including spectral flow cytometry, imaging flow cytometry, mass cytometry, microfluidic platforms, large-particle cytometry, and nanoparticle flow cytometry, have expanded its analytical scope beyond intact cells to include organoids, extracellular vesicles, and complex immune environments. The current challenge lies not only in acquiring multiparametric data but also in ensuring the reproducible design, validation, interpretation, reporting, and clinical integration of these datasets. Future developments are expected to prioritize full-spectrum acquisition, artificial intelligence-supported automated analysis, miniaturization for point-of-care use, standardized quality management, and the integration of multimodal approaches with genomic, proteomic, metabolomic, and imaging techniques. This review summarizes the past, present, and future of flow cytometry, highlighting its system architecture, analytical workflows, current platforms, clinical and research applications, quality requirements, limitations, and emerging role in precision diagnostics.

Keywords: Flow cytometry, immunophenotyping, single-cell analysis, spectral flow cytometry

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Flow cytometry is an advanced analytical method that enables rapid, high-throughput, multiparametric evaluation of the physical and biological properties of cells and particles at the single-cell level. Unlike conventional biochemical assays, which average signals from heterogeneous populations, flow cytometry records each individual cell or particle and therefore provides a more detailed analysis. This capacity for discrete event analysis enables heterogeneous samples to be decomposed into biologically and clinically significant subpopulations, facilitating the detection, quantification, and phenotypic characterization of rare populations [1–3].

To understand the analytical capabilities and historical development of flow cytometry, it is first necessary to delineate the system architecture and signal-generation principles that transform a cellular suspension into quantitative multiparametric data.

Principles, System Architecture, and Signal Generation in Flow Cytometry

The analytical power of flow cytometry depends on the coordinated operation of fluidic, optical, detection, electronic, and software-based signal-processing systems (Fig. 1). The flu-

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idic system transports the sample to the interrogation points and aligns the cells in a single file. This is typically achieved through laminar sheath flow and hydrodynamic or acoustic focusing. Controlled alignment is essential because reliable signal generation requires each cell to intersect the excitation beam individually and reproducibly. Consequently, disturbances in sample quality, cell aggregation, flow stability, pressure regulation, or nozzle geometry can directly affect event rate, coincidence, sensitivity, and data quality [4, 5].

The optical system provides excitation and signal-collection capabilities. Lasers with well-defined wavelengths, typically ranging from violet to near-infrared, excite endogenous fluorophores and fluorochrome-conjugated probes. Forward scatter is commonly used as a relative indicator of cell size, whereas side scatter reflects internal complexity or granularity. Fluorescence signals indicate the presence or intensity of surface antigens, intracellular proteins, nucleic acids, viability dyes, enzyme substrates, calcium indicators, cytokines, phosphoproteins, and other functional probes. The efficiency with which emitted light is separated and transmitted to appropriate detection channels is determined by optical components, including lenses, collection optics, dichroic mirrors, band-pass filters, optical fibers, and detector arrays [4–6].

The detection and electronic modules convert optical signals into digital data. Photomultiplier tubes, photodiodes, avalanche photodiodes, and other detector technologies transform scattered light or fluorescence signals into electrical pulses. These pulses are then amplified, filtered, digitized, and stored as list-mode data. Pulse area, height, and width provide information that can be used to quantify signal intensity, discriminate doublets, and improve the reliability of downstream gating. Consequently, the final dataset is not an image of a cell but a structured digital representation of multiple physical and fluorescence-derived variables measured for every event [1–6].

A distinguishing feature of flow cytometry is its capacity to integrate cellular phenotypes and functions within a unified analytical framework. A carefully designed panel used in a single tube can combine multiple biological markers, including lineage indicators, maturation markers, activation antigens, intracellular transcription factors, viability stains, proliferation tracers, cytokines, and signaling readouts. This multiparametric structure is particularly valuable in immunology, hematology, oncology, transplantation, infectious diseases, cardiovascular research, cell and gene therapy, vaccine development, and translational biomarker discovery. The method is compatible with various biological samples,

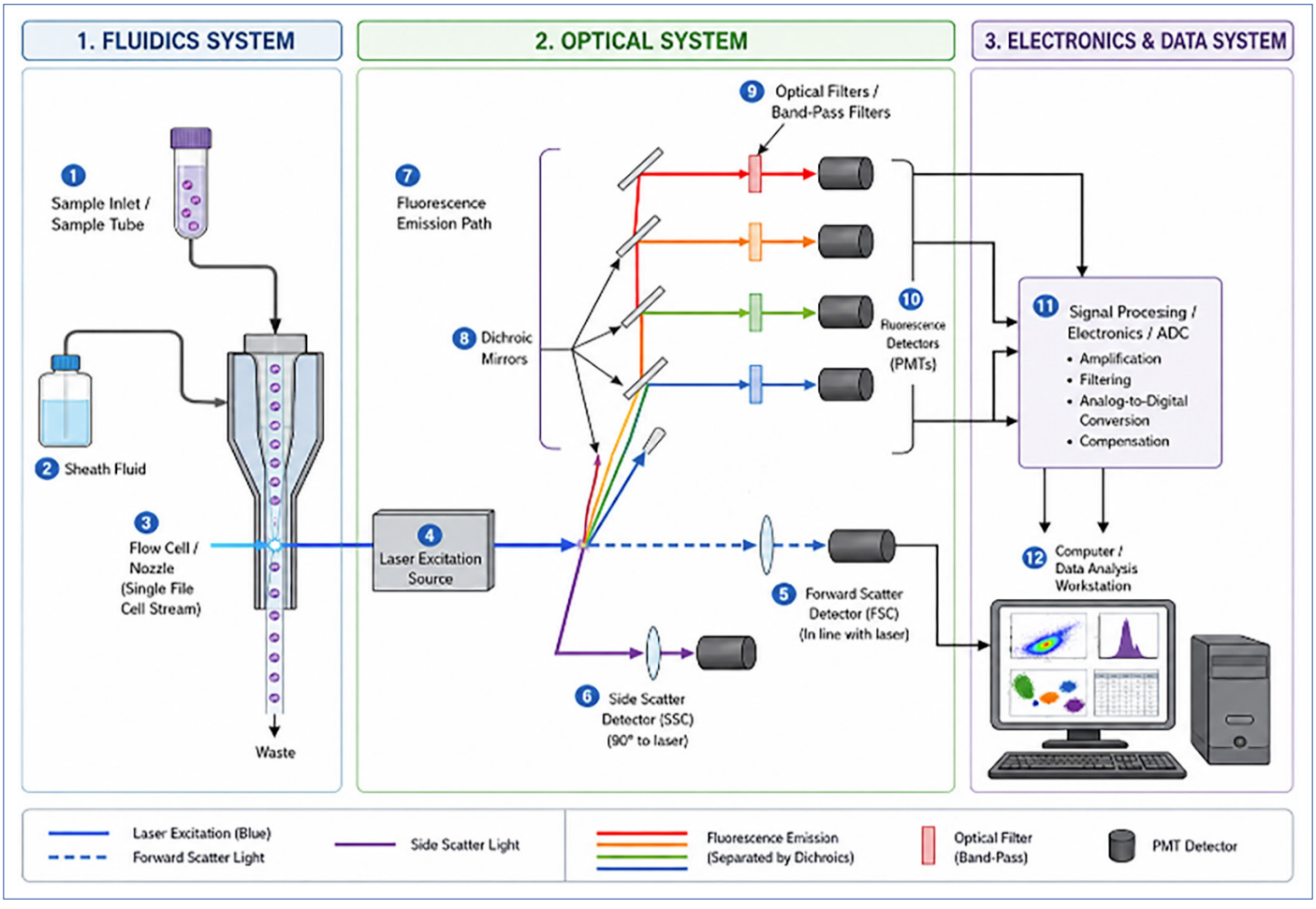

Figure 1. Main components and signal-generation pathways of a flow cytometer.

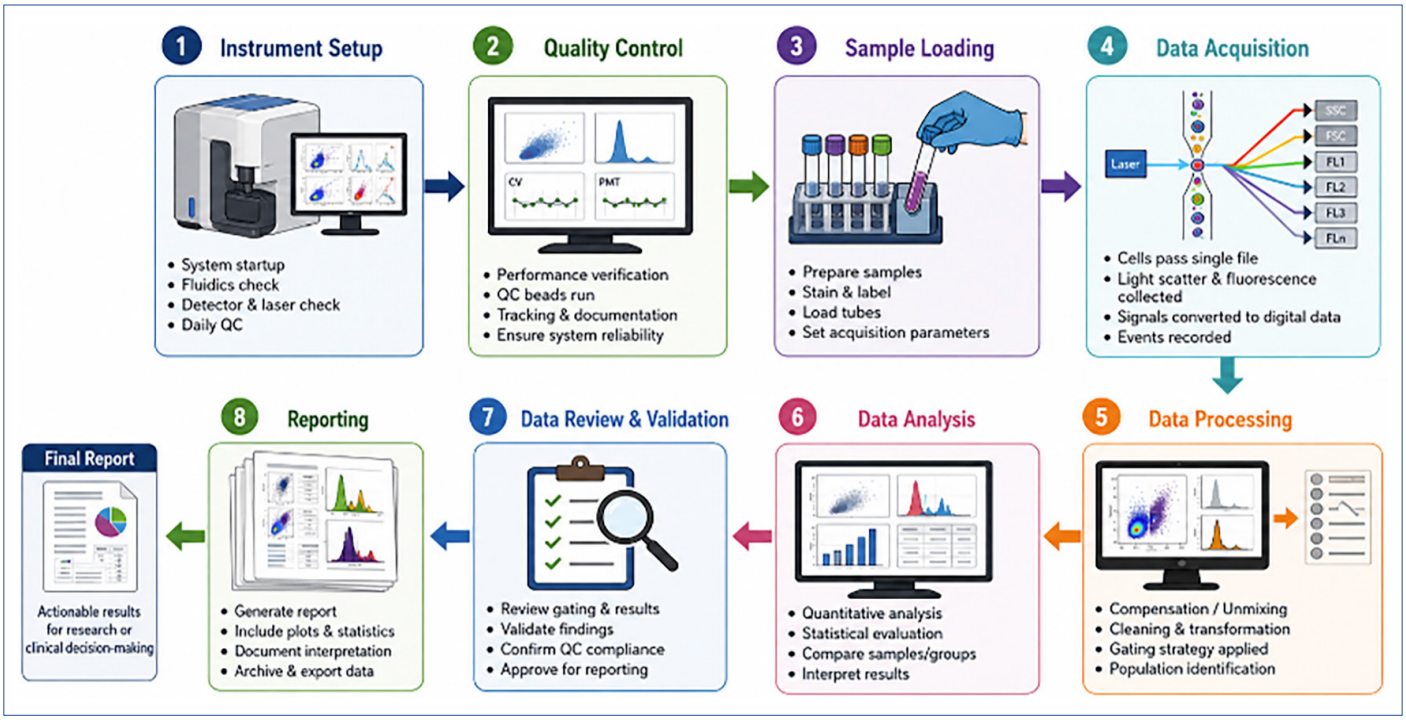

Figure 2. Workflow of flow cytometric analysis from instrument setup to reporting.
FL: Fluorescence channels; FSC: Forward scatter; PMT: Photomultiplier tube; QC: Quality control; SSC: Side scatter.

including peripheral blood, bone marrow, body fluids, cultured cells, tissue-derived single-cell suspensions, microorganisms, extracellular vesicles, and other particles within the analytical limits of the instrument [1, 7, 8].

Flow cytometry generates high-dimensional, event-based data, and its interpretation requires a structured analytical workflow. To convert raw signals into biologically and clinically meaningful results, several processes must be implemented, including gating, compensation, spectral unmixing, data transformation, quality control review, and expert interpretation.

Workflow and Analytical Process

Flow cytometric analysis follows a sequential workflow that begins with instrument setup and ends with clinically or experimentally interpretable reporting. Before sample acquisition, the instrument should undergo preparatory procedures, including system startup, fluidics inspection, laser and detector verification, and daily quality control. This step ensures that the optical, fluidic, and electronic components function within acceptable analytical limits. Quality control (QC) is then performed using standardized QC beads or equivalent reference materials to verify instrument performance, monitor detector sensitivity, assess coefficients of variation, and document day-to-day stability (Fig. 2) [1, 7, 9].

After instrument readiness is confirmed, prepared and stained samples are loaded into the cytometer. During acquisition, cells are hydrodynamically focused and pass through the laser interrogation point in single file. Each cell generates three main signal types: forward scatter, side scatter, and fluorescence. These signals are determined by the physical characteristics of the cell and by the specific fluorochrome-labeled markers present. The optical signals are detected, converted into electronic pulses, digitized, and recorded as distinct events. The resulting dataset is then processed through compensation or spectral unmixing, signal transformation, exclusion of debris, doublets, and nonviable cells, and application of predefined gating strategies [1, 7, 10, 11].

Following data processing, quantitative analysis is performed to identify target populations, determine marker expression, calculate frequencies or absolute counts, and compare samples or experimental groups when required. Analytical findings must then be reviewed and validated by assessing gating accuracy, result consistency, QC compliance, and biological or clinical plausibility. In the final step, validated results are reported with appropriate plots, numerical data, interpretative comments, and relevant methodological information. Data files and analysis outputs are archived or exported in accordance with established laboratory policy. This structured workflow integrates instrument performance, sample quality, data acquisition, analysis, validation, and reporting, thereby ensuring reproducible and clinically reliable results. From a laboratory medicine perspective, the reliability of results also depends on multiple preanalytical variables, including anticoagulant selection, specimen transport conditions, the interval between collection and analysis, sample preparation, and antibody titration [1, 7, 10–13].

With this operational framework in mind, the historical development of flow cytometry can be understood as a stepwise

Table 1. Selected milestones in the development of flow cytometry

Era	Representative developments	Conceptual impact
1930–1960s	Photoelectric cell counting, fluorescent antibody methods, impedance-based cell sizing, hydrodynamic focusing	Established the principles of single-particle alignment, optical/electrical detection, and antigen-specific labeling
1960–1970s	Cell spectrophotometry, electronic separation, laser illumination, fluorescence-activated cell sorting	Converted flow-based counting into analytical and preparative cytometry
1980–1990s	Commercial multi-laser instruments, multicolor immunophenotyping, computerized acquisition	Enabled routine clinical and research use
2000–2010s	Violet lasers, tandem dyes, high-parameter panels, digital electronics, early spectral concepts	Expanded immune profiling and rare-population detection
2020s and beyond	Full-spectrum cytometry, AI-supported analysis, microfluidics, nanoparticle cytometry, multimodal single-cell workflows	Moves cytometry toward precision medicine and integrated diagnostics

improvement in the same core domains: fluidic alignment, optical interrogation, signal detection, cell sorting, and computational data analysis.

Past: From Cell Counting to High-dimensional Cytometry

The conceptual origins of flow cytometry can be traced to the early twentieth-century need to count and classify cells in suspension rapidly and objectively (Table 1). In 1934, Moldavan described a photoelectric technique for enumerating microscopic cells, introducing the concept that cells could be aligned in a fluid stream and detected in succession [14]. In the 1940s, photoelectronic counters for colloidal particles demonstrated the feasibility of using light-scattering signals to detect and classify particles in a fluid environment [15]. Concurrently, the fluorescent antibody methods established by Coons and Kaplan laid the immunochemical foundation for antigen-specific cell identification [16]. The Coulter principle, based on electrical impedance fluctuations generated by particles traversing an aperture, introduced another analytical approach for cell counting and sizing and directly influenced the automation of hematology [17].

A further milestone was Crosland-Taylor's development of controlled particle alignment along a defined optical axis, which established an important fluidic basis for reproducible optical interrogation [18]. This was a significant technical advance. In the 1960s, rapid cell spectrophotometry and electronic cell separation shifted flow-based measurements from simple counting to more sophisticated analytical frameworks. Kamentsky et al. [19] introduced ultrarapid spectrophotometric cell analysis, enabling the measurement of spectrophotometric properties and nucleic acid-related cellular characteristics at the single-cell level. Fulwyler subsequently demonstrated the electronic separation of biological cells by volume, while Sweet's electrostatic deflection principle provided an important engineering basis for droplet-based sorting [20, 21]. These advances contributed to the transition from cell counting to analytical and preparative cytometry.

The 1970s were a period of major transformation driven by the convergence of analysis and sorting. Bonner et al. [22] described fluorescence-activated cell sorting (FACS), which integrated the optical recognition of labeled cells with droplet formation and electrostatic deflection. The ability to identify, isolate, and recover viable subpopulations transformed flow cytometry from a descriptive analytical technique into an experimental platform. Subsequently, Herzenberg et al. [23] popularized the term "FACS" as a method for isolating immunologically defined lymphocyte subsets. The advent of two-color immunofluorescence and early multiparameter systems enabled researchers to perform simultaneous measurements of cell lineage and functional markers [24].

From the late 1970s through the early 1990s, major advances in instrumentation and technology made flow cytometry widely accessible in clinical and research laboratories. These developments included commercial instruments, argon lasers, advanced photomultiplier tubes, improved optical filters, and computerized data acquisition systems. Multilaser instruments increased panel complexity, while digital data handling enabled more reproducible compensation, gating, and storage of list-mode data. The subsequent introduction of violet lasers, tandem dyes, quantum dot nanocrystals, and increasingly bright fluorochromes supported 11-color, 17-color, and higher-parameter immunophenotyping. In this historical arc, flow cytometry evolved from a cell-counting method into a multifaceted tool for immune system mapping, cancer diagnosis, rare-population detection, and therapeutic guidance [24–27].

Present: Flow Cytometry Platforms, Applications, and Challenges

Contemporary flow cytometry encompasses a diverse range of analytical platforms, driven by advances in fluidics, laser optics, fluorochrome chemistry, detector technology, digital electronics, and computational data analysis. Despite differences in optical design, labeling strategy, detection principle, throughput, and data architecture, these platforms share a common analytical logic: the interrogation of single cells or particles and the transformation of optical, fluorescence-based, image-based, or mass-based signals into quantitative, multiparametric data [2–8, 27, 28].

This technological diversity is best conceptualized as a flow cytometry ecosystem rather than as a collection of isolated

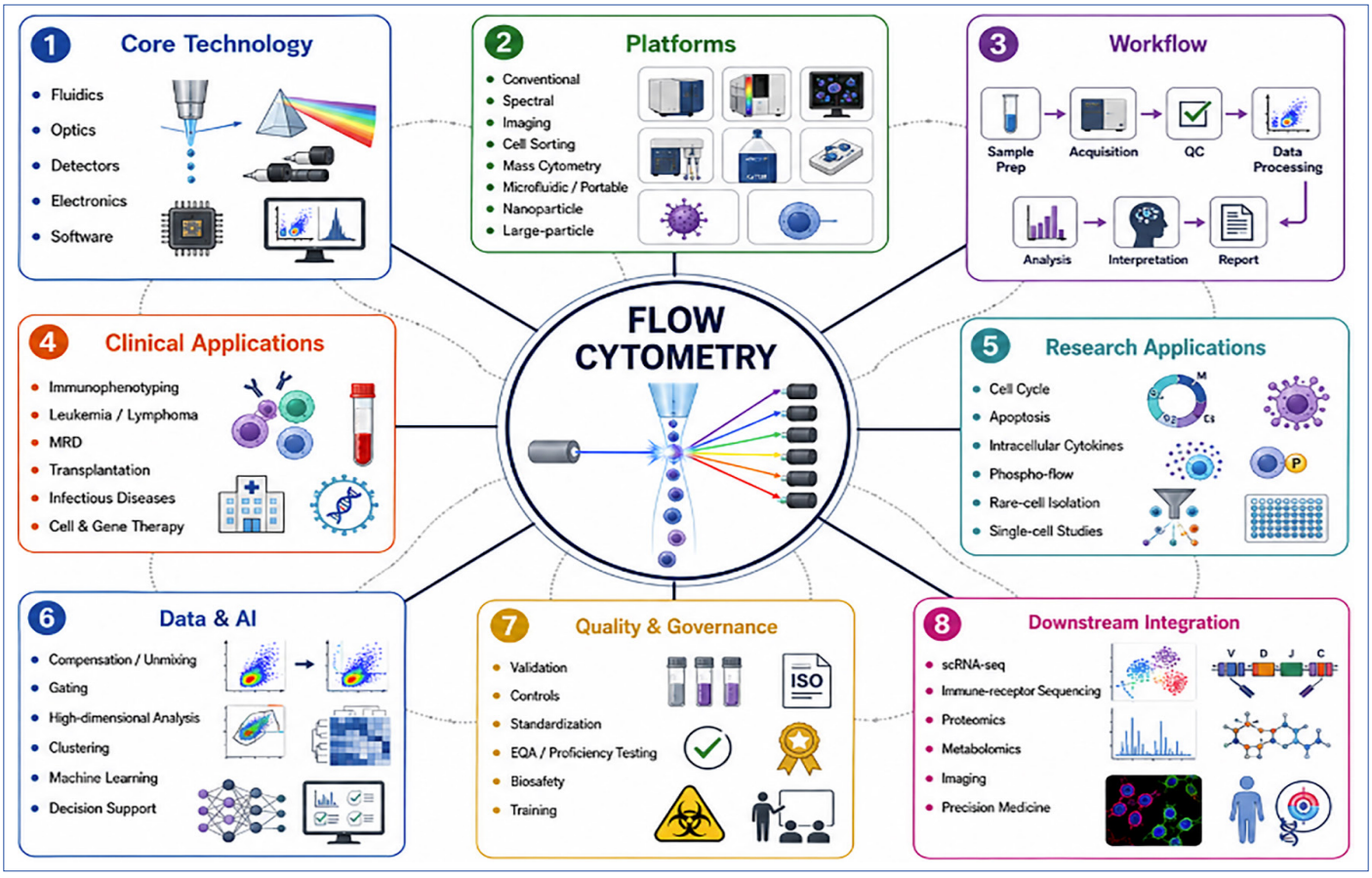

Figure 3. Flow cytometry ecosystem.

AI: artificial intelligence; EQA: external quality assessment; ISO: International Organization for Standardization; MRD: measurable residual disease; QC: quality control; scRNA-seq: single-cell RNA sequencing.

instruments. As shown in Figure 3, contemporary flow cytometry integrates core technological components, analytical platforms, standardized workflows, clinical and translational applications, research assays, computational analysis, quality management, and downstream multimodal approaches. Within this ecosystem, instrument architecture, sample preparation, data acquisition, interpretation, validation, biosafety, training, and integration with single-cell and multi-omics technologies are closely interconnected. Accordingly, the current role of flow cytometry extends beyond signal acquisition and requires the coordinated use of platform-specific capabilities within clinically and biologically meaningful analytical frameworks [7, 27].

Current flow cytometry platforms and technologies

Conventional flow cytometry: The clinical backbone

Conventional flow cytometry remains the most widely used platform in clinical and research laboratories worldwide (Table

Table 2. Current cytometry platforms, major strengths, and limitations

Platform	Current strength	Main limitation
Conventional flow cytometry	Robust, widely available, clinically familiar	Compensation, spectral overlap, limited panel complexity
Spectral flow cytometry	High-dimensional panels and autofluorescence extraction	Unmixing complexity and reference quality requirements
Cell sorting	Viable isolation of defined populations	Biosafety, aerosol control, operator dependence
Imaging flow cytometry	Morphology plus phenotype in high throughput	Large data volume and analysis complexity
Microfluidic/portable cytometry	Low sample volume and point-of-care potential	Lower multiplexing and sensitivity in many systems
Mass cytometry	High multiplexing without fluorescence overlap	Destructive analysis and no live sorting
Nanoparticle cytometry	Extracellular vesicle and nanoscale particle analysis	Calibration, background, and standardization difficulties

2). Cells are labeled with fluorochrome-conjugated antibodies or probes, hydrodynamically focused, and excited by one or more lasers. Detectors and band-pass filters capture forward scatter, side scatter, and fluorescence signals. Conventional instruments commonly support multicolor immunophenotyping, cell cycle assessment, apoptosis assays, proliferation monitoring, intracellular cytokine staining, phosphoprotein analysis, and routine clinical diagnostic workflows. Owing to its robustness, accessibility, and established interpretive frameworks, conventional flow cytometry remains the foundation of diagnostic immunophenotyping and many translational applications [2–8].

Spectral flow cytometry and high-dimensional immunophenotyping

spectral flow cytometry is one of the most important recent advances in this field. Instead of assigning emitted light to a limited number of filtered channels, spectral systems collect the complete emission fingerprint across a broad wavelength range and use reference spectra and mathematical unmixing to resolve fluorochromes. This strategy enhances panel flexibility, facilitates more precise discrimination of fluorochromes with overlapping emission spectra, and enables autofluorescence to be treated as a distinct spectrum. Spectral flow cytometry allows higher-dimensional immunophenotyping, particularly in immune monitoring, rare-population detection, tumor microenvironment analysis, and clinical panels requiring the simultaneous measurement of multiple lineage and functional markers [28–30].

Cell sorting cytometry: From analysis to isolation

Cell sorting extends cytometric analysis from measurement to the physical isolation of specific cell populations. In droplet-based sorters, cells undergo optical analysis, are enclosed in droplets, charged according to predefined gates, and electrostatically deflected into collection tubes or plates. High-purity sorting is used in stem cell biology, functional immunology, single-cell sequencing workflows, clustered regularly interspaced short palindromic repeats (CRISPR) screening, tumor cell isolation, and manufacturing-related processes in cell and gene therapy. However, this process requires careful attention to biosafety, sterility, aerosol containment, viability preservation, instrument optimization, and operator expertise [9, 13, 31].

Imaging, microfluidic, and portable cytometry

Imaging flow cytometry combines quantitative analysis with microscopic imaging. It acquires high-throughput images of cells together with fluorescence and scatter measurements. This methodological framework enables the analysis of cellular phenomena such as cell shape, nuclear morphology, apoptosis, mitosis, internalization, nuclear translocation, cell-cell interaction, and subcellular localization. Its value is greatest when intensity alone is insufficient and the spatial distribution of the signal has biological significance [32, 33].

Concurrently, microfluidic cytometry and magnetic or impedance-based cytometric systems have emerged as compact platforms for low-volume analysis and point-of-care testing. These systems are attractive for focused immune monitoring, infectious disease applications, emergency settings, and resource-limited environments. However, compared with central laboratory cytometers, many remain limited in parameter number, optical sensitivity, throughput, and standardization [34–37].

Large-particle, mass, and nanoparticle cytometry

Several contemporary and emerging platforms have extended cytometry beyond conventional single-cell immunophenotyping. Large-particle cytometry enables the analysis and sorting of large cells, adipocytes, and model organisms in the micrometer-to-millimeter range [38, 39]. Mass cytometry replaces fluorochromes with metal isotope tags and uses time-of-flight mass spectrometry after inductively coupled plasma ionization. Its primary advantage is the avoidance of fluorescence spectral overlap, enabling highly multiplexed single-cell proteomic profiling [40, 41]. Recent advances, such as nanoparticle flow cytometry and high-sensitivity scatter/fluorescence approaches, have enabled the analysis of extracellular vesicles and other nanoscale particles, which had previously been difficult to examine using conventional cytometers [2–7, 42]. Collectively, these platforms illustrate the progression of cytometry from immunophenotyping to the broader fields of single-cell and single-particle science.

This technological diversity forms the basis for the broad clinical, translational, and research applications of flow cytometry.

Clinical and translational applications

The most mature clinical application of this technology is immunophenotyping [42, 43]. Flow cytometry defines hematopoietic and immune cell populations using combinations of cluster of differentiation (CD) antigens, intracellular markers, maturation patterns, and functional readouts. In routine laboratories, CD45-based gating and lineage markers such as CD3, CD4, CD8, CD19, CD20, CD16/56, CD14, and CD34, together with disease-specific aberrant patterns, support diagnosis, classification, and monitoring. Clinical value is greatest when panel design, antibody titration, sample processing, instrument setup, compensation or unmixing, gating strategy, and interpretive reporting are harmonized [1–7, 43].

The diagnosis of hematological malignancies illustrates the diagnostic power of multiparameter flow cytometry (MFC). In acute leukemias, chronic lymphoproliferative disorders, plasma cell neoplasms, and lymphomas with accessible cell suspensions, flow cytometry helps define lineage, maturation arrest, aberrant antigen expression, and clinically relevant subclones [45–50]. Measurable residual disease (MRD) assessment requires much higher sensitivity than diagnostic immunophenotyping and depends on large-event acquisition, validated antibody panels, carefully controlled backgrounds, standardized analyses, and expertise in distinguishing regen-

erating hematopoiesis from residual neoplastic cells. In acute leukemia and multiple myeloma, MRD detection by flow cytometry is increasingly used to evaluate response depth, refine relapse risk, and support therapeutic decisions [51, 52].

Flow cytometry is also becoming a practical tool for cell and gene therapy, where identity, purity, viability, potency-related markers, activation state, and contaminating populations must be measured during development, release testing, and post-infusion monitoring [53]. Targeted therapies have created new opportunities and challenges. Therapeutic monoclonal antibodies can alter target antigen assessment or immune-cell profiles, whereas chimeric antigen receptor T-cell (CAR-T-cell) therapy requires assays that document target antigen expression, CAR-T expansion, persistence, exhaustion, and residual malignant populations [54, 55].

In solid tumors, flow cytometry contributes to the analysis of tumor-infiltrating lymphocytes, regulatory T cells, myeloid-derived suppressor cells, macrophage phenotypes, circulating tumor cells, and extracellular vesicles [8,42]. Although tissue dissociation can alter marker expression and cause loss of spatial information, multiparameter cytometry provides functional and phenotypic details that complement histopathology and spatial methods. Tumor-derived exosomes and programmed death-ligand 1 (PD-L1)-positive extracellular vesicles are increasingly being investigated as mediators of immune escape and potential liquid biopsy biomarkers [56].

Additional clinical applications include transplantation-related immune-cell phenotyping, infectious disease immune monitoring, and cardiovascular or thromboinflammatory research [57–59]. In transplantation, flow cytometry can characterize immune-cell phenotypes and cytokine-production profiles associated with rejection-related immune states [57]. In infection-related workflows, cytometry can support selected immune-monitoring applications, particularly CD4+ T-cell assessment and experimental host-response assays [58]. In cardiovascular research and emerging diagnostics, platelet activation, platelet-leukocyte aggregates, and related thromboinflammatory markers are measurable by flow cytometry, although broader clinical standardization is still needed [59].

Research applications beyond routine phenotyping

In research laboratories, flow cytometry is valued because it can connect phenotypes with cellular behavior. Cell cycle analysis uses DNA-binding dyes such as propidium iodide, 4′,6-diamidino-2-phenylindole (DAPI), 7-aminoactinomycin D (7-AAD), and Hoechst dyes to assign cells to the G0/G1, S, and G2/M phases and to detect aneuploidy, sub-G1 events, or treatment-induced changes in proliferative status. Dye-dilution assays using carboxyfluorescein succinimidyl ester (CFSE), cell trace violet, or related probes follow generational proliferation because fluorescence intensity is halved with each cell division. These methods are widely used in T-cell activation studies, vaccine research, stem cell biology, cancer pharmacology, and toxicology [13, 60].

Functional immune monitoring has become a defining research application. Intracellular cytokine staining identifies antigen-specific or stimulus-induced cytokine production at single-cell resolution, whereas phospho-flow cytometry measures rapid activation of signaling pathways such as the Janus kinase–signal transducer and activator of transcription (JAK-STAT) pathway, mitogen-activated protein kinase (MAPK) pathway, phosphoinositide 3-kinase–protein kinase B (PI3K-AKT) pathway, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and T-cell receptor pathways. These approaches are especially useful when small functional subsets are masked in bulk assays. They support vaccine immunogenicity studies, autoimmune and inflammatory disease research, immunotherapy monitoring, and early drug development, where mechanism of action, target engagement, and off-target effects must be assessed before large clinical studies [13, 42].

Apoptosis and cell death assays illustrate the advantages of multiparametric analysis. Annexin V binding identifies externalized phosphatidylserine, viability dyes distinguish membrane integrity, mitochondrial dyes measure membrane potential, and intracellular markers can capture caspase activation or DNA fragmentation. By combining these markers with lineage or activation antigens, researchers can determine which cell subset is dying, at which stage, and in response to which stimulus. Imaging flow cytometry adds morphological confirmation, allowing apoptosis, necrosis, mitosis, and nuclear translocation events to be discriminated within the same analytical framework [32, 61–63].

Further research applications include the generation of purified or index-linked cellular material for downstream assays. Sorting rare populations, depositing single cells into plates, and index sorting before sequencing permit a direct link between cytometric phenotype and transcriptomic or genomic profile [64, 65]. This bridge is increasingly important because flow cytometry can rapidly enrich biologically meaningful populations, whereas sequencing technologies can define transcriptional programs, clonal architecture, and receptor repertoire. The most informative studies will be those in which cytometry is used not as an isolated measurement but as part of a planned single-cell experimental ecosystem that includes other techniques. The expansion of flow cytometry into clinical diagnostics, translational studies, and high-dimensional research applications has made quality assurance, standardization, and reproducible data interpretation central requirements in this field [1–7].

Quality, standardization, and data interpretation

the central challenge of contemporary cytometry no longer lies in parameter generation alone but in producing interpretable, comparable, and clinically actionable results. Preanalytical variation is influenced by numerous factors, including specimen type, anticoagulant, transport time, ambient temperature, cell viability, red-cell lysis, fixation conditions, stimulation conditions, and sample stability. Analytical vari-

ation encompasses laser alignment, detector performance, voltage or gain settings, compensation or unmixing matrix quality, antibody clone and fluorochrome selection, lot-to-lot variation, reagent titration, nonspecific binding, autofluorescence, spectral spread, doublets, debris, and rare-event statistics [13, 44, 66–68].

Panel design has evolved into a distinct discipline. Bright fluorochromes should be used for dim or critical antigens, whereas highly expressed markers can be paired with less bright fluorochromes. Spreading error and autofluorescence should be minimized, and controls should match the analytical question. Appropriate controls include single-stain controls, fluorescence-minus-one controls, biological controls, viability dyes, doublet discrimination, and standardized gating templates [65]. In spectral flow cytometry, high-quality single-stain references and verification of unmixing performance are essential [28, 66]. For MRD and rare-event assays, the lower limit of detection depends on event number, background, sample quality, and analytical reproducibility rather than solely on the theoretical sensitivity of the instrument [51, 52, 68].

Clinical implementation requires rigorous validation procedures to ensure reliability and relevance. Each laboratory must define the intended use, specimen requirements, reportable populations, analytical sensitivity and specificity when applicable, precision, carryover, interference, stability, reference or decision intervals, and acceptance criteria. Standard operating procedures should include instrument setup, daily quality control, reagent management, gating hierarchy, review process, result reporting, and corrective actions. External quality assessment (EQA) and interlaboratory harmonization (ILH) are particularly important because the clinical significance of a cytometric result depends on both numerical output and expert interpretation [3, 65, 68].

Limitations and unresolved problems

Several important limitations remain despite the analytical strength of flow cytometry. The method requires single-cell or single-particle suspensions. Consequently, tissue architecture and spatial context are partially compromised unless the process is integrated with imaging or spatial technologies. Enzymatic dissociation can alter surface antigen expression, selectively lose fragile populations, or induce activation artifacts. Preanalytical handling of fragile cells, platelet-derived particles, extracellular vesicles, and bacteria must be specialized, as protocols optimized for leukocytes cannot simply be transferred to nanoscale or microbial targets [3, 67, 69].

High-dimensional cytometry creates a series of interpretive challenges. Manual gating is transparent and clinically familiar; however, its performance depends on operator expertise and may not be optimal for rare populations or complex panels [70]. Unsupervised algorithms can reveal hidden structures, but they may also generate clusters that are statistically distinct yet clinically irrelevant. Batch effects, spectral spread, compensation errors, inconsistent controls, and insufficient event numbers can generate patterns that appear biological but are technical in origin. Consequently, computational analysis must be integrated with experimental design, biological validation, and clinically anchored interpretation [69–71].

Cost and workforce limitations are also significant. Sophisticated instruments require trained staff, preventive maintenance, biosafety infrastructure, reagent validation, and continuing personnel education. In many settings, the availability of a cytometer is not the limiting factor; rather, the critical issue is the ability to design, validate, troubleshoot, and interpret assays. Therefore, the credibility of cytometry in clinical laboratories will depend on harmonized training, multidisciplinary consultation, and reports that translate complex immunophenotypic patterns into clear diagnostic or management-relevant statements [65, 71, 72].

Future: Automation, AI, Portability, and Multimodal Integration

The future of flow cytometry will be shaped by four overlapping directions: higher-dimensional acquisition, intelligent analysis, decentralization, and integration with other single-cell technologies. Full-spectrum flow cytometry will continue to expand clinical and translational panels. However, the practical limit will be determined by reagent quality, biological interpretability, spectral spread, standardization, cost, and user training rather than by parameter number alone. The integration of lineage, activation, exhaustion, proliferation, metabolic, signaling, and viability markers into a unified assay represents a significant advance. This integration enables a transition from enumeration to functional immune profiling and provides a more comprehensive approach to immune assessment [2–7, 28–30, 66].

Automation will influence each stage of the workflow. Robotic sample preparation, standardized staining modules, automated instrument setup, digital quality-control dashboards, template-based analysis, and automated report generation can reduce operator-dependent variation. The literature indicates that artificial intelligence (AI) and machine learning (ML) are particularly promising for pattern recognition in leukemia and lymphoma immunophenotyping, MRD detection, high-dimensional clustering, anomaly detection, and decision support. Nevertheless, clinical AI cannot be treated as a black box. Curated training data, external validation, locked and version-controlled algorithms, transparent performance metrics, bias assessment, audit trails, and human oversight are essential. Artificial intelligence should support, but not replace, cytometry expertise and clinicopathological correlation [7, 66, 68, 72].

Portable and microfluidic cytometers have the potential to bring selected assays closer to patients. Low-volume cartridges, magnetic enrichment, impedance detection, and compact optics are promising for immune status monitoring, infectious disease triage, emergency settings, and resource-limited environments. The probable near-term model is not complete

replacement of central laboratory cytometry but rather tiered testing. In this model, portable platforms will support screening or focused monitoring, while central laboratories will perform complex panels, MRD assessment, cell therapy testing, and high-dimensional analyses [34–37].

Multimodal integration will also redefine the field. Sorted or indexed cells can be linked to single-cell RNA sequencing, immune-receptor sequencing, and related molecular or imaging-based workflows. Cytometry will increasingly serve as both a diagnostic method and a gateway technology that enriches relevant populations for downstream molecular analysis. In this context, clinical laboratories will require data structures that connect cytometry files, gating metadata, molecular results, clinical phenotypes, treatment history, and longitudinal outcomes. Such integration is essential for precision medicine, but it also introduces challenges related to data governance, interoperability, privacy, reproducibility, and long-term storage [10, 31, 53, 65, 73].

Consequently, the future of cytometry will be constrained by quality and workforce realities. Advanced lasers, large parameter arrays, sophisticated algorithms, and automated outputs do not automatically improve clinical care. Successful integration of these technological advances into medical practice requires assay validation, personnel training, instrument maintenance, interpretation of complex patterns, and communication of results in language that clinicians can readily understand. Therefore, the next phase of progress will depend not only on hardware innovation but also on harmonized education, laboratory accreditation, and collaborative reference frameworks [5, 7, 10, 67, 74].

Conclusion

Flow cytometry has evolved from photoelectric cell-counting methods into high-dimensional, clinically integrated single-cell analysis. The clinical reliability of the method depends on the integration of instrument architecture, standardized analytical workflow, validated data interpretation, and clear communication of results. The history of the field is characterized by the development of hydrodynamic focusing, fluorescence labeling, lasers, monoclonal antibodies, and cell sorting. The present era includes conventional and spectral cytometry, imaging, microfluidic and mass-based systems, nanoparticle analysis, and expanding roles in immunophenotyping, MRD detection, transplantation immunology, infection-related immune monitoring, oncology, drug development, and cell therapy. The future of the field will be determined by the integration of artificial intelligence into interpretation, the development of portable platforms, the advancement of multimodal single-cell approaches, and the implementation of rigorous standardization. For laboratory medicine, the essential message is clear: flow cytometry is no longer only an instrument-based technique; it is a complete diagnostic discipline requiring analytical validation, quality management, expert interpretation, and clinical integration.

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