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REVIEW ARTICLE

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THE ROLE OF PROINFLAMMATORY CYTOKINES IL-6 AND IL-17A AND ANTI-INFLAMMATORY CYTOKINE IL-10 IN COLORECTAL CANCER

Lana Sarajlić[1] Edin Hodžić[1] Alma Mekić Abazović[2] Samir Muhović[1] Amina Mehić[1] Emsad Halilović[1] Salem Bajramagić[1] Adnan Kulo[1] Ramajana Šukić-Karalić[1]

[1]Clinic for General and Abdominal Surgery, Clinical Center University of Sarajevo, Sarajevo, Bosnia and Herzegovina [2]Clinic for Oncology, Clinical Center University of Zenica, Zenica, Bosnia and Herzegovina

Colorectal cancer (CRC) remains the leading cause of cancer-related mortality worldwide, with chronic inflammation recognized as a critical factor in its pathogenesis. This review focuses on the roles of pro-inflammatory cytokines interleukin-6 (IL-6) and interleukin-17A (IL-17A), alongside the anti-inflammatory cytokine interleukin-10 (IL-10), in the development and progression of CRC. The reason we chose these cytokines among others is that we found a certain number of similar studies in recently published literature for comparison, given that this topic is quite rare. Elevated levels of IL-6 and IL-17A have been linked to enhanced tumor proliferation, survival, invasion, and metastasis, highlighting their contribution to a tumor-promoting microenvironment. Conversely, IL-10 exhibits a dual role by suppressing inflammation yet potentially facilitating immune evasion and tumor progression in certain contexts. Understanding the complex interplay and signalling pathways of these cytokines may improve the CRC risk assessment, diagnosis, prognosis, and offer new avenues for targeted therapies. This review synthesizes current evidence from recent literature to elucidate the molecular mechanisms and clinical implications of IL-6, IL-17A, and IL-10 in colorectal cancer.

Keywords: colorectal cancer, interleukin-6 (IL-6), interleukin-17A (IL-17A), interleukin-10 (IL-10), inflammation

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Correspondence to:
Lana Sarajlić
Clinic for General and Abdominal Surgery
Clinical Center University of Sarajevo
Bolnička 25, 71000 Sarajevo, Bosnia and Herzegovina
E-mail: lanasarajlic@yahoo.com

INTRODUCTION

Colorectal cancer (CRC) represents one of the leading causes of cancer-related mortality worldwide (1). The pathogenesis of CRC involves several key genetic alterations, including mutations in tumor protein 53 (TP53), adenomatous polyposis coli (APC), Kirsten rat sarcoma viral oncogene homolog (KRAS), and genes responsible for DNA mismatch repair (2). Mutations in TP53 are frequently linked to tumor progression and poor prognosis, whereas mutations in APC constitute early events triggering malignant transformation of intestinal epithelial cells (2,3). KRAS mutations are also common and vary according to tumor stage and patient population (4–7).

Chronic inflammation plays a pivotal role in CRC carcinogenesis. Patients suffering from inflammatory bowel diseases (IBD), such as ulcerative colitis and Crohn's disease, are at a significantly increased risk of CRC (8). Persistent inflammation drives continuous regeneration and repair of the intestinal mucosa, potentially leading to the accumulation of genetic and epigenetic alterations that favor malignancy (9–13). Moreover, inflammation shapes a tumor-supportive microenvironment by stimulating epithelial proliferation, angiogenesis, and recruitment of immune cells, further promoting genetic instability (10).

Inflammation mediators such as tumor necrosis factor-alpha (TNF-α), IL-6, and interleukin-1 beta (IL-1β) activate signalling pathways like NF-κB and STAT3, which enhance tumor proliferation, survival, invasion, and metastasis (14–17). Systemic inflammation, influenced by lifestyle factors including obesity, smoking, and alcohol use, correlates closely with CRC risk and progression (18–20). Conversely, physical activity and diets rich in anti-inflammatory components (fruits, vegetables, omega-3 fatty acids) may reduce systemic inflammation and CRC risk (2,14,15,21,22).

Despite advances, many aspects of cytokines' complex roles in CRC tumor biology remain to be elucidated. This study aims to investigate the roles of pro-inflammatory cytokines IL-6 and IL-17A, as well as the anti-inflammatory cytokine IL-10, in the CRC pathogenesis and progression. Understanding the circulating and tissue levels of these cytokines may improve CRC prevention, diagnosis, and personalized therapy.

METHODS

This work is a narrative literature review with elements of a systematic approach, aimed at synthesizing the current knowledge on the roles of the pro-inflammatory cytokines IL-6 and interleukin-17A (IL-17A), as well as the anti-inflammatory cytokine interleukin-10 (IL-10), in the pathogenesis and progression of CRC. The review summarizes key molecular mechanisms, biological functions, and the clinical relevance of these cytokines in relation to CRC development and progression. The included studies involved human subjects diagnosed with CRC, spanning various disease stages and treatment settings. Extracted data were qualitatively analyzed and thematically organized, with an emphasis on molecular pathways involving IL-6, IL-17A, and IL-10, their influence on tumor biology, and their potential roles as diagnostic or prognostic biomarkers and therapeutic targets.

A comprehensive literature search was conducted using three major electronic databases: PubMed, Scopus, and Web of Science. The search strategy combined relevant keywords and Medical Subject Headings (MeSH) terms, including "IL-6," "IL-17A," "IL-10," "colorectal cancer," and "cytokines." The search was restricted to articles published in English between January 2010 and April 2024.

Inclusion criteria encompassed original research articles, systematic reviews, and meta-analyses that addressed the roles of IL-6, IL-17A, and IL-10 in CRC etiology, progression, diagnosis, or treatment. Studies were excluded if they were unavailable in full text, not published in English, or presented as conference abstracts, case reports, editorials, or articles not directly related to cytokine involvement in CRC.

Study selection was conducted independently by two reviewers who screened titles and abstracts for relevance. Full-text articles of potentially eligible studies were retrieved and reviewed. Disagreements were resolved through discussion or consultation with a third reviewer. Extracted data included study design, population characteristics, cytokine measurement methods, key findings, and clinical implications.

Cytokines and their role in colorectal cancer

Cytokines are secreted proteins that mediate immune and inflammatory responses. Primarily produced by leukocytes (macrophages, T lymphocytes), they influence various cell types, including tumor cells, promoting malignant transformation and tumor progression (23,24). Tumor cells themselves can secrete cytokines to activate oncogenic pathways supporting growth (25). Chronic inflammation involves elevated pro-inflammatory cytokines, such as IL-6, IL-17A, TNF-α, and IFN-γ, which contribute to tumor

growth and metastasis (26). The CRC microenvironment contains increased concentrations of these cytokines; for example, IL-6 not only promotes tumor proliferation but also metastatic potential (27). TNF-α is associated with advanced stages and enhances tumor invasiveness and metaplasia (28,29).

Conversely, the anti-inflammatory cytokine IL-10 has a complex dual role in the tumor microenvironment. While it suppresses inflammation and protects tissue, excessive IL-10 production may inhibit anti-tumor immune responses by dampening cytotoxic T lymphocyte and macrophage activity, facilitating immune evasion by tumors (30–32). Elevated IL-10 levels have been correlated with poor prognosis in CRC and other cancers (29).

Some cytokines, such as IL-12, possess anti-tumor effects by activating NK cells and T lymphocytes but have limited therapeutic use due to stability and potential side effects (33,34). Elevated pro-inflammatory cytokines often reflect aggressive tumor phenotypes and poor prognosis, making cytokine signaling a promising therapeutic target (35,36).

Roles of IL-6, IL-17A (Pro-inflammatory) and IL-10 (Anti-inflammatory) in CRC

Cytokines are classified into pro-inflammatory (e.g., IL-1β, IL-6, IL-17A, TNF-α), anti-inflammatory (e.g., IL-4, IL-10, IL-13), chemokines (e.g., IL-8), and growth factors (e.g., VEGF) (37). The CRC tumor microenvironment is characterized by elevated pro-inflammatory cytokines IL-6, IL-17A, TNF-α, and IFN-γ, as well as anti-inflammatory cytokines like IL-10, which modulate immune responses (38,39).

IL-6, mainly secreted by monocytes and macrophages, plays a multifunctional role by inhibiting apoptosis, promoting tumor cell survival, and regulating reactive oxygen species (ROS) production. Under homeostasis, IL-6 and related cytokines (IL-10, IL-11, IL-23) serve as “alarm” signals resolving inflammation (40-43). IL-1α and IL-1β initiate and amplify local inflammation, while IL-12 and IL-23 drive differentiation of naïve T cells into IFN-γ-producing Th1 cells with antitumor activity (44,45).

IL-10 is an immunosuppressive type 2 cytokine that inhibits type 1 immune responses and host antitumor immunity (46). In advanced CRC, increased serum IL-10 correlates with reduced IL-12 production by stimulated peripheral blood mononuclear cells, promoting immune evasion (46). The role of IL-10 is context-dependent; it may both promote and inhibit tumorigenesis (47), and dysregulated IL-10 expression is implicated in systemic diseases (48).

DISCUSSION

IL-6 has been extensively studied in CRC, with multiple reports confirming elevated serum levels in patients compared to controls. IL-6 enhances tumor progression by promoting proliferation, survival, and differentiation of malignant epithelial cells, thereby facilitating metastasis (49,50). Elevated IL-6 levels correlate positively with tumor size, TNM stage, poor differentiation, and worse prognosis, suggesting its potential as both a diagnostic and prognostic biomarker (50–54). In vitro data support the role of IL-6 in stimulating CRC cell growth (51,52).

IL-10 is crucial for intestinal immune regulation. Therapeutic IL-10 administration in Crohn’s disease demonstrates its ability to suppress excessive immune responses and maintain homeostasis (55). Stanilova et al. reported increased IL-10 gene expression in CRC patients, with higher preoperative IL-10 mRNA levels than postoperative or control levels, suggesting a pro-tumorigenic role (56). Conversely, IL-10 knockout mouse models exhibit increased CRC susceptibility, indicating a protective role in tumor prevention (57). Abtahi et al. highlighted the context-dependent role of IL-10 influenced by the tumor microenvironment (58).

Recent studies identify serum IL-17 as a promising early diagnostic and prognostic biomarker in CRC. Elevated IL-17 correlates with advanced disease and p53 deficiency, reflecting tumor-associated cytokine production and systemic release. Radosavljević et al. confirmed the importance of IL-17 as a potential tumor-specific biomarker in CRC (59). Wang et al. additionally indicated that the combination of CCL20 and IL-17A could serve as reliable biomarkers for early diagnosis and prognosis (60).

Large-scale multicenter studies should be conducted to further elucidate the importance of measuring inflammatory cytokines in CRC patients and their possible role in CRC diagnosis and prognosis. Future research involving larger targeted studies is necessary to thoroughly understand the mechanisms underlying the increase in de novo cytokines in the serum of CRC patients. Long-term, readily available biomarkers could facilitate matching patients to state-of-the-art therapeutic modalities such as blockade with monoclonal anti-cytokine antibodies. The positive results of this study should only serve as a starting point for additional confirmatory research.

Pro-inflammatory cytokines IL-6 and IL-17A contribute to CRC pathogenesis by promoting tumor growth, survival, and metastasis, while the anti-inflammatory cytokine IL-10 plays a complex, context-dependent role in immune regulation and tumor progression. Understanding these cytokines' dynamics offers opportunities for improved CRC diagnosis, prognosis, and targeted therapy development.

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Authors' Contributions

Conceptualization, investigation, writing – original draft, review & editing, L.S., E.H., A.M.A., S.M., A.M., E.H., S.B., A.K., and R.Š.K. All authors have read and approved the published version of the manuscript.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

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No generative AI was used.

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REVIEW ARTICLE

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MEDICAL HISTORY TAKING IN TEACHING ENGLISH FOR MEDICAL PURPOSES: A KEY COMMUNICATION SKILL FOR FUTURE HEALTHCARE PROFESSIONALS

Nataša Šelmić

Department of General Education Subjects, English Language, University of Niš Faculty of Medicine, Niš, Serbia

Effective doctor-patient communication is central to medical practice, and medical history forms a large part of it. Medical history taking, as the most frequently performed task by physicians, is, therefore, referred to as the most effective and adaptable tool in clinical practice. Adequate communication and interpersonal skills include active listening, demonstrating empathy, providing appropriate counseling, delivering clear therapeutic instructions, and building firm, trust-based relationships. Strengthening doctor-patient communication can enhance patient participation, improve adherence to treatment plans, boost satisfaction, and optimize healthcare utilization. In the English for Medical Purposes (EMP) course, medical history taking represents an integrative part of the curriculum and syllabus at the Faculty of Medicine, University of Niš. The course aims to introduce students to Medical English as an aid to patient-doctor communication, help them acquire medical vocabulary, master questioning techniques and linguistic patterns, thus preparing them for effective communication. This paper explores the significance of medical history taking in EMP instruction and highlights linguistic aspects that will assist students in improving their communication skills.

Keywords: medical history taking, English for medical purposes, medical vocabulary, communication skills

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Correspondence to:
Nataša Šelmić
Department of General Education Subjects, English Language
University of Niš Faculty of Medicine
Bulevar dr Zorana Đinđića 81, Niš, Serbia
E-mail: natasa.selmic@medfak.ni.ac.rs

INTRODUCTION

Effective doctor-patient communication is central to medical practice, and medical history forms a large part of it. Medical history taking, as the most frequently performed task by physicians, is, therefore, referred to as the most effective, perceptive, and adaptable tool in clinical practice (1). Evidence has demonstrated that effective history taking increases patient satisfaction, enhances treatment adherence, and results in better health outcomes (2).

A medical history is a structured approach to eliciting information from the patient about their symptoms, past medical conditions, family history, and lifestyle factors. The most prominent functions include establishing rapport with patients, facilitating accurate diagnosis and treatment planning, and enhancing interdisciplinary communication among healthcare teams.(3).

There are three principal stages in a clinical conversation between a doctor and a patient. The first stage refers to the doctor-patient dialogue, involving language for asking questions, confirming details, and exploring the main signs and symptoms presented by the patient. The second stage involves physical examinations, where further instructions and procedures are defined. The third stage includes establishing the diagnosis, adherence to the treatment plan, and use of medications. The development of the language skills required to carry out these interactions contributes to communicative competence, empowering learners to use language for meaningful communication (4).

This paper examines how medical history taking is integrated into EMP teaching and highlights linguistic aspects that will assist students in improving their communication skills.

THE USE OF MEDICAL HISTORY TAKING IN THE ENGLISH FOR MEDICAL PURPOSES COURSE

Medical history taking is an integrative part of the English for Medical Purposes (EMP) course at the Faculty of Medicine, University of Niš. The course aims to help students appreciate the need to use precise medical terminology and language when interacting with patients and colleagues. In addition, it covers the best practices for using Medical English in different healthcare contexts to achieve effective, clear, and courteous communication.

Good communication and interpersonal skills include active listening, demonstrating empathy, providing appropriate counseling, delivering clear therapeutic instructions, and building good, compassionate relationships with patients. Strengthening doctor-patient communication can enhance patient participation, improve adherence to treatment plans, boost satisfaction, and optimize healthcare utilization. Ultimately, effective communication plays a vital role in delivering high-quality care and achieving better health outcomes. These basic clinical skills play a crucial role in medical practice to ensure optimal outcomes and patient satisfaction, which are fundamental aspects of healthcare delivery (5).

For EMP learners, history taking requires both linguistic and communicative competence. They are supposed to acquire specialized terminology, ask open-ended questions, and possess active listening skills. In this way, a connection between specialist subjects and language learning can readily be perceived by students — integration of content and context is achieved, where content provides a source of interest and motivation to learn, and the content is made accessible through language instruction (6).

The structure of medical history taking

A medical history generally consists of the following structural elements:

General information, including name and surname of the patient, date of birth;

The opening in which the doctor greets the patient, builds initial rapport, and identifies the main issues for which the patient is seeking medical attention:

What seems to be the problem?

What has brought you here today?

The history of the present illness in which the physician gathers information by asking a series of questions to prompt the patient to describe their current issues in detail:

How long have they/has it been bothering you?

How long have you had them/ it?

When did they start?

The patient's past medical history, which includes significant illnesses, any previous surgeries or operations, and any ongoing health conditions:

Have you ever had this problem before?

Has there been any change in your health since your last visit?

The review of systems involves gathering information about current or past issues related to the different body systems.

Family history contains questions relating to diseases running in the family:

Does anyone else in your family suffer from this problem?
Are your parents alive and well?
Social history includes details about living arrangements, occupation, marital status, number of children, substance use, recent travel abroad, and exposure to environmental risks through recreational activities or pets:
Do you smoke?
How many cigarettes a day?
Do you use alcohol?
Do you exercise?
Medications, both regular and acute, including those prescribed by doctors, as well as over-the-counter drugs or alternative medicines:
Are you taking any medication at the moment?
Allergies to medications, food, latex, and other environmental factors:
Are you allergic to any medication? (7).

ROLE-PLAY AS A SIMULATION IN-CLASS ACTIVITY

Role-play as a form of simulation is regarded as an effective method for learning communication skills, wherein the importance of the social context of learning is emphasized. For a simulation to occur, the participants should assume their roles and responsibilities and carry out their duties to the best of their abilities within the given circumstances. Consequently, the participants and observers learn about the person or situation being acted out. In essence, each participant is creating part of the social context, establishing an environment in which they can examine their behavior or observe group dynamics (8). During in-class history-taking practice, students are divided into small groups, whereby one group assumes the role of a patient, and another assumes the role of a doctor. Carefully selected case reports provide an ideal ground for practising accurate, concise, and easily understood questions and giving instructions during doctor–patient interactions. The main objective is to allow students to integrate communicative and linguistic functions in a realistic clinical context. An interactive learning environment is a practical tool for clinical skills teaching. It supports the following specific aspects of medical communication:
Active listening: Participants are encouraged to listen actively to the "patient" and respond appropriately to the main complaints. In addition, they need to concentrate on the essential information relating to specific signs and symptoms of the disease.
Problem solving: Different scenarios and thinking of various possibilities of treatment or communication in medical practice enable students to develop critical thinking and decision-making skills.
Empathy: Empathy is an important trait that medical professionals should possess to foster patient confidence and cooperation. Role play is a useful technique for students to develop emotional sensitivity and understanding in real- life situations.
Knowledge acquisition: Role play is an effective way of learning as it makes students use theoretical knowledge in real-life situations and gain practical experience.
Effective communication: Regardless of whether it is delivering a diagnosis, bad news, or simply comforting a patient, role play enhances both verbal and non-verbal communication skills of the participants, particularly in multicultural environments (9).
A key linguistic requirement is the ability to appropriately use medical or professional terms and their non-professional equivalents. When communicating with patients, doctors must use clear and appropriate language while avoiding medical jargon, as this plays a crucial role in effective doctor-patient interactions. The way information is conveyed directly impacts patient satisfaction and adherence to treatment. By incorporating language tasks that emphasize linking technical and everyday terms, we can enhance students' awareness of the appropriate register for different situations and better equip them for real-life interactions.
Teaching students structured question frameworks like the SOCRATES or OLDCART patterns helps them develop a systematic approach to patient interviews in Medical English. These frameworks guide students in gathering comprehensive and relevant clinical information, ensuring clarity and precision in history taking.
SOCRATES (commonly used for pain assessment) stands for:
Site – Where is the pain located?
Onset – When did it start?
Character – What is the pain like (sharp, dull, burning, etc.)?
Radiation – Does the pain spread anywhere?
Associations – Are there other symptoms (nausea, fever, etc.)?
Time course – Has it changed over time?
Exacerbating/relieving factors – What makes it worse or better?
Severity – How bad is the pain on a scale of 1–10?

OLDCART (another pain assessment tool) stands for:
Onset – When did the symptoms begin?
Location – Where is the pain/symptom?

Duration – How long has it lasted?
Character – What does it feel like?
Aggravating factors – What makes it worse?
Relieving factors – What makes it better?
Temporal factors – Has it changed over time? (10).
By incorporating these language tasks into medical English training, students can enhance their ability to ask structured and correct questions, improving their communication skills and patient interactions.
Integrating medical history into EMP education increases students' motivation and improves communication, analytical, and critical thinking skills that positively affect learning practice. Because of the familiarity with the subject matter, active participation in the learning process is achieved. This approach is student-centred and content-based, enhancing students' interest in using different learning strategies. In addition, they can practise all language skills and the grammatical and semantic features of Medical English. Moreover, they acquire crucial social and communication skills indispensable for future training and professional development.

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Statement of Competing Interest

The author declares no conflicts of interest.

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REVIEW ARTICLE

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SUSTAINABILITY OF HEALTHCARE SYSTEMS: INTEGRATION OF GREEN TECHNOLOGY IN HOSPITAL FACILITIES

Marko Kimi Milić[1] **Šćepan Sinanović**[1] **Tanja Prodović**[1] **Saša Bubanj**[2]

[1]High Medical College of Professional Studies „Milutin Milanković", Belgrade, Serbia [2]University of Niš Faculty of Sport and Physical Education, Niš, Serbia

The healthcare sector represents a substantial contributor to global environmental degradation and resource depletion, necessitating the urgent implementation of sustainable operational solutions. This study investigates the integration of green technologies—specifically renewable energy systems, advanced waste management practices, and water conservation strategies—within hospital facilities. The research aims to evaluate the environmental, economic, and operational impacts of these technologies while identifying critical barriers to their widespread adoption. A mixed-methods approach was employed, combining a systematic literature review following PRISMA guidelines with case study analysis and advanced statistical modeling. Data from 48 peer-reviewed studies and diverse international case studies were synthesized to quantify trends in energy, waste, and water efficiency. The findings demonstrate significant ecological benefits, including a 30% to 50% reduction in energy consumption and a 40% to 50% decrease in medical waste volumes. Furthermore, hospitals implementing water conservation protocols achieved up to 35% reductions in usage, enhancing operational resilience. Economic analysis revealed annual savings ranging from $50,000 to $120,000 USD per institution, primarily driven by optimized resource systems. However, barriers such as high initial capital requirements and technical expertise gaps remain significant challenges. This study underscores the transformative potential of green technologies in minimizing the healthcare sector's ecological footprint while simultaneously optimizing economic performance. The results suggest that targeted policy interventions and capacity-building initiatives are essential to foster more resilient and environmentally responsible healthcare infrastructure.

Keywords: green technologies, healthcare sustainability, renewable energy, waste management, water conservation

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Correspondence to:
Šćepan Sinanović
High Medical College of Professional Studies
"Milutin Milankovic"
Crnotravska 27, Belgrade, Serbia
E-mail: scepan.sinanovic@gmail.com

INTRODUCTION

The healthcare sector represents a substantial source of global environmental pollution and resource exhaustion, creating a critical necessity for the integration of sustainable practices within medical infrastructure. Hospital facilities, as the functional core of these systems, operate as energy-intensive and resource-demanding institutions. Their daily operations require vast energy inputs, produce significant volumes of medical waste, and rely heavily on stable water resources, positioning them as primary targets for strategic sustainability interventions. The environmental impact of these facilities extends well beyond simple consumption; it encompasses toxic emissions from medical waste incineration, water pollution resulting from inadequate disposal protocols, and a persistent reliance on non-renewable energy sources. Globally, the healthcare industry is responsible for approximately 4.4% of net greenhouse gas emissions, with the hospital infrastructure accounting for the largest share of this ecological footprint (1). Such emissions not only accelerate climate change but also present a direct paradox to the medical mission by exacerbating respiratory illnesses and other environmentally-linked health conditions.

In response to these systemic challenges, the concept of sustainable healthcare has emerged, prioritizing the minimization of environmental harm without compromising clinical outcomes. This approach integrates environmental stewardship directly into hospital operations through the adoption of green technologies and the optimization of resource efficiency. Such a transition is increasingly viewed as both a moral and a practical imperative for institutions dedicated to the holistic protection of human and planetary health. Within this framework, green technologies provide innovative solutions for energy efficiency, waste management, and water conservation. For instance, the implementation of renewable energy sources—such as solar arrays—and high-efficiency lighting has demonstrated a capacity to significantly reduce both operational overhead and environmental degradation (2). Similarly, advanced waste management systems designed for recycling or the safe neutralization of medical waste have proven effective in mitigating regional pollution risks (3), while water conservation strategies like rainwater harvesting and low-flow plumbing systems support hygiene standards and reduce total consumption (4).

Despite the evident advantages of these technologies, several structural barriers continue to impede their widespread adoption. High initial capital requirements, limited access to funding, and a lack of robust regulatory frameworks often hinder the transition toward sustainability. Furthermore, while the implementation of specialized waste management systems has been shown to minimize risks such as toxic emissions and groundwater contamination (5), overcoming the broader systemic inertia requires a coordinated effort among policymakers, healthcare administrators, and environmental scientists.

The present study explores the transformative potential of integrating these green technologies within the hospital environment. The primary focus is to evaluate the environmental impact of such implementations, specifically regarding the reduction of carbon emissions, waste production, and water usage. Furthermore, the research assesses the economic implications—including long-term financial benefits and operational cost savings—while investigating how sustainable practices influence the overall efficiency of healthcare delivery. By identifying the prevailing technical and financial barriers, this inquiry proposes evidence-based strategies and policy recommendations to foster sustainability at local, national, and global levels.

This research is guided by the hypothesis that the integration of green technologies significantly diminishes energy consumption and environmental pollution without compromising the quality of patient care. It is further posited that green waste management and water conservation practices effectively improve resource efficiency and regulatory compliance. Ultimately, the study operates on the premise that overcoming current financial and regulatory hurdles is essential for the successful modernization of healthcare facilities. By reducing the environmental footprint of hospital operations, these practices not only align with global climate objectives but also enhance the long-term economic viability and operational resilience of the healthcare sector.

METHODS

This study employed a multi-faceted methodology to evaluate the integration of green technologies within hospital facilities, specifically focusing on their environmental, economic, and operational impacts. To ensure the highest level of reliability and reproducibility, the research design was structured into three interconnected components: a systematic literature review, case study analysis, and the synthesis of secondary data. This triangulated approach was chosen to

strengthen the validity of the conclusions and provide a holistic view of the subject.

The first phase involved a systematic literature review conducted in strict accordance with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (6). A comprehensive search was executed across major academic databases, including PubMed, Scopus, Web of Science, and Google Scholar, using targeted keywords such as "green hospitals," "renewable energy in healthcare," and "sustainable healthcare systems." The search was limited to peer-reviewed articles published between 2012 and 2023 that provided measurable outcomes regarding energy, waste, or water efficiency. Following an initial screening of 1,200 abstracts and a subsequent review of 110 full-text articles, 48 studies were selected for final analysis based on their methodological rigor and the presence of sufficient quantitative data (7,8).

Complementing the literature review, a case study analysis was performed to obtain contextual insights into the practical implementation of these technologies. Selection criteria for these cases emphasized geographical diversity—to capture the nuances of both developed and developing regions—as well as the availability of robust pre- and post-intervention data. Notable examples included a rural hospital in Kenya that achieved a 35% reduction in grid dependency through solar energy, and a German urban facility that reduced medical waste by 40% through advanced recycling programs (9,10). In parallel, secondary data were synthesized from international organizations such as the World Health Organization (WHO) and the Global Green and Healthy Hospitals (GGHH) network to quantify broader trends in sustainability metrics and associated cost benefits (11,12).

Data collection and preparation were handled through a rigorous extraction process where key metrics, such as energy use and waste reduction, were standardized to ensure cross-case comparability. This involved a cleaning phase to address missing values and inconsistencies, followed by normalization to account for variations in hospital size and geographic context; for instance, energy consumption was standardized to kilowatt-hours per square meter (kWh/m^2).

The analytical phase utilized a suite of statistical methods to validate the research findings. Descriptive statistics were first used to summarize the core characteristics of the data, including means, medians, and measures of variability for resource consumption. Subsequently, inferential statistics, such as paired t-tests, were applied to assess the significance of reductions in carbon emissions and other metrics following the adoption of green technologies (13). Analysis of variance (ANOVA) was further employed to compare the relative effectiveness of different technological solutions, such as solar energy versus energy-efficient lighting, thereby identifying statistically significant differences between groups (14). Furthermore, a meta-analysis was performed on the aggregated data from the literature review to calculate pooled effect sizes, with heterogeneity among studies assessed using the I^2 statistic (15). Linear regression models were also implemented to explore the relationships between technology adoption and sustainability outcomes, while simultaneously assessing cost-benefit trade-offs (16). All statistical procedures were executed using SPSS (v28) and R (v4.2.3), specifically utilizing the meta and lm() packages for advanced modeling.

To ensure the robustness of these results, cross-validation was conducted on the case study data by partitioning it into training and test sets. Additionally, sensitivity analyses were performed to evaluate the impact of potential outliers, such as hospitals with exceptionally high baseline energy consumption, on the overall estimates. Finally, the study adhered to strict ethical principles, including transparency in data usage and the proper acknowledgment of all primary sources to maintain intellectual integrity, while avoiding selection bias to ensure the objectivity of the findings (17).

RESULTS

The findings from the systematic review, case study analysis, and secondary data synthesis reveal that the adoption of green technologies in hospital facilities consistently yields substantial improvements in sustainability metrics. Specifically, in the domain of energy efficiency, the implementation of renewable energy systems—most notably solar arrays—and energy-efficient HVAC systems emerged as the most transformative interventions. Hospitals that integrated solar energy reported an average reduction in energy consumption ranging from 30% to 45%, depending on geographic location and baseline demand. Furthermore, the transition to energy-efficient lighting, such as LED systems, accounted for a 20% to 25% decrease in electricity usage across the analyzed facilities. As demonstrated in the research, the synergy between renewable sources and

efficient hardware resulted in annual operational savings between $50,000 and $120,000 in prominent case studies from Germany and Kenya (Table 1).

These technological shifts also directly impacted environmental outputs; for instance, the integration of solar and wind energy was associated with a 20% to 35% reduction in hospital-related carbon emissions over a three-year period. Facilities utilizing active energy monitoring systems achieved the most consistent reductions by identifying and mitigating inefficient consumption patterns (Figure 1).

Parallel to energy gains, advanced waste management systems significantly enhanced the efficiency of medical waste segregation and disposal. The systematic separation of hazardous and non-hazardous waste streams reduced the total volume of incinerated medical waste by 40% to 50% (Table 2). Beyond the ecological impact, these programs contributed to a 15% to 25% reduction in overall waste disposal costs (Figure 2). Geographic trends were evident here as well; hospitals in urban centers in Germany and Australia saved an average of $30,000 USD annually through recycling initiatives, while smaller rural facilities reported more modest but significant savings through composting and eco-friendly treatment methods.

Similarly, water conservation strategies proved vital for operational resilience. The implementation of rainwater harvesting systems led to a 20% to 35% reduction in water consumption in rural hospitals, particularly within water-scarce regions of sub-Saharan Africa (Table 3).

Table 1. *Energy consumption before and after green technology implementation across hospitals*

Hospital	Location	Technology implemented	Energy consumption before (kWh/year)	Energy consumption after (kWh/year)	Reduction (%)
Hospital A	Germany	Solar panels + LED lighting	1,200,000	720,000	40%
Hospital B	Kenya	Solar panels	500,000	325,000	35%
Hospital C	Australia	Energy-efficient HVAC systems	1,000,000	700,000	30%
Hospital D	Canada	Solar panels + energy monitoring	800,000	480,000	40%
Hospital E	India	LED lighting	300,000	240,000	20%

Table 2. *Volume of medical waste before and after implementing waste management systems*

Hospital	Location	Waste management system implemented	Waste volume before (kg/month)	Waste volume after (kg/month)	Reduction (%)
Hospital A	Germany	Advanced waste recycling system	1,200	720	40%
Hospital B	Australia	Waste segregation and composting	900	540	40%
Hospital C	Kenya	Basic waste segregation	500	300	40%
Hospital D	India	Eco-friendly incineration system	1,500	900	40%
Hospital E	Canada	Combined recycling and composting	800	480	40%

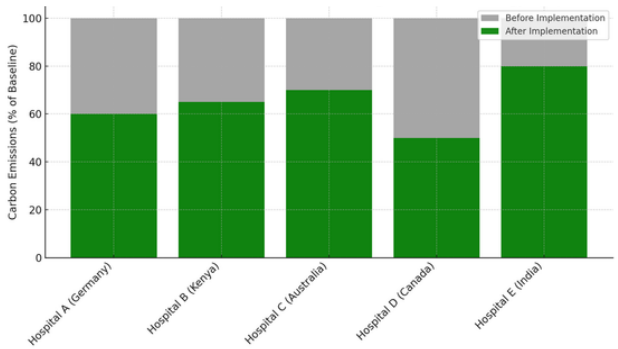

Figure 1. *Carbon emissions reduction achieved through renewable energy adoption*

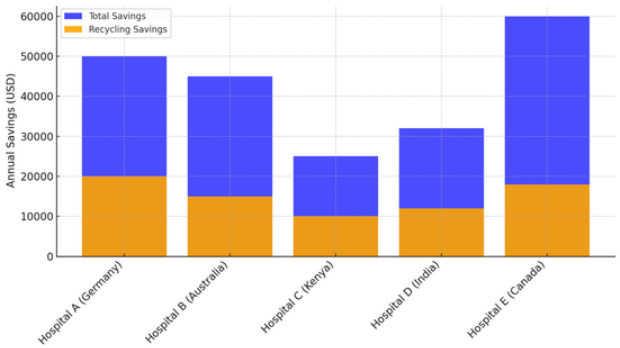

Figure 2. *Economic benefits of recycling and waste management in hospitals*

Table 3. *Water usage reductions in hospitals with rainwater harvesting and efficient plumbing*

Hospital	Location	Water conservation technology implemented	Water usage before (liters/month)	Water usage after (liters/month)	Reduction (%)
Hospital A	Kenya	Rainwater harvesting	100,000	70,000	30%
Hospital B	Australia	Low-flow faucets	80,000	64,000	20%
Hospital C	Germany	Rainwater harvesting + efficient plumbing	120,000	84,000	30%
Hospital D	India	Efficient plumbing	90,000	72,000	20%
Hospital E	Canada	Rainwater harvesting	110,000	77,000	30%

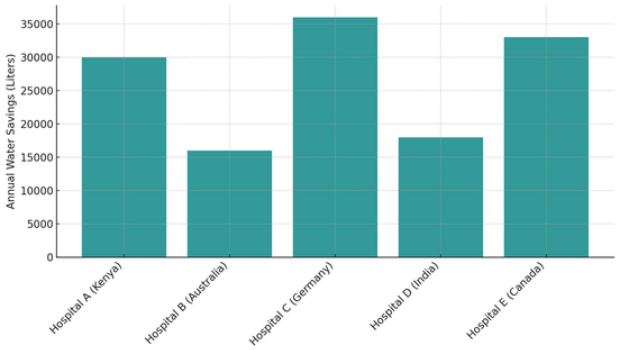

Figure 3. *Annual water savings achieved through conservation strategies*

Urban facilities, meanwhile, achieved savings of 15% to 25% through the installation of low-flow plumbing fixtures. These interventions not only reduced costs but also increased operational continuity by 20% during periods of crisis or shortage, with cumulative annual water savings often exceeding 50,000 liters (Figure 3).

When examining the combined environmental and economic impact, hospitals that adopted an integrated approach—combining energy, waste, and water solutions—observed compound benefits, including carbon footprint reductions of 50% to 60% over five years and annual operational cost savings ranging from $80,000 to $200,000 USD (Table 4). However, the effectiveness of these technologies was notably influenced by regional factors; rural facilities in developing countries derived the most benefit from water and energy interventions, whereas urban hospitals in developed regions saw higher returns from sophisticated waste management systems (Figure 4).

Table 4. *Combined environmental and economic outcomes of green technology adoption in hospitals*

Hospital	Location	Green technologies implemented	Carbon footprint reduction (%)	Annual energy savings (USD)	Annual waste management savings (USD)	Total annual savings (USD)
Hospital A	Germany	Solar panels + LED lighting	40%	50,000	20,000	70,000
Hospital B	Kenya	Solar panels + rainwater harvesting	35%	25,000	10,000	35,000
Hospital C	Australia	Advanced HVAC + waste recycling systems	30%	45,000	15,000	60,000
Hospital D	Canada	Energy monitoring + rainwater harvesting	50%	60,000	18,000	78,000
Hospital E	India	LED lighting + eco-friendly incineration	20%	20,000	12,000	32,000

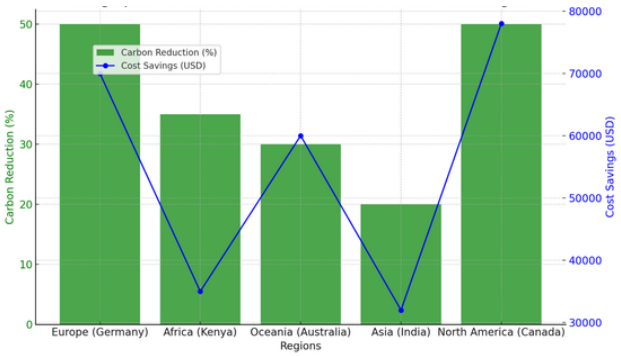

Figure 4. *Geographic variations in the effectiveness of green technologies*

Despite these benefits, several persistent barriers to implementation were identified. Financial constraints remain the most significant hurdle, with approximately 75% of hospitals reporting that high upfront investment costs deter the adoption of green technologies (Table 5). Additionally, limited technical expertise for the maintenance of advanced systems and regulatory gaps regarding sustainability policies continue to hinder the widespread transition toward green hospital infrastructure.

Table 5. *Key barriers to green technology implementation in healthcare facilities*

Barrier	Description	Percentage of hospitals reporting
Financial Constraints	High upfront costs of installing green technologies, such as solar panels and advanced HVAC systems	75%
Lack of Technical Expertise	Limited knowledge and skills for operating and maintaining green technologies	60%
Regulatory Gaps	Absence of supportive policies or incentives for adopting sustainable practices	55%
Infrastructure Limitations	Inadequate infrastructure to support new technologies, particularly in rural hospitals	50%
Resistance to Change	Reluctance among hospital staff and administrators to adopt new systems and workflows	40%

DISCUSSION

The integration of green technologies in healthcare facilities offers a transformative opportunity to align environmental stewardship with operational efficiency and fiscal responsibility. The results of this study underscore the profound capacity of renewable energy sources, particularly solar energy, to mitigate the environmental footprint of hospitals by reducing carbon emissions by up to 50%. This finding is consistent with contemporary research suggesting that a reduced dependence on fossil fuels is essential for the healthcare sector to meet global climate goals (1, 2). Furthermore, the success of water conservation strategies in resource-limited settings—such as the 35% reduction in usage observed in sub-Saharan African hospitals—highlights the dual importance of these technologies in promoting sustainability while ensuring service continuity during periods of environmental stress (4, 17).

From an economic perspective, the data challenge the common perception that sustainable infrastructure is prohibitively expensive. While initial capital requirements are high, energy-efficient systems such as LED lighting and advanced monitoring tools were shown to reduce operational costs by 30% to 40%, generating annual savings of up to $120,000 USD in larger institutions. These outcomes reinforce global evidence that investments in sustainable infrastructure yield significant long-term financial dividends (7, 16). Similarly, the economic viability of green waste management is clear, as recycling programs can reduce disposal costs by nearly half, providing a compelling financial argument for the adoption of comprehensive waste strategies (3, 5, 10). Beyond these quantifiable metrics, the study also observed qualitative operational improvements.

However, the path to widespread adoption is complicated by several structural barriers. Financial constraints remain a primary obstacle, particularly in low-income settings where the initial costs of renewable energy or advanced waste systems often exceed available budgets. Overcoming this inertia requires decisive policy interventions, including subsidies and targeted financial incentives (7, 3). Furthermore, the lack of specialized technical expertise often leads to system inefficiencies or failures, emphasizing a critical need for capacity-building initiatives and training programs for both healthcare administrators and technical staff (4, 17). These challenges are further influenced by geographic and socioeconomic disparities; while hospitals in developed regions benefit from supportive regulatory frameworks, those in developing regions must often contend with infrastructural limitations that necessitate more tailored, region-specific technological solutions (1, 11).

To address these challenges, a multifaceted policy approach is recommended. Governments and international organizations should prioritize financial support through grants and low-interest loans to offset the high entry costs of green technologies (7, 3). This must be coupled with the development of clear regulatory frameworks that establish mandatory sustainability targets, such as waste segregation protocols and renewable energy quotas (12, 4). Additionally, fostering public-private partnerships can drive innovation and resource mobilization, while dedicated training programs can ensure the long-term viability of installed systems. Looking forward, future research should focus on the long-term impact of these technologies on patient outcomes and explore the potential of integrating digital tools, such as artificial intelligence and the Internet of Things (IoT), to further optimize resource management.

This study demonstrates the profound impact of green technologies on improving the sustainability of healthcare systems, offering measurable environmental, economic, and operational benefits. A detailed synthesis of the findings reveals that these interventions can simultaneously address pressing challenges in energy efficiency, waste management, and water conservation while laying the groundwork for long-term institutional resilience. Beyond the immediate metrics, the results carry broader societal and global implications, positioning the modernization of hospital infrastructure as a critical component of public health and environmental stewardship.

The environmental achievements identified in this research are particularly striking, as hospitals adopting renewable energy systems achieved carbon emission reductions ranging from 30% to 50%. Such data underscore the transformative potential of green technology to mitigate the healthcare sector's contribution to greenhouse gas emissions. These ecological gains are further supported by waste management programs that reduced medical waste volumes by nearly half, alongside rainwater harvesting systems that conserved up to 35% of monthly water usage. These outcomes have direct implications for public health, as reducing the environmental footprint of medical facilities limits the pollutants known to exacerbate respiratory and waterborne illnesses, thereby protecting both the environment and the surrounding communities.

Complementing these environmental gains, the economic outcomes of the study challenge the traditional perception that sustainability initiatives are prohibitively expensive. The integration of renewable energy and monitoring tools resulted in annual savings of up to $120,000 USD, providing a clear pathway to financial sustainability with payback periods as short as three to five years. Furthermore, the efficiency of waste and water management protocols generated additional cost reductions of up to 40% through decreased disposal fees and the potential for material resale. These findings demonstrate that green technologies are not only ecologically responsible but are also economically feasible investments that yield substantial long-term financial returns.

The operational advantages of these technologies were equally evident, particularly regarding institutional resilience and continuity of care. Real-time energy monitoring allowed for the optimization of consumption and ensured a reliable power supply during peak demand, while advanced waste segregation reduced the logistical burden on hospital personnel. In regions prone to drought or resource scarcity, water conservation systems proved essential for maintaining uninterrupted medical services. This enhanced reliability is critical for the core mission of healthcare, especially in rural or resource-limited settings where operational disruptions can have severe consequences for patient safety.

On a global scale, the adoption of sustainable practices aligns healthcare infrastructure with the United Nations Sustainable Development Goals, specifically regarding health, well-being, and climate action. By leading in environmental stewardship, the healthcare sector can serve as a role model for other industries, creating a ripple effect of sustainable transition across society. However, the study emphasizes that these strategies must be carefully tailored to local contexts; for instance, while solar energy is highly effective in high-insolation regions, rainwater harvesting remains a priority in areas with erratic water supplies. Recognizing these regional disparities is essential for ensuring that the benefits of green technology are distributed equitably.

The transition toward sustainable healthcare should ideally begin with small-scale pilot projects, such as auxiliary solar installations or basic recycling initiatives, which build the necessary momentum for larger investments. Successful implementation further requires the active engagement of stakeholders—including staff, patients, and local communities—to foster ownership of these initiatives. Additionally, leveraging external technical and financial support from governmental and private partners can help offset initial costs. Continuous monitoring and transparent reporting are also vital to track performance and support ongoing improvement. Ultimately, achieving a sustainable future for the healthcare sector requires coordinated action; it is an imperative that demands supportive policy frameworks, institutional commitment, and a shared vision of environmental responsibility to ensure the continued well-being of both humanity and the planet.

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Author Contributions

Conceptualization & investigation, M.K.M., Š.S., T.P., and S.B.; Writing – original draft, review & editing, M.K.M., Š.S., T.P., and S.B. All authors have read and approved the published version of the manuscript.

Statement of Competing Interests

The authors declare no relevant conflicts of interest.

Statement of Generative AI Use

No generative AI was used.

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ORIGINAL ARTICLE

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COMPARATIVE STUDY OF THE ANTIBACTERIAL, ANTI-INFLAMMATORY, AND ANTIOXIDANT EFFECTS OF HONEY, PROPOLIS, AND ROYAL JELLY PRODUCED BY THE APIS MELLIFERA BEE

Mabrouka Bouacha[1,2] **Ines Boudiar**[1]

[1]Laboratory of Microbiology and Molecular Biology, Badji Mokhtar University Sciences Faculty, Annaba, Algeria [2]Laboratory of Biochemistry and Environmental Toxicology, Badji Mokhtar University Sciences Faculty, Annaba, Algeria

Honey, propolis, and royal jelly are natural products of bees that have been used for medicinal purposes for centuries. The aim of this study was to investigate and compare the therapeutic effects of two samples of honey, two of propolis, and two of royal jelly. All hive products were produced by the *Apis mellifera* bee and collected from two regions of Algeria. The study evaluated the antibacterial activity of hive products using agar well-diffusion and microbroth dilution assays. The agar well-diffusion assay involved inoculating agar plates with ten different bacterial strains, while the micro-broth dilution assay involved serial dilutions of hive products and bacterial cultures. The antioxidant potential was assessed using scavenging assays of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric-reducing antioxidant power (FRAP) assays, which measured the product's ability to donate electrons and neutralize DPPH free radicals. The albumin denaturation method evaluated the anti-inflammatory properties of the hive products by measuring the extent of inhibition of protein denaturation under heat stress. The results demonstrated significant differences in the antibacterial effects of the tested products; honey samples showed greater antibacterial activity than propolis and royal jelly samples. Gram-positive bacteria were more susceptible to the antibacterial effects than Gram-negative bacteria. Regarding antioxidant and anti-inflammatory properties, honey samples were more effective than propolis and royal jelly samples. Propolis sample 1 had the highest concentration of polyphenols and flavonoids. All hive products exhibited potent antibacterial, antioxidant, and anti-inflammatory effects, making them a promising natural supplement for combating various health issues associated with bacterial infections, oxidative stress, and inflammation.

Keywords: *Apis mellifera*, honey, propolis, royal jelly, therapeutic properties

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Correspondence to:
Mabrouka Bouacha
Laboratory of Microbiology and Molecular Biology
Badji Mokhtar University Sciences Faculty
Annaba, Algeria
E-mail: bouacha.mabrouka@yahoo.fr

INTRODUCTION

The *Apis mellifera* bee is a social insect belonging to the genus *Apis* and the family *Apidae*. It is of considerable economic and cultural importance for human society as its role in crop pollination is essential for global food production (1). In addition, the *Apis mellifera* bee provides natural products such as propolis, pollen, venom, royal jelly, and honey with many potential healthy qualities, including antimicrobial (2,3), antimutagenic (4), antioxidant (5), and antitumoral effects (6). Commercialization and export of *Apis mellifera*'s products are a source of income for several countries worldwide, especially for honey production (1,7). Moreover, due to their composition of several bioactive substances, natural bee products are increasingly used in research to develop new treatment alternatives for several diseases (8).

Several properties of honey contribute to its antimicrobial effects. It has a pH between 3.2 and 4.5, which inhibits the growth of most pathogenic microorganisms (9,10). It contains a very high concentration of sugar (glucose and fructose), which dehydrates bacteria and other microorganisms. It also contains glucose oxidase, an enzyme that produces hydrogen peroxide (H_2O_2), which is responsible for the inhibition of bacterial growth. Other factors contribute to the antibacterial effect of honey, such as lysozymes, flavonoids, polyphenols, and other aromatic substances derived from foraged plants (11). In addition, honey has antioxidant activity, preventing oxidation by neutralizing free radicals in the body, which can damage cells, including DNA being also responsible for cell aging. The antioxidant properties of honey are attributed to many biological substances, such as phenolic acids, flavonoids, vitamins, enzymes, and minerals (12). Moreover, honey could also have anti-inflammatory and antioxidant properties, which are associated with each other through multiple cellular signaling pathways (13). Honey has been found to inhibit inflammation, oxidative stress, and gene expression pathways. Inflammation is a crucial part of the body's immune response, but chronic inflammation can lead to diseases like arthritis and cardiovascular disorders. Honey's polyphenolic and flavonoid content can modulate these processes by acting as agonists or inhibitors of key pro-inflammatory cytokines. Honey's antioxidants help counteract oxidative stress by scavenging free radicals and enhancing the body's antioxidant defenses. By addressing both inflammation and oxidative stress, honey not only addresses individual issues but also disrupts the cycle that perpetuates their coexistence, contributing to its therapeutic potential in preventing and managing chronic inflammatory and oxidative stress-related diseases (5,6,13).

Royal jelly is the most elaborate substance of the hive. It is intended for the feeding of queens. It is a creamy, whitish-to-yellowish secretion composed of water (about 66%), carbohydrates (about 14.5%), fatty acids (about 4.5%), including essential fatty acids, and proteins (about 13%), as well as essential amino acids. The antibacterial activity of royal jelly is mainly due to its acidic pH, trans-10-hydroxy-2-decenoic acid, and proteins with glucose-oxidase activity, such as major royal jelly proteins, defensin, royalisin, jelleins, and apisimin (14,15).

Propolis, a resinous substance collected by bees from various botanical sources, is used as a building material to fortify hives and protect them from environmental threats. It fills gaps, seals cracks, and minimizes heat loss during cold days. Propolis also exhibits antimicrobial properties, reducing infection risk and inhibiting the growth of fungi and bacteria, thereby creating a hygienic environment for bees (16). Propolis contains polyphenols, terpenoids, flavonoids, and aromatic acids, which are responsible for its strong antioxidant activity and help prevent the development of bacterial resistance (15,16). In addition to reducing bacterial mobility, propolis may disturb membrane potential, interfere with permeability of the microbe's cell membrane, and inhibit ATP generation (16).

Few studies were conducted to compare the therapeutic effects of beehive products; therefore, in this study, we aimed to evaluate the antibacterial, antioxidant, and anti-inflammatory effects of beehive products (honey, propolis, and royal jelly) from two regions of Algeria.

METHODS

The study analyzed two samples each of honey, propolis, and royal jelly produced by *Apis mellifera* bees from a semi-arid region in central Algeria and a humid region in eastern Algeria. The floral origin of the samples was determined based on the plant species present in the vicinity of each hive. All samples were kept at 6°C until their analysis. Honey and royal jelly were diluted using distilled water. For propolis, 1 g of propolis powder was homogenized and mixed with 10 mL of 80% (v/v) ethanol/water solution for 72 hours and kept at 70 °C for 1 hour. The resulting mixtures were filtered and then evaporated. Subsequently, all samples were passed through 0.22 µm microfilters to eliminate bacterial contamination. A series of dilutions was then prepared at

Table 1. *Color, pH value, and floral and geographical origin of hive bee products*

Samples	Color	pH	Floral origin	Region	Climate
Honey 1	Dark brown	3.15 ± 0.02	*Ziziphus sp, Brassica sp, Erica arborea, Carduus type, Lotus sp, Xanthium sp.*	Djelfa	Semi-arid
Propolis 1	Dark brown	4.19 ± 0.02			
Royal jelly 1	Cream	3.91 ± 0.02			
Honey 2	Brown	3.47 ± 0.01	*Trifolium sp, Eucalyptus sp, Rosmarinus sp, Hedera helix, Convolvulacea, Chrozophora tinctoria*	Annaba	Humid
Propolis 2	Dark brown	5.26 ± 0.03			
Royal jelly 2	Cream	3.24 ± 0.03			

concentrations of 2.5%, 5%, 10%, 20%, 40%, 60%, 80%, and 100% (v/v).

Antibacterial effect assays

The evaluation of the antibacterial activity of the samples was carried out according to the methods described by Bouacha et al., against ten multidrug-resistant bacteria isolated from infected wounds (8). These included Gram-negative bacteria: *Escherichia coli, Enterobacter aerogenes, Citrobacter koseri, Proteus mirabilis, Klebsiella pneumoniae, K. oxytocae, and Pseudomonas aeruginosa,* and Gram-positive bacteria: *Staphylococcus aureus., S. saprophyticus, and Enterobacter faecalis*. An inoculum of each bacterium was prepared by transferring one to two well-isolated, morphologically identical colonies into nutrient broth (Difco, MD, USA). The optical density was measured and adjusted to 0.08–0.10 at 625 nm.

Agar diffusion assay

The study used a standard technique to evaluate the efficacy of antimicrobial agents, specifically honey. Mueller Hinton agar (Difco, MD, USA) was prepared and poured into sterile Petri dishes, and wells of 6 mm in diameter were created as reservoirs for honey samples. Each plate was inoculated with a bacterial suspension standardized to an appropriate concentration. After inoculation, 50 µL of honey dilution was dispensed into each well, and the plates were incubated at 37°C for 24 hours. The antibacterial activity was assessed by measuring the diameter of clear zones of inhibition around the wells. The size of these zones correlated with the antibacterial potency of a honey sample, with larger zones indicating stronger antibacterial activity.

Microdilution assay

Ninety-six-well microplates (Fisher Scientific, UK) were used to determine the minimum inhibitory concentration (MIC) of honey against antibacterial activity. For this test, 100 µL of bacterial inoculum was mixed in each well with 100 µL of honey at various dilutions. Wells containing inoculum without honey served as positive controls, while broth alone served as the negative control. The microplates were incubated at 37°C for 24 hours to cultivate bacteria.

Bacterial growth was evaluated by absorbance at 620 nm to derive MIC values. Formula for calculating growth inhibition: Inhibition (%) = $\left(\frac{A\ test - A\ control}{A\ test}\right) \times 100$, where A control and A test were the absorbance values of the control and test samples, respectively. The lowest honey dilution that inhibited bacterial growth 100%, showing no well growth, was the MIC.

To determine the minimal bactericidal concentration (MBC), 10 µL from wells without growth was plated onto nutrient agar plates. These plates were incubated at 37°C for 24 hours. The MBC was defined as the lowest honey dilution at which no bacterial growth occurred, demonstrating its bactericidal properties. To compare the honey's bacteriostatic (growth-inhibiting) and bactericidal (bacteria-killing) activities, the MBC/MIC ratio was calculated.

Antioxidant effect

Total polyphenolic content determination

The quantification of total polyphenols was carried out using the Folin-Ciocalteu assay (17). A 5 mL aliquot of a methanolic hive product solution (1g in 5 mL) was mixed with 2.4 mL of distilled water and 200 µL of Folin-Ciocalteu reagent. After a 3-minute reaction period, 0.6 mL of a 20% sodium carbonate solution was added to the mixture. The samples were incubated in the dark at 25 °C for two hours. The absorbance of the reaction was measured at 725 nm using a spectrophotometer. Calibration was performed using various concentrations of gallic acid, and results were expressed in milligrams of gallic acid equivalents per 100 grams of honey (mg GAE/100g).

Flavonoids determination

Flavonoid content was determined using a colorimetric method involving aluminum chloride (18). A 1 mL sample of a 2% $AlCl_3$ solution was mixed with 1 mL of a methanolic solution of hive product (1 mg/mL). The mixture was allowed to react at 24 °C for 40 minutes. Absorbance readings were taken at 430 nm. A calibration curve was constructed using quercetin standards at concentrations of 20, 40, 60, 80, and 100 mg/L. Results were reported as milligrams of quercetin equivalents per gram of hive product (mg QE/g).

Ferric reducing/antioxidant power assay

The Ferric Reducing Antioxidant Power (FRAP) assay was performed following the method outlined by Beretta et al. (17). This colorimetric assay measures the change in absorbance at 593 nm, reflecting the reduction of colorless Fe^{3+}-TPTZ (2,4,6-tripyridyl-s-triazine) to blue Fe^{2+}-TPTZ by electron-donating antioxidants. Hive products were prepared at a concentration of 1 g/mL in double-distilled water. A 200 µL aliquot of the honey solution was combined with 1.8 mL of FRAP reagent, consisting of 10 mmol/L TPTZ in 40 mmol/L HCl, 20 mmol/L $FeCl_3$, and 0.3 mol/L acetate buffer at pH 3.6. After incubating at 37 °C for 10 minutes, absorbance was measured at 593 nm. The antioxidant capacity was quantified in µmol/L of ascorbic acid equivalents per gram of honey (µmol/L AAE/g), using a standard curve prepared with ascorbic acid concentrations from 20 to 700 µmol/L.

1,1-Diphenyl-2-Picrylhydrazyl (DPPH) assay

The DPPH radical scavenging capacity was assessed using a modified method. A 2.7 mL solution of methanolic DPPH (6.10^{-5} mol/L) was mixed with 0.3 mL of honey. After vortexing the mixture at 2500 rpm for 1 minute, the solution was incubated in the dark for one hour. Following this incubation, the absorbance was measured at 517 nm after an additional 15-minute incubation at 25°C. Ascorbic acid served as the positive control. The DPPH radical scavenging activity (%) was calculated by taking the difference between the absorbance of the control and the sample, dividing it by the absorbance of the test, and then multiplying the result by 100. Using a calibration curve ranging from 0 to 10 mg/L of ascorbic acid, the IC50 value, which represents the honey concentration required to scavenge 50% of DPPH radicals, was determined.

Anti-inflammatory effect

The albumin denaturation method, with a slight modification, was used to evaluate the anti-inflammatory activity in vitro, as described by Ali et al. (19). At varying concentrations, the samples were combined with a 1% water-based bovine serum albumin fraction in test tubes. Following a 15-minute incubation period at 37°C, the tubes were subjected to a 10-minute heating cycle at 70°C. Absorbance was measured spectrophotometrically at 660 nm. Distilled water was used as the negative control, while aspirin at the same concentrations (2.5%, 5%, 10%, 20%, 40%, 50%, 80%, and 100%) served as the positive control. The following formula was used to compute the percentage of inhibition of albumin denaturation = $\left(\frac{A2-A1}{A2}\right) \times 100$.

In this case, A1 represents honey's absorbance, and A2 represents the control's absorbance (distilled water).

Data analysis

Results were expressed as mean values ± standard deviation (SD), and the study used triple analyses to guarantee accuracy and reliability. The GraphPad Prism software was used to conduct the statistical analysis. To differentiate between treatments, a one-way analysis of variance (ANOVA) was employed. When comparing two sets of data, Tukey's post hoc test was used. Statistical analysis was performed with significance set at $p < 0.05$, supporting the reliability of the observed differences.

RESULTS

Antibacterial activity

As shown in Figure 1 and Table 2, the antibacterial activities of hive products have been thoroughly investigated. The results showed that the tested products varied significantly; honey samples outperformed royal jelly and propolis. Furthermore, the hive products had a greater impact on Gram-positive bacteria compared to Gram-negative bacteria. According to the MBC/MIC ratios, none of the bee products were bacteriostatic; rather, they were bactericidal. This indicates that the hive products could kill the bacteria and not just inhibit their growth. These findings indicate that honey, royal jelly, and propolis all exhibit antibacterial activity and may serve as natural antibacterial agents, with honey showing the highest efficacy.

Antioxidant effect

The determination of total phenolic content, flavonoid content, and antioxidant effects of hive products is reported in Figure 2. The total polyphenolic content of honey sample 1 was found to be relatively high in comparison with those of propolis and royal jelly. Flavonoid content also varied significantly; propolis collected from the Djelfa region has the highest concentration. Honey samples exhibited the highest antioxidant activity, followed by propolis and royal jelly samples.

Anti-inflammatory effect

The results of the anti-inflammatory activities of hive products determined by the membrane stabilization method are presented in Figure 3. The hive products showed a good anti-inflammatory activity. Honey and royal jelly samples were more effective than propolis.

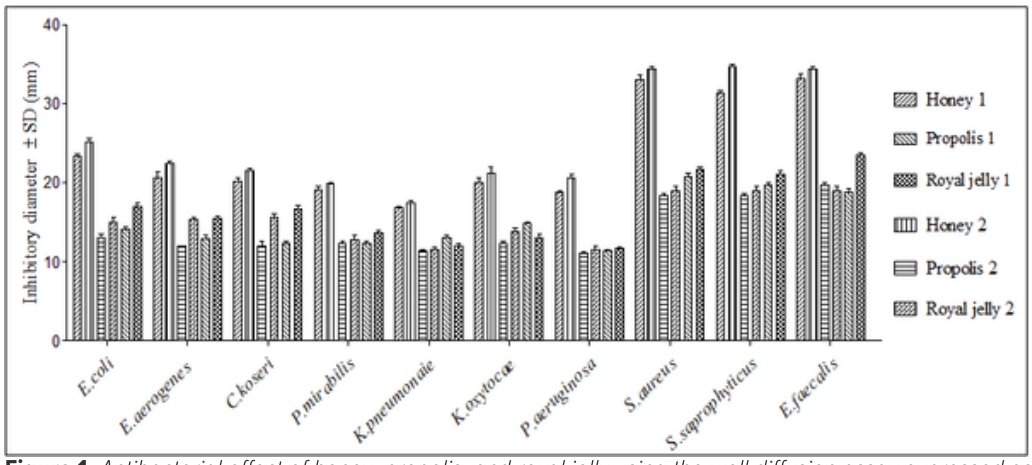

Figure 1. *Antibacterial effect of honey, propolis, and royal jelly using the well diffusion assay expressed as inhibitory diameters ±SD (mm)*

Table 2. *MIC, MBC, and MBC/MIC ratio of honey, propolis, and royal jelly*

	Honey 1		Honey 2		Propolis 1		Propolis 2		Royal jelly 1		Royal jelly 2	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli	20.0	40	20	20	40	60	40	80	40	60	40	80
E. aerogenes	20.0	20	20	20	40	40	40	80	40	60	20	60
C. koseri	20.0	20	20	20	40	40	40	80	60	60	40	80
P. mirabilis	40.0	80	40	60	40	60	40	80	40	60	40	80
K. pneumoniae	40.0	100	40	60	40	100	40	100	40	100	40	100
K. oxytocae	40.0	60	40	80	40	80	40	80	40	100	40	100
P. aeruginosa	40.0	60	40	80	40	60	40	100	60	100	40	100
S. aureus	02.5	05.0	02.5	05.0	10	20	10	20	10	20	20	40
S. saprophyticus	02.5	05.0	02.5	05.0	5	10	10	20	10	20	20	40
E. faecalis	05.0	05.0	05.0	10.0	10	20	10	20	10	20	20	40
MBC/MIC ratio	01.0-02.5		01.0-02.0		01.0-2.0		01.0-02.5		01.0-02.5		01.0-03.0	

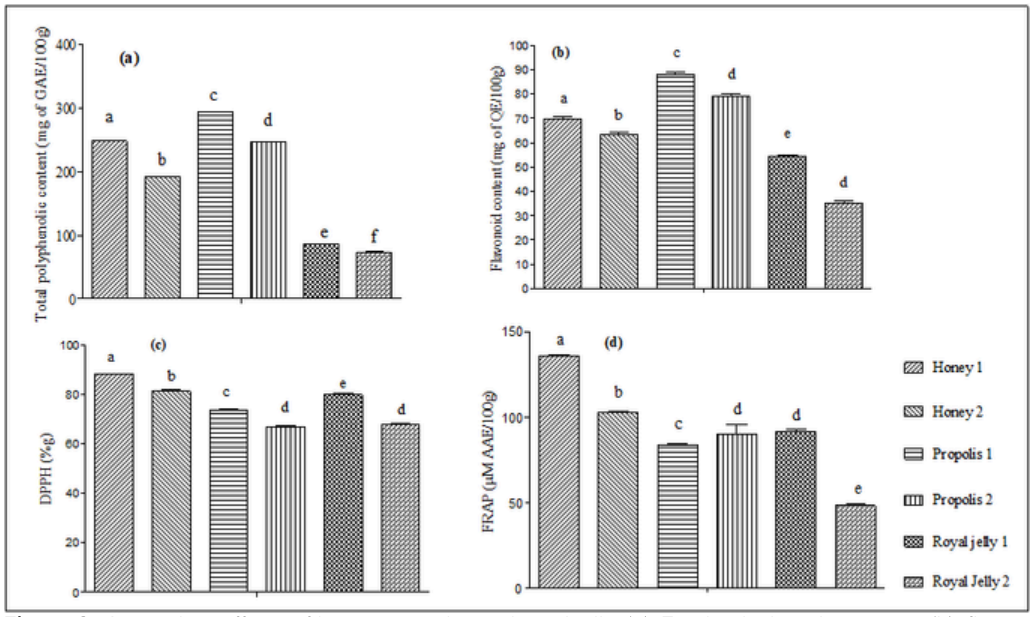

Figure 2. *Antioxidant effects of honey, propolis, and royal jelly. (a): Total polyphenolic content, (b): flavonoid content, (c): DPPH, (d): FRAP. There are significant differences between samples with different letters.*

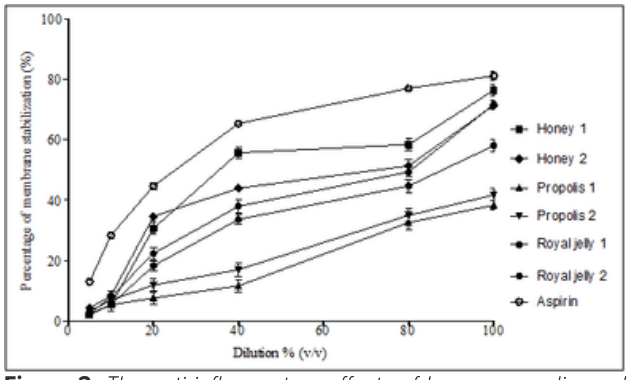

Figure 3. *The anti-inflammatory effects of honey, propolis, and royal jelly.*

DISCUSSION

Hive products showed diversity in their bioactive components, contributing to their effectiveness against a variety of microorganisms, including multidrug-resistant bacteria. As reported in Figure 1 and Table 2, the antibacterial effect of honeybee products varied significantly. Honey samples showed greater efficiency than the other products. Similarly, (20) have also shown that honey exhibited the highest antibacterial activity against *E. coli* and *S. typhimurium* strains. The effectiveness of honey as an antibacterial agent can be attributed to several factors, such as acidity, glucose oxidase, and lysosomes, which may act in a synergetic manner to inhibit the growth and viability of pathogenic microorganisms (10). Similar findings have been previously reported by many authors (2,3,21). The mechanism of the antibacterial action of hive products is not well known. However, some authors suggest that they cause a disruption of membrane potential and permeability, which may contribute significantly to overall cytotoxicity, affecting bacterial viability. In addition, Gram-positive bacteria were more susceptible to hive products than Gram-negative bacteria. This is due to the structure of the outer membrane of Gram-negative bacteria. Indeed, Gram-positive and Gram-negative bacteria have a cell wall that protects the cytoplasmic membrane. In addition, Gram-negative bacteria have additional protection offered by the polysaccharide-rich outer membrane (11). Moreover, the tested hive products exhibit a bactericidal effect on both Gram-negative and Gram-positive bacteria, as their MBC/MIC ratios are between 1 and 3. This ratio is essential for differentiating between a bacteriostatic agent, which prevents bacterial proliferation without killing the bacterium, and a bactericidal agent, which destroys and kills the bacterial cell. According to O'Neill and Chopra (22) an antimicrobial agent is bactericidal when the MBC/MIC ratio is less than or equal to 4; therefore, the tested hive products exhibited a bactericidal effect against multidrug-resistant bacteria (22). Many authors previously reported that honey (3,11,23,24) and propolis (25–27) displayed a bactericidal effect. However, García et al. (14) reported that two royal jelly samples had a bacteriostatic effect.

The determination of the total polyphenolic content, flavonoid content, and antioxidant effects of hive product samples (Figure 2) showed significant differences among them. Propolis had the highest TPC and TFC content when compared with honey and royal jelly. However, honey had the highest antioxidant activity, suggesting that it may contain other compounds with antioxidant effects that can protect against oxidative stress. Similarly, propolis and royal jelly have also been found to have antioxidant properties due to their high phenolic and flavonoid content. Several mechanisms contribute to the antioxidant effects of hive products, including free-radical scavenging, hydrogen donation, metal ion chelation, quenching of singlet oxygen, and acting as a substrate for superoxide and hydroxyl radicals. Many authors report a strong correlation between phenolic compounds and the antioxidant activity of bee products, which is influenced by botanical source, geographic and entomological origin, and climatic conditions. (3,11,28,29). However, hive products also contain other substances with antioxidant effects, such as minerals, amino acids, peptides, proteins, organic acids, and enzymes (15,30). Similar results were previously reported by Nagai et al. (31). In this study, commercial honey, royal jelly, and propolis were found to have a significant antioxidant activity, as measured by their ability to scavenge free radicals and inhibit lipid peroxidation (32). Mouhoubi-Tafinine et al. also found that honey samples had a higher antioxidant activity than propolis samples (12). In contrast, Postali et al. reported that propolis showed the highest antioxidant activity (33). Buratti et al. also found that propolis samples had a higher antioxidant capacity than honey (34). Indeed, many factors could affect the similarity or divergence in the antioxidant effect of hive products, including bee species, geographic region, plant species, forage, harvest, and storage conditions. Additionally, the methods used to evaluate antioxidant activity can significantly impact results. Variations in sample dilution, extraction methods, and other conditions can affect the concentration of bioactive compounds and their potency. Criteria for reporting findings can also cause discrepancies in results.

Therefore, a standardized approach is crucial for reliable and comparable results in antioxidant activity evaluations.
Based on the results in Figure 3, all hive products exhibited the anti-inflammatory effect; however, honey samples were found to have the most potent anti-inflammatory effects, followed by royal jelly and propolis. The anti-inflammatory activity of honey is attributed to its capacity to block the synthesis of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β) (35). The antioxidant and anti-inflammatory properties of hive products are closely connected and often interdependent. These effects are largely attributed to the presence of polyphenols and flavonoids, which can modify the immune response, decrease pro-inflammatory cytokine production, scavenge free radicals, and alleviate oxidative stress (15,21).
The study demonstrated that honey, propolis, and royal jelly have significant bactericidal effects against a variety of microorganisms, including multidrug-resistant bacteria. All hive products showed good antioxidant and anti-inflammatory properties due to their phenolic and flavonoid content. Honey samples exhibited the strongest antibacterial, antioxidant, and anti-inflammatory properties. Overall, bee products show potential as natural therapeutic alternatives for various health conditions. However, further research is needed to better understand their mechanisms of action and to establish optimal dosages and formulations for effective use.

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Authors' Contributions

Conceptualization, M.B. and I.B.; Methodology, M.B. and I.B.; Investigation, M.B.; Formal Analysis, I.B.; Writing – original draft, M.B.; Writing – review & editing, M.B. and I.B. Both authors have read and approved the published version of the manuscript.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availability

The datasets generated and analyzed during the current study are available from the corresponding author.

Statement of Generative AI Use

No generative AI was used.

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AFMN BIOMEDICINE

ORIGINAL ARTICLE

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RELIABILITY OF ELISA TEST IN THERAPEUTIC DRUG MONITORING OF ADALIMUMAB AND INFLIXIMAB IN PEDIATRIC INFLAMMATORY BOWEL DISEASE

Jasmina Katanić[1,2] **Dejan Dobrijević**[1,2] **Mirjana Stojšić**[1,2]

[1]Institute for Children and Youth Health Care of Vojvodina, Novi Sad, Serbia [2] University in Novi Sad Faculty of Medicine, Novi Sad, Serbia

Inflammatory bowel disease (IBD) is a significant cause of morbidity and disability in pediatric patients, characterized by chronic intestinal inflammation. Biologic therapies, such as adalimumab and infliximab, are widely used for induction and maintenance of remission. Therapeutic drug monitoring (TDM) enables individualized treatment by optimizing drug dosage based on serum concentrations. The aim of this study was to evaluate the reliability of the enzyme-linked immunosorbent assay (ELISA) for determining serum levels of adalimumab and infliximab in pediatric patients with IBD. This retrospective study analyzed serum samples of 40 pediatric patients, divided into two groups: 20 receiving adalimumab or infliximab therapy and 20 controls. Serum levels were measured using the RIDASCREEN ELISA kits (R-Biopharm AG) and the Dynex DS2 analyzer. Method reliability was assessed through precision, accuracy, sensitivity, and specificity. Calibration curves demonstrated high reliability, with coefficients of determination (R^2) of 0.998 (adalimumab) and 0.999 (infliximab). Precision, indicated by coefficients of variation, was 4.3% for adalimumab and 4.1% for infliximab. Accuracy, measured by bias, was 3.75% for adalimumab and 0.05% for infliximab. Sensitivity and specificity were both 100%, confirming the test's ability to accurately detect or exclude drug presence in serum samples. R-Biopharm AG ELISA-based TDM provides reliable, precise, and accurate results for monitoring adalimumab and infliximab levels in pediatric patients. These findings support its use as a gold standard for individualized treatment optimization in IBD.

Keywords: inflammatory bowel disease, biologic therapies, adalimumab, infliximab, therapeutic drug monitoring, ELISA

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Correspondence to:
Dejan Dobrijević
Institute for Children and Youth Health Care of Vojvodina
Hajduk Veljkova 10, Novi Sad, Serbia
E-mail: dejan.dobrijevic@mf.uns.ac.rs

INTRODUCTION

Inflammatory bowel disease (IBD) is a group of chronic, relapsing-remitting inflammatory conditions affecting the gastrointestinal tract, primarily encompassing Crohn's disease (CD) and ulcerative colitis (UC). The prevalence of pediatric IBD onset has been increasing globally, particularly in developed countries, suggesting a strong interplay between genetic predisposition and environmental factors. Although the precise etiology remains unknown, IBD is believed to result from an inappropriate immune response to intestinal microbiota in genetically susceptible individuals (1, 2).

The incidence of pediatric IBD varies across different geographic regions. In Europe, the incidence of IBD in children is estimated at 23 per 100,000, whereas in North America, it is approximately 15 per 100,000. The rising prevalence in developing countries suggests that environmental factors such as diet, hygiene, and antibiotic use play a crucial role in disease pathogenesis (1-4).

Genetic factors also contribute significantly to IBD susceptibility. Genome-wide association studies have identified multiple risk loci, including genes involved in immune regulation, epithelial barrier integrity, and microbial interactions. However, environmental factors such as early antibiotic exposure, a diet rich in processed foods, and reduced microbial diversity due to improved hygiene are believed to modulate disease expression.

IBD pathogenesis involves a complex interplay of immune dysregulation, genetic susceptibility, and environmental triggers. The disruption of the gut barrier function allows microbial antigens to activate immune cells, leading to chronic intestinal inflammation. Key cytokines involved in this inflammatory cascade include tumor necrosis factor-alpha (TNF-α), interleukin-12 (IL-12), and interleukin-23 (IL-23) (5,6).

Clinically, pediatric IBD presents with a wide spectrum of symptoms. CD can affect any segment of the gastrointestinal tract and is characterized by transmural inflammation, leading to complications such as strictures and fistulae. In contrast, UC primarily involves the colon and is limited to the mucosal layer, often presenting with bloody diarrhea and urgency. Systemic manifestations, including growth retardation, weight loss, and extraintestinal manifestations (e.g., arthritis, uveitis, and erythema nodosum), are common in pediatric patients (7-10).

The diagnosis of IBD relies on a combination of clinical assessment, laboratory tests, imaging, and endoscopic evaluation with histopathological confirmation. Laboratory markers such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and fecal calprotectin are useful in assessing disease activity. Endoscopic findings, including skip lesions in CD and continuous colonic involvement in UC, aid in differentiation (11-16).

The therapeutic approach to pediatric IBD has evolved significantly with the introduction of biologic therapies targeting specific inflammatory pathways. TNF-α inhibitors such as infliximab and adalimumab have demonstrated efficacy in inducing and maintaining remission in moderate-to-severe cases. The optimization of biologic therapy through therapeutic drug monitoring (TDM) ensures adequate drug exposure while minimizing immunogenicity and adverse effects (17-20).

Due to the central role of TNF-α in the pathogenesis of IBD, it has become a key target for biological therapy. Biological drugs such as infliximab and adalimumab are monoclonal antibodies that specifically inhibit TNF-α activity, preventing its binding to receptors, thereby reducing inflammation. This allows for healing of the intestinal mucosa, reduction of symptoms, and long-term maintenance of remission in patients with IBD (21-27).

The quality of life in children with IBD varies depending on several factors, including the severity of the disease, response to therapy, presence of complications, and the child's overall health condition. CD and UC are chronic and often unpredictable diseases, carrying a certain degree of disability. However, with timely diagnosis, appropriate treatment, and adequate psychological support, long periods of remission and good quality of life can be achieved (28,29).

The enzyme-linked immunosorbent assay (ELISA) is widely utilized for measuring serum drug levels and detecting anti-drug antibodies in IBD patients receiving biologic therapy. ELISA is based on antigen-antibody interactions and provides a cost-effective and reliable method for quantifying therapeutic agents such as adalimumab and infliximab. The application of ELISA in TDM allows clinicians to adjust drug dosing based on serum concentrations, thereby optimizing treatment outcomes (30).

Recent studies have highlighted the clinical benefits of proactive TDM using ELISA. Maintaining optimal drug levels is associated with better mucosal healing rates, prolonged remission, and reduced need for corticosteroids. Furthermore, early detection of antidrug antibodies enables timely intervention, such as dose escalation or switching to alternative therapies (31,32).

Given the increasing reliance on biologic therapies in pediatric IBD, accurate and reliable methods for serum drug monitoring are essential. The aim of this study was to assess the reliability of an ELISA-based method for determining adalimumab and infliximab serum concentrations in pediatric patients. By evaluating calibration, precision, accuracy, sensitivity, and specificity, we aimed to validate the clinical utility of ELISA in therapeutic drug monitoring.

METHODS

Study design and participants

This retrospective study was conducted at the Institute for Health Protection of Children and Youth of Vojvodina. It included 40 pediatric patients, of whom 20 were receiving adalimumab or infliximab therapy, while the remaining 20 served as controls. Inclusion criteria were pediatric patients (<18 years) with confirmed IBD treated with adalimumab or infliximab, with at least one ELISA-based serum drug level available, complete medical records, and documented clinical data; control patients were age-matched, not receiving biologic therapy, and without inflammatory or autoimmune diseases. Exclusion criteria included incomplete documentation, missing serum levels, use of other biologics, known immunodeficiencies or severe comorbidities, and, for controls, presence of IBD or chronic inflammatory conditions.

Sample collection and processing

Serum samples were collected using vacuum tubes with clot activators (Becton Dickinson, New Jersey, USA). The samples were centrifuged at 4000 rpm for 5 minutes (Rotofix 32A, Hettich, Tuttlingen, Germany), and the serum was separated and stored at -20°C until analysis, with a maximum storage period of three months.

ELISA analysis

Serum concentrations of adalimumab and infliximab were determined using RIDASCREEN ADM Monitoring and RIDASCREEN IFX Monitoring kits (R-Biopharm AG, Darmstadt, Germany) on a DYNEX DS2 automated ELISA analyzer (Dynex Technologies, Chantilly, Virginia, USA). The method was based on a sandwich ELISA technique where TNF-α molecules were immobilized on microtiter wells. The analysis included the following steps:

1. Incubation of serum samples (100 µL per well) at 37°C for 1 hour, allowing the drug to bind to TNF-α.
2. Washing and addition of enzyme-conjugated antibody (100 µL per well) followed by incubation at 37°C for 30 minutes.
3. Addition of substrate solution (hydrogen peroxide) and incubation for 10 minutes, producing a color reaction.
4. Stopping the reaction with sulfuric acid (50 µL per well), followed by spectrophotometric measurement at 450 nm with a 620 nm reference filter.

Calibration and quality control

Calibration was performed using six standard solutions to generate a sigmoidal calibration curve. The accuracy of the calibration was assessed using the coefficient of determination (R2). Quality control was conducted using control sera with predefined low (8–16 µg/mL) and high (20–40 µg/mL) concentration ranges.

Statistical analysis

Method reliability was assessed through precision, accuracy, sensitivity, and specificity. Precision was expressed as the coefficient of variation (CV), accuracy as bias percentage, and diagnostic performance via sensitivity and specificity calculations. Statistical analysis was performed using Microsoft Excel 2021 Professional Plus.

Ethical approval

This study was approved by the Ethics Committee of the Institute for Health Protection of Children and Youth of Vojvodina (Approval No. 3987-8, August 15, 2024). All procedures were conducted in accordance with the Declaration of Helsinki.

RESULTS

The reliability of the method for determining serum concentrations of adalimumab and infliximab was examined through precision, accuracy, sensitivity, and specificity, after calibration and quality control were performed.

Calibration

Calibration curves for determining the serum concentrations of adalimumab and infliximab were successfully created, with a coefficient of determination of 0.998 for adalimumab (Figure 1) and 0.999 for infliximab (Figure 2).

Quality control

The obtained concentrations of adalimumab and infliximab in control sera are shown in Table 1.

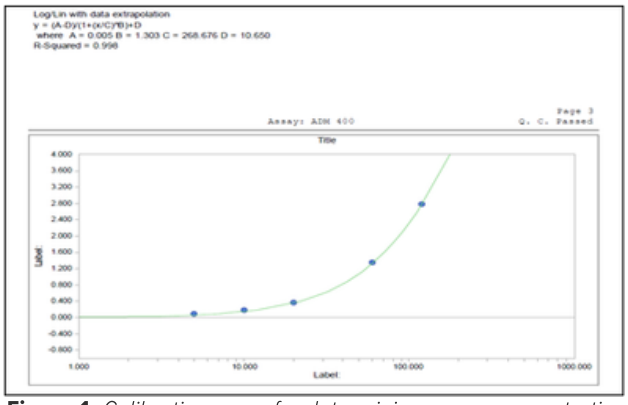

Figure 1. *Calibration curve for determining serum concentration of adalimumab*

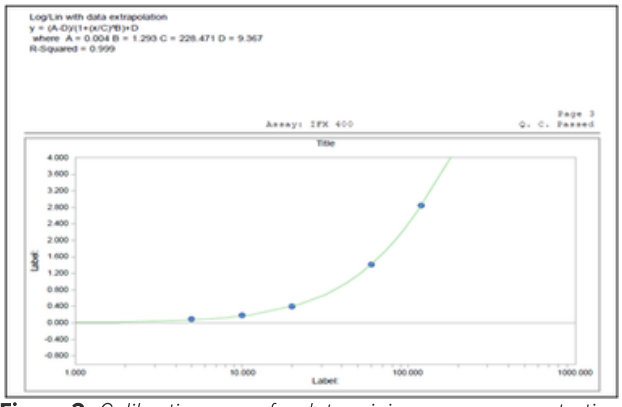

Figure 2. *Calibration curve for determining serum concentration of infliximab*

Precision

The coefficient of variation for determining serum concentration of adalimumab using the ELISA method in the Laboratory Diagnostics Department of the Institute for Health Care of Children and Youth Vojvodina was 4.3% (x = 13.229, SD = 0.572).

The coefficient of variation for determining serum concentration of infliximab using the ELISA method in the Laboratory Diagnostics Department of the Institute for Health Care of Children and Youth Vojvodina was 4.1% (x = 22.165, SD = 0.904).

Accuracy

The inaccuracy, or dispersion (d), in determining serum concentration of adalimumab using the ELISA method in the Laboratory Diagnostics Department of the Institute for Health Care of Children and Youth Vojvodina was 3.75%.

The inaccuracy, or dispersion (d), in determining serum concentration of infliximab using the ELISA method in the Laboratory Diagnostics Department of the Institute for Health Care of Children and Youth Vojvodina was 0.05%.

Sensitivity

In all samples from children receiving adalimumab, the drug was successfully detected (with no false negative results), confirming the test's sensitivity of 100%.

In all samples from children receiving infliximab, the drug was successfully detected (with no false negative results), confirming the test's sensitivity of 100%.

Specificity

In no sample from children not receiving adalimumab was the drug detected (with no false positive results), confirming the test's specificity of 100%.

In no sample from children not receiving infliximab was the drug detected (with no false positive results), confirming the test's specificity of 100%.

DISCUSSION

The determination of serum concentrations of adalimumab and infliximab plays a crucial role in the individualization of therapy for patients with inflammatory bowel diseases.

The metabolism of adalimumab and infliximab primarily occurs in the liver through the reticuloendothelial system, but a significant portion of these drugs may be lost in the stool, especially in patients with active inflammatory bowel disease. This loss of the drug through stool represents one of the most important factors contributing to subtherapeutic drug levels in serum, which directly impacts the effectiveness of therapy. Such losses can be attributed to increased intestinal permeability and augmented protein turnover due to active inflammation, which necessitates adjustments to the therapeutic regimen.

Table 1. *Control serum concentrations for adalimumab and infliximab*

Control	Obtained value (μg/mL)	Target value (μg/mL)	Range (μg/mL)
Adalimumab – low control	11.137	12	8-16
Adalimumab – high control	27.786	28	20-40
Infliximab – low control	11.745	12	8-16
Infliximab – high control	26.275	28	20-40

According to recommendations, drug concentration should be measured in the following scenarios:
•After induction therapy: This measurement helps assess the primary response to therapy and identify patients who do not respond to treatment.
•Before maintenance therapy: This measurement enables dose adjustments before transitioning to long-term therapy, ensuring optimal drug levels.
•At any point when secondary loss of response occurs: Secondary loss of response may arise from the development of antibodies against the drug or due to reduced drug concentrations in serum.
Interpretation of serum drug and antibody levels is as follows:
•High drug level / Low antibody level: The drug is present in adequate concentrations with no significant development of antibodies. Optimization may involve switching to a drug outside the anti-TNF class if necessary.
•Low drug level / High antibody level: This indicates immune resistance. Switching to another anti-TNF drug within the same class is recommended.
•Low drug level / Low antibody level: This suggests an insufficient dose, prompting an increase in dosage or shortening the dosing interval.
The goal of therapy optimization is to achieve maximum therapeutic results without losing the response to treatment. This can be achieved through careful dosing that avoids episodic administration, which could lead to the development of antibodies. Additionally, combining with immunomodulators like azathioprine or methotrexate may reduce the risk of antibody development and enhance therapeutic effectiveness. Eliminating other negative factors, such as smoking, may further improve therapeutic outcomes.
Proactive monitoring through regular assessment of drug levels in serum (trough levels) enables early dose optimization, prevents biological relapses, and minimizes the development of immunogenicity, thus ensuring long-term improved therapeutic outcomes. This approach significantly reduces the risk of therapeutic failure and enhances the quality of life for patients (33-37).
Thanks to its simplicity and reliability, ELISA testing has become the gold standard for monitoring the levels of biological drugs. The aim of this study was to evaluate the reliability of the method for determining serum concentrations of adalimumab and infliximab in terms of precision, accuracy, sensitivity, and specificity at the Laboratory Diagnostics Department of the Institute for Health Care of Children and Youth of Vojvodina in Novi Sad.
Calibration curves for determining serum concentrations of adalimumab and infliximab showed extremely high precision, with a coefficient of determination of 0.998 for adalimumab and 0.999 for infliximab. The concentrations of the tested drugs in control sera were within the expected ranges, with minimal deviations from target values.
The precision of the ELISA test, measured by the coefficient of variation, was 4.3% for adalimumab and 4.1% for infliximab. These values indicate high consistency in repeated measurements, which is particularly important in clinical practice where tests are expected to be reliable and reproducible. Compared to other studies where the coefficient of variation for ELISA tests typically ranges from 5% to 10%, our results demonstrate superior precision in measurements, a critical factor for clinical decision-making.
The accuracy of measurements, expressed by a dispersion of 3.75% for adalimumab and 0.05% for infliximab, is of a high standard. This level of accuracy defines our tests as ideal choices for routine monitoring of serum concentrations of adalimumab and infliximab.
The obtained sensitivity of 100% indicates the excellent ability of the ELISA test to detect adalimumab and infliximab in all serum samples from children undergoing therapy. This result is crucial for avoiding false-negative results and ensuring accurate clinical decisions.
The specificity of the test was also 100%, indicating no false-positive results in children not receiving therapy, which further confirms the reliability of the ELISA method. Specificity is one of the most important aspects of diagnostic tests, and our results demonstrate that the tested ELISA assays can precisely differentiate between patients receiving therapy and those not receiving it, thus minimizing the risk of incorrect treatment (38-40).
Proactive monitoring of serum concentrations of adalimumab and infliximab plays a pivotal role in the personalized treatment approach for patients with inflammatory bowel diseases (IBD). The results of this study demonstrate that the method for determining serum concentrations of these drugs using the investigated ELISA test is highly reliable, as confirmed by the parameters of precision, accuracy, sensitivity, and specificity. This underscores the utility of the ELISA method as an effective tool for optimizing therapy and ensuring the best possible outcomes in IBD management.

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Authors' Contributions

Conceptualization, J.K. and D.D.; Methodology, J.K. and D.D.; Investigation, J.K., D.D. and M.S.; Formal Analysis, J.K., D.D. and M.S.; Writing – original draft, J.K.; Writing – review & editing, D.D. and M.S. All authors have read and approve the published version of the manuscript.

Statement of Ethics

This study was approved by the Ethics Committee of the Institute for Health Protection of Children and Youth of Vojvodina (Approval No. 3987-8, August 15, 2024). All procedures were conducted in accordance with the Declaration of Helsinki.

Statement of Competing Interest

The authors declare no relevant financial or non-financial conflicts of interest.

Statement of Data Availability

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

Statement of Generative AI Use

The authors declare that no generative artificial intelligence tools were used in the writing or preparation of this manuscript.

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AFMN BIOMEDICINE

ORIGINAL ARTICLE

MONITORING MICROCIRCULATION IN THE PALATAL MUCOSA BENEATH AN UPPER COMPLETE DENTURE USING A LASER DOPPLER FLOWMETRY: A PRELIMINARY STUDY

Petar Tekić[1] Stefan Vulović[2] Marija S. Milić[3] Marko Igić[4,5] Milena Kostić[4,5] Aleksandra Popovac[2] Ivica Stančić[2] Aleksandra Milić Lemić[2]

[1]University of Belgrade Faculty of Dental Medicine, Belgrade, Serbia [2]Department of Prosthodontics, University of Belgrade Faculty of Dental Medicine, Belgrade, Serbia [3]Department of General and Oral Physiology, University of Belgrade Faculty of Dental Medicine, Belgrade, Serbia [4]Department of Prosthodontics, Clinic of Dental Medicine Niš, Niš, Serbia [5]University of Niš Faculty of Medicine, Niš, Serbia

Older edentulous adults often rely on mucosa-supported complete dentures (CDs) due to limitations that preclude implant therapy. Although an adequate retention of maxillary CD is essential for functional efficiency, compression of the palatal mucosa during impression making may compromise palatal mucosal microcirculation during denture wear. The aim of the study was to evaluate short-term changes in palatal mucosal blood perfusion in elderly individuals during adaptation to newly fabricated maxillary CDs. Ten fully edentulous participants (mean age 67.3 years) received conventionally fabricated maxillary CDs and custom-made thermoplastic splintsreplicating the denture base. Palatal microcirculation was measured using laser Doppler flowmetry (LDF) through perforations in the splints at three regions (frontal, premolar, and molar), and at four time points: before denture insertion (T0), and at 30 min (T1), one week (T2), and six weeks (T3) post-insertion. Significant reductions in blood perfusion units (BPU) were observed over time in the premolar and molar regions ($p < 0.05$), while changes in the frontal region were not significant ($p > 0.05$). At each time point, the molar region exhibited higher BPU values than the other regions. The greatest reductions were noted between the initial (T0/T1) and later (T2/T3) stages. Short-term use of maxillary CDs was associated with reduced palatal mucosal blood perfusion, particularly in posterior regions. These findings underscore the importance of monitoring tissue response during maxillary complete denture adaptation to improve therapy outcomes in elderly patients.

Keywords: complete denture, laser Doppler flowmetry, microcirculation, palatal mucosa

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Correspondence to:
Stefan Vulović
Department of Prosthodontics
University of Belgrade
Faculty of Dental Medicine
Rankeova 4, Belgrade, Serbia
E-mail: stefan.vulovic@stomf.bg.ac.rs

INTRODUCTION

Older adult edentulous patients often face socioeconomic constraints, systemic health challenges, and limited bone support for implant placement, which can preclude implant-based treatment options. As a result, prosthodontic rehabilitation in this population predominantly relies on the fabrication of mucosa-supported complete dentures (CDs) (1), with the maxillary CD often meeting both esthetic and functional demands to a satisfactory extent (2).

To fulfill functional requirements, a maxillary complete denture (CD) must achieve adequate retention and stability. A well-retained, stable prosthesis significantly improves mastication and encourages the consumption of healthier, fiber-rich food, including raw fruits and vegetables. This diet has been shown to enhance both oral and systemic health in older adults (3). The retention and, more critically, the stability of the maxillary CD depend largely on the intimate contact between the denture base and the underlying mucosa. This relationship underscores the importance of precise impression techniques and denture base design to accurately capture the edentulous ridge morphology and border extensions, as well as to properly displace soft tissues to establish an effective mucosal seal (4). Retention is further enhanced by slight compression of the palatal mucosa, especially in the posterior palatal seal area and along the lateral regions adjacent to the midline, where the mucosa houses the greater palatine nerve and blood vessels (5).

During mastication, occlusal forces are predominantly distributed across the peripheral seal and the supporting tissues, which must be resilient enough to withstand functional loading (6). However, intermittent or continuous pressure exerted by the maxillary CD can potentially compromise the microcirculation within the palatal mucosa. Prolonged disturbances in blood perfusion may lead to localized ischemia, the accumulation of metabolic by-products (7), and the alveolar ridge resorption (8). Furthermore, mechanical stress transmitted through the denture base and the resulting vascular alterations have been associated with the accumulation of microorganisms, especially fungal species, and the development of pathological conditions such as denture stomatitis (9).

Considering all the aforementioned, the present study aimed to evaluate changes in palatal mucosal microcirculation microcirculation in older adults during the adaptation period to a newly fabricated maxillary CD. The null hypotheses were that: 1. No significant changes in blood perfusion would be found across different time points within the same anatomical region. 2. No significant changes in blood perfusion would be found among anatomical regions at the same time point.

METHODS

A total of 10 edentulous participants (n = 10; 7 females and 3 males), aged 65–72 years (mean age 67.3 years), were included in the study between October and November 2024. Eligibility was determined according to predefined systemic and local inclusion and exclusion criteria. Systemic inclusion criteria comprised age ≥65 years, stable general health, and non-smoking status. Local inclusion criteria included complete edentulism in both the upper and lower jaws, absence of pathological changes in the oral mucosa, and no prior experience wearing complete dentures.

Systemic exclusion criteria included uncontrolled diabetes or hypertension, a history of head and neck radiation therapy, ongoing chemotherapy, psychiatric disorders, use of bisphosphonates, acute anemia, alcohol abuse, and refusal to provide informed consent. Local exclusion criteria included the presence of active pathology in the upper or lower jaw and the presence of a median palatal torus. Experimental procedures were conducted in full accordance with the ethical principles outlined in the 1964 Declaration of Helsinki, and the study protocol was reviewed and approved by the institutional Ethics Committee of the School of Dental Medicine, University of Belgrade (approval number 36/7, issued on 12.03.2024). Prior to participation, all participants provided written informed consent, and, during the study, no dropouts were recorded.

Denture fabrication

For each participant, maxillary CD was fabricated following standardized clinical and laboratory protocols. A selective pressure impression technique was employed, utilizing border molding with extended custom trays and an impression compound (Impression compound green, Harvard, Hoppegarten, Germany). Final impressions were made using a zinc oxide eugenol paste (Cavex Outline, Cavex Holland BV, Haarlem, The Netherlands) to ensure precise anatomical detail reproduction. Following denture insertion, any clinical issues, such as pressure spots, were identified and promptly adjusted. A balanced occlusal scheme was established to promote functional efficiency and comfort, whereas occlusion was carefully evaluated to

ensure uniform bilateral contacts and to eliminate any deflective interferences.

Splint fabrication

To enable accurate microcirculation assessment, a transparent, custom-made splint was fabricated to match the base of the maxillary CD. The splint was constructed on a duplicated master model before denture flasking, using a 3 mm thermoplastic foil (Erkoloc Pro 3.0 × 120 mm, Erkodent, Pfalzgrafenweiler, Germany) adapted with a vacuum-forming device (Erkoform 3D Motion, Erkodent, Pfalzgrafenweiler, Germany). The splint design ensured that no pressure was applied to the maxillary mucosa during microcirculation measurements. This was achieved by creating a relief space using two layers of baseplate wax during the splint fabrication process. To facilitate probe stabilization and enable reproducible positioning for repeated measurements, the splint was perforated at three specific anatomical landmarks in the frontal, premolar, and molar regions to accommodate the probe holder (Figure 1) along the course of the greater palatine artery (10). During the procedure, the probe was positioned perpendicular to the mucosal surface to ensure consistent and accurate contact throughout the measurements.

Microcirculation measurements were performed at four time points: prior to denture insertion (T0), 30 min after insertion (T1), one week after insertion (T2), and six weeks after insertion (T3) (11). Following initial insertion, participants were instructed on proper denture usage, including evenly distributed mastication and removing the denture overnight. Follow-up appointments for measurements were scheduled accordingly. Microcirculation was assessed using a laser Doppler flowmeter (PeriFlux PF 5001, Perimed, Järfälla, Sweden), operating with red laser light at a wavelength of 632.8 nm. Red light was produced by a 1 mW helium-neon laser diode within the flowmeter and transmitted to the tissue surface along the fiber-optic conductor inside a round probe (407-2, Perimed), with a cross-sectional diameter of 1 mm. The probe of the flowmeter, stabilized using a probe holder (PH 07-6, Perimed), simultaneously received the reflected and scattered light via an afferent optical fiber, which was then registered by a photodetector in the flowmeter. According to the Doppler phenomenon, the light reflecting from moving particles (red blood cells) shifted in frequency, while the frequency of light reflecting from static structures remained unchanged. Frequency shifts were used to calculate the concentration and velocity of moving particles, and the result was proportional to tissue blood flow, expressed in semiquantitative blood perfusion units (BPU). To ensure measurement accuracy, the device was calibrated prior to each measurement session using a latex particle colloidal suspension (Perimed Motility Standard, Perimed), and recordings were obtained for a minimum of 3 min at each designated point to ensure signal stability and reliability. Data acquisition and analysis were performed using the associated software (PeriSoft v.2.50, Perimed). All measurements were performed by the same experienced, trained operator, under consistent ambient conditions (room temperature, at 10:00 AM), with participants seated in a semi-reclined position to minimize body movements.

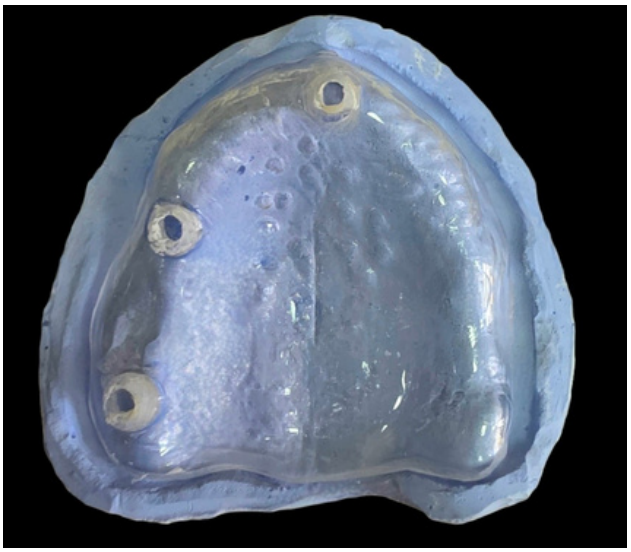

Figure 1. *Transparent, custom-made splint made of thermoplastic foil used for microcirculation measurements*

Statistical analysis

All statistical analyses were conducted using statistical software (SPSS v.22.0, Chicago, IL, USA), with the threshold for statistical significance set at $\alpha = 0.05$. The normality of the data was verified using the Kolmogorov-Smirnov test, which confirmed that all variables followed a normal distribution. To assess differences in BPU between anatomical regions at a given time point, a one-way analysis of variance (ANOVA) was applied, followed by Tukey's post hoc test for pairwise comparisons. Temporal changes in BPU within the same anatomical region were examined using repeated measures ANOVA, with Bonferroni correction applied to adjust for multiple

comparisons. Results were reported as mean values with their corresponding standard deviations (mean ± SD).

RESULTS

Comparison across time points within the same region

The results of the microcirculation measurements are presented in Tables 1 and 2. When evaluating changes in BPU across different time points within each region, a statistically significant difference was observed in both the premolar and molar regions ($p < 0.05$), whereas no significant variation was found in the frontal region ($p > 0.05$). In the frontal region, although a decreasing trend in BPU was observed over time, the changes were not statistically significant ($p > 0.05$). BPU values were slightly higher at T0 and T1 compared to T2 and T3, though the differences remained non-significant ($p > 0.05$).In the premolar region, a significant decline in BPU was observed across the time points ($p < 0.001$). No significant difference was found between T0 and T1 ($p > 0.05$); however, statistically significant reductions were detected when comparing T0 and T1 with T2 and T3, respectively (T0 vs T2: $p = 0.012$; T0 vs T3: $p = 0.002$; T1 vs T2: $p = 0.004$; T1 vs T3: $p < 0.001$). In the molar region, BPU values also significantly decreased over time ($p < 0.001$). BPU at T0 was significantly higher compared to BPU at T1 ($p = 0.021$), T2 ($p = 0.027$), and T3 ($p = 0.002$). No significant difference was found between BPUs at T1 and T2 ($p > 0.05$), whereas both were significantly higher than BPU at T3 (T1 vs T3: $p = 0.002$; T2 vs T3: $p = 0.001$).

Comparison among regions at the same time point

Significant differences in BPU were found among regions at each time point ($p < 0.05$). At baseline (T0), interregional differences were significant ($p = 0.001$). The molar region exhibited the highest BPU value (47.81 ± 18.25), which was significantly higher compared to the frontal (20.22 ± 9.63; $p = 0.001$) and premolar regions (30.29 ± 14.32; $p = 0.031$). Although BPU in the premolar region was higher than in the frontal region, the difference was not statistically significant ($p > 0.05$). A similar trend was observed 30 min post-insertion (T1), with statistically significant differences among the regions ($p = 0.004$). The molar region again demonstrated the highest BPU value (40.47 ± 17.50), significantly higher than the BPUs in frontal (20.02 ± 9.28; $p = 0.004$) and premolar regions (25.80 ± 9.61; $p = 0.040$). The difference between the frontal and premolar regions remained non-significant ($p > 0.05$), with the premolar

Table 1. *Blood perfusion units (BPU) were measured for each participant and expressed as mean ± SD*

Participant	Frontal region				Premolar region				Molar region			
	T0	T1	T2	T3	T0	T1	T2	T3	T0	T1	T2	T3
1	7.64	7.86	6.74	6.27	11.11	9.34	8.21	3.94	30.05	15.21	14.09	6.73
2	14.24	14.27	14.03	13.79	20.92	20.56	9.10	4.70	44.13	31.86	22.51	13.60
3	29.53	28.25	22.96	22.82	55.57	37.64	18.09	14.26	54.42	44.31	22.88	16.75
4	16.85	16.80	12.29	11.77	25.45	26.95	8.83	9.30	49.47	41.81	32.38	22.13
5	18.39	18.36	16.09	15.72	31.77	28.27	11.64	6.56	77.71	60.64	32.10	27.16
6	14.81	14.78	14.22	13.81	21.48	15.22	8.91	7.45	30.11	28.10	25.05	20.01
7	12.31	12.09	10.64	9.45	18.92	19.09	16.24	10.55	24.45	23.31	20.08	18.62
8	35.32	34.85	38.72	36.12	52.11	38.56	23.24	14.15	61.11	59.52	53.24	39.91
9	34.91	34.24	28.78	26.43	36.74	33.22	29.15	21.01	71.11	67.72	60.34	55.54
10	18.22	18.75	16.54	15.71	28.83	29.11	18.43	10.23	35.56	32.22	33.11	21.11

T0 - baseline measurements; T1 - measurements 30 min after denture insertion; T2 - measurements 1 week after denture insertion; T3 - measurements 6 weeks after denture insertion.

Table 2. *Blood perfusion units (BPU) were measured for each anatomic region and time point and expressed as mean ± SD*

Region	Time point			
	T0	T1	T2	T3
Frontal	20.22 ± 9.63 $^{B, a}$	20.02 ± 9.28 $^{B, a}$	18.10 ± 9.54 $^{B, a}$	17.19 ± 8.89 $^{AB, a}$
Premolar	30.29 ± 14.32 $^{B, a}$	25.80 ± 9.61 $^{B, a}$	15.18 ± 7.13 $^{B, b}$	10.21 ± 5.16 $^{B, b}$
Molar	47.81 ± 18.25 $^{A, a}$	40.47 ± 17.50 $^{A, b}$	31.58 ± 14.67 $^{A, b}$	24.16 ± 14.04 $^{A, c}$

T0 - baseline measurements; T1 - measurements 30 min after denture insertion; T2 - measurements one week after denture insertion; T3 - measurements six weeks after denture insertion. Different uppercase letters indicate significant difference inside the columns ($p < 0.05$; Tukey's post hoc test), whereas different lowercase letters indicate significant difference inside the rows ($p < 0.05$; Bonferroni post hoc test).

region showing slightly higher values. After one week (T2), significant interregional differences in BPU persisted ($p = 0.005$). The molar region showed the highest BPU value (31.58 ± 14.67), significantly greater than both the frontal (18.10 ± 9.54; $p = 0.027$) and premolar regions (15.18 ± 7.13; $p = 0.006$). Although the frontal region exhibited a higher BPU value than the premolar region, the difference was not statistically significant ($p > 0.05$). After six weeks (T3), the trend continued with statistically significant differences across regions ($p = 0.016$). The molar region remained dominant (24.16 ± 14.04), showing significantly higher BPU value than the premolar region (10.21 ± 5.16; $p = 0.012$), and a higher, but not statistically significant BPU value compared to the frontal region (17.19 ± 8.89; $p > 0.05$). As observed at T2, the frontal region demonstrated a slightly higher BPU value than the premolar region, though without statistical significance ($p > 0.05$).

DISCUSSION

The present study was designed to observe changes in palatal mucosal blood perfusion over a six-week period of adaptation to CDs. The procedure was performed using LDF, a non-invasive and efficient method for assessing capillary blood flow, volume, and velocity. This technique detects the Doppler shift caused by moving red blood cells within the tissue illuminated by the laser beam, producing measurements in blood perfusion units (BPU). LDF has been safely used in several studies since the 1980s for evaluating blood perfusion in various oral tissues, including the tongue, gingiva, periodontium, masseter muscle, and denture-supporting mucosa (12-16).

The results of the present study revealed a consistent decline in blood perfusion from baseline to subsequent measurement stages across all three anatomical regions, which supports the opinion that maxillary CDs impose mechanical stress, thereby contributing to reduced blood perfusion beneath the denture-bearing palatal mucosa. This reduction aligns with what occurs during impression-making and reflects the functional dynamics of the denture during mastication, as also reported in the previous study (17). Furthermore, it was also observed that individuals with reduced reactive hyperemia exhibited lower mucosal blood perfusion under their dentures, suggesting that denture use can influence microvascular behavior (16). Biomechanically, the oral mucosa acts as a cushion, distributing occlusal forces from the denture to the underlying bone, and its vascular network plays a critical role in supplying nutrients to the supporting bone. Therefore, excessive mechanical loading can impair this function, compromising tissue health (18). Moreover, a poorly designed or ill-fitting denture may exacerbate these effects and diminish the success of prosthetic treatment. However, the results of this study suggest that a properly fabricated maxillary CD can gradually conform to the supporting tissues and allow redistribution of occlusal forces in line with the mucosa's physiological capacity to adapt. Although the duration of this study was limited, the findings align with previous research, in which a reduction in mucosal perfusion following denture insertion was reported, with improvement observed by the end of a six-month period, suggesting tissue adaptation over time (19). Contrarily, other studies report minimal effects of loading forces on palatal blood perfusion (17) or no differences in palatal blood perfusion between long-term denture wearers and edentulous individuals who had never worn dentures, indicating that age-related vascular changes may play a more dominant role than prosthesis use alone (20, 21). Considering all the aforementioned, the first null hypothesis that no significant changes in blood perfusion would be found across different time points within the same anatomical region was rejected.

When analyzing regional differences in blood perfusion at the same time point, the molar region consistently exhibited

significantly higher perfusion compared to the frontal and premolar regions throughout the entire observation period. This observation may be explained by the fact that the molar region is primarily supplied by the greater palatine artery and is located closer to the main vascular entry point, thereby receiving a richer, more direct blood supply compared to the anterior region. In addition, the palatal mucosa in the molar area is generally thicker and more vascularized than the mucosa in the anterior palate, which is more firmly attached to the underlying periosteum, particularly in the region of the palatal rugae. However, immediately following denture insertion, a significant decline in blood perfusion was observed in the molar region. One plausible explanation is that the posterior palatal seal area is often intentionally compressed during final impression procedures to enhance denture retention. This compression may contribute to localized disturbances in blood perfusion. Conversely, one study reported that although blood perfusion initially decreased in both the canine and molar regions after denture placement, vascular recovery occurred over a six-month period, particularly in the molar region, suggesting region-specific adaptation (22). Similarly, another group of authors observed greater vascular compromise in the anterior maxilla following denture insertion, potentially due to increased occlusal forces generated by mandibular implant-retained overdentures opposing maxillary CDs (11). These conflicting findings may be attributed to differences in study design, specifically, the inclusion of participants with natural teeth or various dental restorations in the opposing arch, unlike the present study, which included only fully edentulous participants in both the maxilla and mandible. Nevertheless, the second null hypothesis that no significant changes in blood perfusion would be found among regions at the same time point was also rejected.

One of the main limitations of this study is the relatively small sample size. However, as a preliminary investigation, the findings provide indicative value and serve as a foundation for future research. Subsequent studies should aim to include larger cohorts and control groups with varying prosthetic or implant-supported configurations in the opposing arch. Another limitation pertains to the methodology itself, specifically the use of LDF. While LDF is widely accepted and has undergone technological refinement, it remains technique-sensitive. Although the method is highly successful for measuring microcirculatory blood flow, it cannot distinguish between different vessel types (arterioles, capillaries, venules) and measures only superficial perfusion, typically within 1-2 mm of tissue depth (23). Therefore, deeper tissue changes may go undetected. Moreover, several methodological factors may also influence LDF measurements, including probe type, use of a stabilization splint, measurement duration, and operator skill (21). In addition, various local and systemic factors, such as temperature variation (24), smoking (25), local anesthesia (26), mechanical compression (27), blood pressure, heart rate, and auto-nomic nervous system activity (28), may affect measurement reliability. Thus, strict control of these parameters is essential to ensure valid and reproducible results when using LDF in oral soft tissue evaluation.

Nevertheless, the findings of the present study underscore the importance of monitoring microvascular responses in the supporting tissues of denture wearers. Further research is warranted to explore the relationship between microcirculatory changes and conditions such as denture stomatitis, and to investigate how clinical variables, including impression techniques, radiotherapy, bisphosphonate therapy, and systemic diseases like diabetes, may influence tissue responses beneath dentures. Understanding the interplay between aging and palatal mucosal blood flow remains essential for optimizing denture design and ensuring long-term oral health in the edentulous population.

Within the limitations of this preliminary study, it can be concluded that the short-term use of maxillary complete dentures is associated with a measurable decrease in palatal mucosal blood perfusion. The alterations were evident one week after denture insertion, more prominent in the molar region, compared with both the frontal and premolar regions.

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Authors' Contribution

Conceptualization, P.T., M.S.M, A.P., and A.M.L.; Data curation, P.T., S.V., M.I., and A.P.; Formal analysis, S.V., M.S.M., and M.I.; Funding acquisition, M.K., I.S., and A.M.L.; Investigation, P.T., S.V., M.S.M., and M.I.; Methodology, P.T., M.I., A.P., and A.M.L.; Project administration, M.S.M., I.S., and A.M.L. Resources, M.K., I.S., and A.M.L. Software, P.T., S.V. Supervision, M.K., A.P., I.S., and A.M.L. Validation, I.S., and A.M.L. Visualization, P.T., M.I., and I.S. Writing – original draft, P.T, S.V., M.S.M., and M.I.; Writing – review & editing, M.K., A.P., I.S., and A.M.L. All authors have read and approved the published version of the manuscript.

Statement of Ethics

This study protocol was reviewed and approved by the Ethics Committee of School of Dental Medicine, University of Belgrade (approval number 36/7, issued on 12.03.2024). Complete written informed consent was obtained from the patient for the publication of this study and accompanying images.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availibility

Not applicable.

Statement of Generative AI Use

No generative AI was used.

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Olu Israel Oyewole **Ayoola Mary Bukoye**

Department of Biochemistry, Osun State University Faculty of Basic and Applied Sciences, Osogbo, Nigeria

Aluminium is a metal that disrupts both the pro-oxidant and antioxidant balance in tissues, leading to systemic biochemical dysfunction. The present study evaluated the protective effect of rutin against aluminium chloride ($AlCl_3$)-induced hepatorenal toxicity in Wistar rats. Twenty male Wistar rats were divided into four groups: Group 1- control; Group 2 received $AlCl_3$ (100 mg/kg b.wt); Group 3 received $AlCl_3$ (100 mg/kg b.wt) plus rutin (50 mg/kg b.wt); Group 4 received rutin (50 mg/kg b.wt). Body weight changes, liver and kidney functions, antioxidant capacities, and histopathology of the liver and kidney were measured at the end of the twenty-one-day experimental period. The obtained results indicated that $AlCl_3$ caused significant weight loss in the rats. The toxicant also disrupted liver and kidney functions, characterized by significant depletion of antioxidant levels, distortion of histoarchitecture, and elevation of serum parameters. Conversely, treatment with rutin ameliorated the observed imbalances in body weight and biochemical indices and improved the histological alterations induced by $AlCl_3$ in the liver and kidneys of the rats. The study concludes that rutin exerts protective effects on the hepatorenal structure and function and may serve as a useful nutraceutical for managing liver and kidney damage in humans.

Keywords: aluminium toxicity, antioxidants, lipid peroxidation, enzyme biomarkers

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Correspondence to:
Olu Israel Oyewole
Department of Biochemistry
Osun State University
Faculty of Basic and Applied Sciences
Osogbo, Nigeria
E-mail: ioluoye@yahoo.com

INTRODUCTION

Aluminium exposure is ubiquitous among humans and animals due to its extensive use and environmental presence. It occurs naturally in the Earth's crust as insoluble aluminosilicate (1) and in various combined forms such as aluminium chloride ($AlCl_3$). It is found in drinking water due to water treatment processes or leaching from weathered rocks and soils (2). Its metallic properties lead to its widespread use in cooking utensils and products such as deodorants, food additives, and antacids (3). This increasing exposure has significant implications for various organs, due to its diverse routes of uptake in the body, with dietary ingestion being the most prevalent.

Oxidative damage has been claimed to occur as a result of Al toxicity due to its increased free radical production (4). It also stimulates inflammatory cytokine production and induction of endoplasmic reticulum stress in the liver and kidneys (5,6). The simultaneous damage of these organs, known as hepatorenal injury, results in severe complications, including liver cirrhosis, renal failure, and eventually systemic organ dysfunction (7).

Rutin (3,3′,4′,5,7-pentahydroxy flavone-3-rutinoside) is a bioflavonoid antioxidant. It is naturally abundant in various plants, such as grapes, buckwheat, tea, apples, tobacco, Forsythia, fruits, vegetables, and grains (8). It possesses a strong reactive oxygen species (ROS) scavenging activity [9], activates antioxidant enzymes, and shows potential to transport electrons, thereby reducing oxidative stress [10]. It also alleviates hepatorenal injuries caused by toxic agents, including mercuric chloride and lead acetate (11-13). As shown experimentally, rutin has anti-carcinogenic, cardioprotective, antithrombotic, and neuroprotective activities, and enhances liver health and integrity (14-16). Owing to this identified characteristic, this research focuses on hepatorenal injuries caused by Al toxicity and the potential role of rutin in alleviating oxidative damage, thereby improving liver and kidney function.

METHODS

Rutin (rutoside) was obtained from Bulk Supplements (Nevada, USA), and analytical kits from Randox Laboratories Ltd., County Antrim, BT29 4QY, United Kingdom. Aluminium chloride and all other necessary reagents of analytical grade were obtained from Sigma-Aldrich Chemicals Co., St. Louis, MO, USA.

Experimental design and animal grouping

Twenty male Wistar rats (average weight 140 g) were obtained and housed in netted plastic cages at the Central Animal House of Osun State University, Osogbo, Nigeria. The animals were kept in a well-ventilated environment at a temperature of 25 ± 2 °C and 55 ± 5 % relative humidity under a 12/12 h light-dark cycle, and provided with standard rat chow and water *ad libitum*. They were randomly assigned to four experimental groups of five rats each: Group 1 (control) received distilled water; Group 2 received 100 mg/kg body weight (b.wt) of aluminium chloride; Group 3 received aluminium chloride plus 50 mg/kg b.wt of rutin, while Group 4 received 50 mg/kg b.wt of rutin only. Treatments were administered using oral gavage for twenty-one days.

Preparation of serum and tissue homogenates

At the end of the experimental period, blood samples were collected from the orbital venous plexus of the rats into plain bottles. The blood samples were left to clot and were then centrifuged at 4000 rpm for 15 minutes. The serum obtained was refrigerated at -20 ºC until required for analysis. The rats were then sacrificed using cervical dislocation and dissected to excise the liver and kidneys. The excised organs were rinsed in ice-cold 1.15% KCl, blotted with filter paper, and weighed. The tissues were then homogenized in 5 volumes of ice-cold 0.1 M phosphate buffer (pH 7.4), using a Teflon homogenizer. The resulting homogenates were centrifuged at 10,000 x g for 20 minutes in a cold centrifuge at 4 ºC to obtain the post-mitochondrial fractions. The supernatant was separated and stored in the refrigerator at 4 ºC for antioxidant analysis.

Biochemical analysis

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were analyzed following the protocols of Reitman and Frankel [17]. Gamma-glutamyl transferase (GGT) activity and total bilirubin concentration were assayed using the methods of Schumann et al. (18) and Jendrassik and Grof [19], respectively. Serum creatinine was estimated using Jaffe's picrate method (20). Finally, serum urea levels were estimated using the Berthelot method (21).

Lipid peroxidation in the tissue homogenates was assessed by measuring the formation of thiobarbituric acid reactive substances (TBARS), expressed as malondialdehyde (MDA) equivalents, following the method

of Preuss et al., (22), while nitrate concentration was determined colorimetrically using the Griess reaction (23), and intracellular reactive oxygen species (ROS) formation was evaluated using the oxidation-sensitive dye DCFH (24). Reduced glutathione (GSH) levels were measured according to Beutler et al. (25). Glutathione peroxidase (GPx) and glutathione-S-transferase (GST) activities were assayed using modified methods of Matkovics et al. (26) and Habig et al. (27), respectively. Superoxide dismutase (SOD) activity was determined by the procedure of Misra and Fridovich (28), and catalase (CAT) activity was analyzed following the method described by Claiborne [29].
All measurements were performed spectrophotometrically, using a Multiskan FC Microplate Reader Spectrophotometer.

Histopathological examination
The liver and kidney tissues were excised and fixed in 10% formalin solution immediately after removal. The specimens were dehydrated in ascending grades of alcohol, cleared in xylene, and embedded in paraffin. Serial sections were cut, stained with hematoxylin and eosin, and examined under a light microscope for the evaluation of histopathological changes [30].

Statistical analysis
GraphPad Prism (GraphPad Software v. 7.1, USA) was used for statistical analysis. Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test, and results were presented as mean ± standard error of the mean (S.E.M.). A priori power analysis was performed, and statistical significance was set at $p < 0.05$.

RESULTS

The body weight changes in the experimental rats are shown in Table 1. One-way ANOVA showed a significantly decreased body weight ($F(3,16) = 11.29$, $p = 0.0003$) in the $AlCl_3$-administered experimental animals, with Tukey's post hoc test showing a decline in body weight ($p = 0.0002$, $q = 7.86$; $df = 16$) compared to the control group. However, co-administration of $AlCl_3$ and rutin significantly improved the body weight gain ($p = 0.0206$, $q = 4.69$).
The serum levels of AST, ALT, GGT, bilirubin, urea, and creatinine are shown in Table 2. One-way ANOVA showed a significant toxicity effect on AST ($F(3, 16) = 8.33$, $p = 0.0015$) and ALT ($F(3, 16) = 7.65$, $p = 0.0022$). Tukey's post hoc test showed that $AlCl_3$ significantly elevated AST and ALT compared with the control (AST: $p = 0.0034$, $q = 5.94$; ALT: $p = 0.0068$, $q = 5.46$; $df = 16$), while rutin co-treatment significantly attenuated these increases (AST: $p = 0.0128$, $q = 5.02$; ALT: $p = 0.0031$, $q = 6.00$). GGT and bilirubin were also significantly affected (GGT: $F(3, 16) = 122.90$, $p < 0.0001$; bilirubin: $F(3, 16) = 44.00$, $p < 0.0001$) with $AlCl_3$ exposure significantly elevating GGT and bilirubin levels (GGT: $p < 0.0001$, $q = 23.410$; bilirubin: $p < 0.0001$, $q = 15.12$; $df = 16$). However, co-treatment with rutin significantly reversed these elevations (GGT: $p < 0.0001$, $q = 18.80$; bilirubin: $p < 0.0001$, $q = 12.20$). Similarly, serum urea and creatinine levels showed significant group differences (urea: $F(3, 16) = 50.79$, $p < 0.0001$; creatinine: $(F(3,16) = 58.54$, $p < 0.0001$) with the administration of $AlCl_3$, significantly increasing urea and creatinine concentrations (urea: $p < 0.0001$, $q = 14.78$; creatinine: $p < 0.0001$, $q = 16.20$; $df = 16$). However, co-treatment with rutin significantly mitigated these elevations (urea: $p < 0.0001$, $q = 15.270$; creatinine: $p < 0.0001$, $q = 10.32$).
Antioxidant and lipid peroxidation analyses for the kidney and liver are summarized in Tables 3 and 4, respectively. One-way ANOVA showed that $AlCl_3$ administration significantly increased MDA (kidney: $F(3, 16) = 32.11$, $p < 0.0001$; liver: $F(3, 16) = 89.04$, $p < 0.0001$) and ROS (kidney: $F(3, 16) = 46.53$, $p < 0.0001$; liver: $F(3, 16) = 55.99$, $p < 0.0001$) compared to the control, with Turkey's post hoc test showing significantly elevated levels of MDA (kidney: $p < 0.0001$, $q = 12.91$; liver: $p < 0.0001$, $q = 21.56$; $df = 16$) and ROS (kidney: $p < 0.0001$, $q = 14.53$; liver: $p < 0.0001$, $q = 18.26$; $df = 16$). However, co-treatment with rutin significantly decreased the MDA (kidney: $p = 0.47$, $q = 2.10$; liver: $p < 0.0001$, $q = 9.55$) and ROS (kidney: $p = 0.0002$, $q = 8.05$; liver: $p = 0.0003$, $q = 7.72$) concentrations toward levels comparable to the control group.

Table 1. *Body weight changes in Wistar rats exposed to $AlCl_3$ and rutin*

Group	Average initial body weight (g)	Average final body weight (g)	Average weight changes (g)
Control	141.20 ± 8.93	156.80 ± 9.26	+15.60
$AlCl_3$	140.80 ± 6.90	131.80 ± 5.50	-9.00[a]
Rutin + $AlCl_3$	140.70 ± 4.67	146.20 ± 5.33	+5.50[a,b]
Rutin only	140.40 ± 7.30	151.00 ± 7.60	+10.60[b]

Values are expressed as mean ± SEM of 5 replicates; [a]$p < 0.05$ vs control and [b]$p < 0.05$ vs $AlCl_3$.

Table 2. *Kidney and liver function parameters in Wistar rats exposed to $AlCl_3$ and rutin*

Analysis	Control	$AlCl_3$	Rutin + $AlCl_3$	Rutin only
AST (U/L)	83.48 ± 3.71	104.24 ± 5.69[a]	78.25 ± 3.22[b]	72.55 ± 2.34[b]
ALT (U/L)	74.82 ± 2.80	95.54 ± 1.86[a]	75.56 ± 4.38[b]	85.20 ± 3.43
GGT (U/L)	41.74 ± 3.26	184.71 ± 4.27[a]	75.84 ± 1.12[a,b]	37.58 ± 4.91[b]
Bilirubin (µmol/L)	50.47 ± 2.02	72.08 ± 1.03[a]	54.64 ± 1.49[b]	62.77 ± 0.92[a,b]
Urea (mmol/L)	23.92 ± 1.07	39.53 ± 1.39[a]	23.4 ± 0.78[b]	27.33 ± 0.89[b]
Creatinine (mmol/L)	44.60 ± 4.90	131.60 ± 2.64[a]	82.20 ± 3.58[a,b]	44.40 ± 5.91[b]

Values are expressed as mean ± SEM of 5 replicates; [a]$p < 0.05$ vs control and [b]$p < 0.05$ vs $AlCl_3$

Table 3. *Kidney antioxidants and lipid peroxidation markers in Wistar rats exposed to $AlCl_3$ and rutin*

Analysis	Control	$AlCl_3$	Rutin + $AlCl_3$	Rutin only
Catalase (µM/ml/mins)	11.75 ± 0.13	9.94 ± 0.17[a]	9.53 ± 0.34[a]	9.85 ± 0.37[a]
Glutathione (mM)	0.37 ± 0.003	0.27 ± 0.008[a]	0.45 ± 0.02[a,b]	0.44 ± 0.03[a,b]
Glutathione-S-transferase (µM/min/ml)	0.046 ± 0.001	0.02 ± 0.003[a]	0.04 ± 0.0004[b]	0.04 ± 0.001[b]
Glutathione peroxidase (mM)	16.15 ± 0.19	11.68 ± 0.23[a]	119.07 ± 0.028[a,b]	20.62 ± 0.22[a,b]
Superoxide dismutase (U/ml)	0.67 ± 0.08	0.20 ± 0.007[a]	0.66 ± 0.021[b]	0.57 ± 0.03[b]
Nitric oxide (µM)	8.70 ± 0.67	1.06 ± 0.23[a]	7.12 ± 0.06[b]	6.56 ± 0.36[a,b]
Malondialdehyde (µM)	7.59 ± 0.03	10.86 ± 0.3[a]	8.12 ± 0.36[b]	9.03 ± 0.19[a,b]
Reactive oxygen species	0.46 ± 0.002	0.87 ± 0.04[a]	0.68 ± 0.03[a,b]	0.48 ± 0.03[b]

Values are expressed as mean ± SEM of 5 replicates; [a]$p < 0.05$ vs control and [b]$p < 0.05$ vs $AlCl_3$

Table 4. *Liver antioxidants and lipid peroxidation marker in Wistar rats exposed to $AlCl_3$ and rutin*

Analysis	Control	$AlCl_3$	Rutin + $AlCl_3$	Rutin only
Catalase (µM/ml/mins)	7.16 ± 0.21	4.36 ± 0.05[a]	7.43 ± 0.18[b]	7.91 ± 0.20[a,b]
Glutathione (mM)	0.37 ± 0.01	0.24 ± 0.009[a]	0.33 ± 0.005[a,b]	0.37 ± 0.004[b]
Glutathione-S-transferase (µM/min/ml)	0.21 ± 0.008	0.072 ± 0.006[a]	0.092 ± 0.003[a]	0.17 ± 0.007[a,b]
Glutathione peroxidase (mM)	57.91 ± 3.29	33.02 ± 0.82[a]	66.6 ± 0.59[b]	110.59 ± 3.00[a,b]
Superoxide dismutase (U/ml)	1.11 ± 0.04	0.53 ± 0.017[a]	1.32 ± 0.032[a,b]	1.22 ± 0.05[b]
Nitric oxide (µM)	9.64 ± 0.19	3.85 ± 0.16[a]	9.57 ± 0.22[b]	10.53 ± 0.06[a,b]
Malondialdehyde (µM)	7.59 ± 0.18	13.23 ± 0.13[a]	10.09 ± 0.46[a,b]	8.55 ± 0.13[b]
Reactive oxygen species	0.34 ± 0.01	1.14 ± 0.017[a]	0.68 ± 0.06[a,b]	0.72 ± 0.07[a,b]

Values are expressed as mean ± SEM of 5 replicates; [a]$p < 0.05$ vs control and [b]$p < 0.05$ vs $AlCl_3$

The inflammatory biomarker nitric oxide was significantly reduced (kidney: $F(3, 16) = 69.24$, $p < 0.0001$; liver: $F(3, 16) = 334.00$, $p < 0.0001$) in the $AlCl_3$-administrated group as shown by one-way ANOVA and supported by Turkey's post hoc test (kidney: $p < 0.0001$, $q = 19.11$; liver: $p < 0.0001$, $q = 34.52$; $df = 16$). Co-treatment with rutin significantly attenuated this effect (kidney: $p = 0.0573$, $q = 3.95$; liver: $p = 0.9900$, $q = 0.39$) when compared with the control group.

Furthermore, one-way ANOVA showed a significant reduction of SOD (kidney: $F(3, 16) = 27.55$, $p < 0.0001$; liver: $F(3, 16) = 101.80$, $p < 0.0001$) and catalase (kidney: $F(3, 16) = 13.46$, $p = 0.0001$; liver: $F(3, 16) = 86.70$, $p < 0.0001$) activities in the liver and kidney following $AlCl_3$-exposure with Turkey's post hoc test showing significantly decreased SOD (kidney: $p < 0.0001$, $q = 11.19$; liver: $p < 0.0001$, $q = 16.51$; $df = 16$) and catalase (kidney: $p = 0.0013$, $q = 6.62$; liver: $p < 0.0001$, $q = 16.29$; $df = 16$) activities. These activities were significantly increased following co-

treatment with rutin for SOD (kidney: p = 0.9947, q = 0.35; liver: p = 0.0034, q = 5.95) and catalase (kidney: p = 0.0002, q = 8.105; liver: p = 0.6921, q = 1.562) to levels indistinguishable from the control group.

Also, one-way ANOVA of GPx (kidney: F(3, 16) = 453.20, p < 0.0001; liver: F(3,16) = 97.19, p < 0.0001) and GST (kidney: F(3, 16) = 64.80, p < 0.0001; liver: F(3, 16) = 44.50, p < 0.0001) activities, together with GSH (kidney: F(3, 16) = 26.43, p < 0.0001; liver: F(3, 16) = 70.52, p < 0.0001) concentration, revealed significant group differences. Administration of $AlCl_3$ significantly increased GPx (kidney: p < 0.0001, q = 24.22; liver: p = 0.0006, q = 7.18; df = 16) and GST (kidney: p < 0.0001, q = 17.88; liver: p < 0.0001, q = 13.87; df = 16) with a concomitant decrease in the GSH concentration (kidney: p = 0.0020, q = 6.32; liver: p < 0.0001, q = 18.21; df = 16). However, co-treatment with rutin significantly mitigated GPx (kidney: p < 0.0001, q = 15.78; liver: p = 0.3215, q = 2.51) and GST (kidney: p = 0.1960, q = 2.97; liver: p < 0.0001, q = 11.71) elevations and GSH reduction (kidney: p = 0.0157, q = 4.88; liver: p = 0.0017, q = 6.44) to levels comparable to the control group.

Figure 1 reveals the liver histopathological examination using H and E staining. $AlCl_3$-exposed animals showed cellular profile derangement characterized by severe loss of liver parenchyma, severe hemorrhage, and infiltration of inflammatory cells within and around the central vein and sinusoids, and dilatation of the hepatic portal triad. Co-administration of rutin in $AlCl_3$-exposed rats showed mild alterations in cytoarchitecture, revealing the protective effect of rutin.

Similarly, renal tissue histology showed preserved renal architecture and normal renal corpuscles in control rats, severe focal sclerosis of the glomerulus, hemorrhagic changes, and observable red inflammatory cells in $AlCl_3$-exposed rats. The concomitant administration of rutin and $AlCl_3$ preserved renal histoarchitecture with mild fibrosis and hemorrhage, characterized by the presence of red inflammatory cells, as shown in Figure 2.

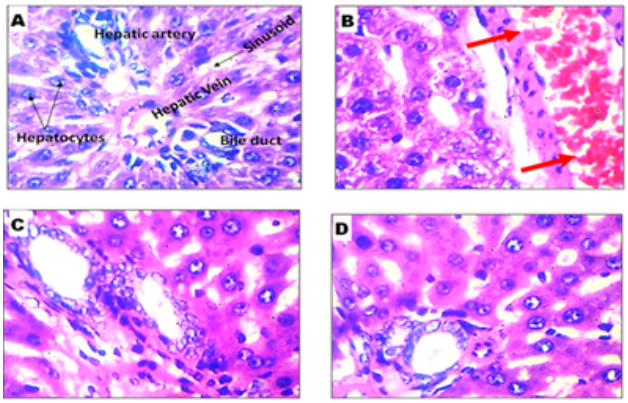

Figure 1. *Photomicrographs of hematoxylin and eosin (H&E)-stained hepatic cells (×400) from rats treated with $AlCl_3$ and rutin*

A: Control group showed a well-outlined cellular profile with no altered panoramic morphological presentation, with hepatocytes well distributed across the general cytoarchitecture. B: Animals treated with $AlCl_3$ only showed derangement in cellular profiles characterized by severe loss of liver parenchyma, severe hemorrhage, infiltration of inflammatory cells within and around the central vein and sinusoids, and dilatation of the hepatic portal triad. (red thick arrows). C: Animals co-administered $AlCl_3$ and rutin showed a mild alteration in cytoarchitecture, less conspicuous than seen in B. D: Animals treated with rutin only also showed a well outlined cellular profile and well distributed hepatocytes across the general cytoarchitecture.

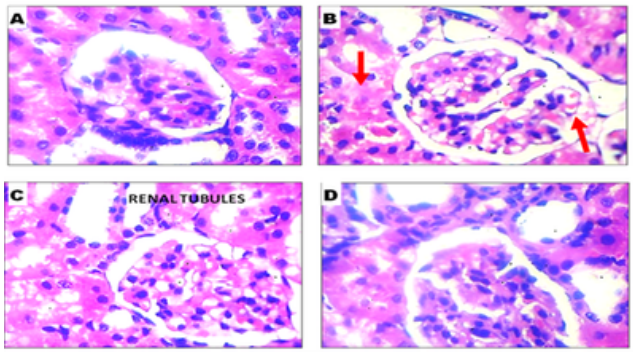

Figure 2. *Photomicrographs of hematoxylin and eosin (H&E)-stained kidney cells (×400) from rats administered $AlCl_3$ and rutin*

A: Control group showed no observable degenerative changes, and well-arranged renal corpuscles, glomeruli, macula densa, and convoluted tubules. B: Animals treated with $AlCl_3$ only showed severe focal sclerosis of the glomerulus with hemorrhagic changes, together with observable red inflammatory cells. C: Animals co-administered $AlCl_3$ and rutin showed mild fibrosis and hemorrhage characterized by the presence of red inflammatory cells, with no other varying degrees of renal injury. D: Animals treated with rutin showed no observable degenerative changes, while the renal corpuscles, glomeruli, macula densa, and convoluted tubules were well arranged.

DISCUSSION

Previous research has shown that weight gain is a reliable marker for assessing substance toxicity (31). A significant decrease in body weight gain in Al-treated rats, as seen in this study, affirms this notion. This decrease may be due to depletion of body fluids and adipose tissue, or reduction of food intake, leading to nutritional malabsorption (32). Our findings thus provide evidence of Al toxicity, as it impairs body weight gain. However, rutin, a naturally occurring flavonoid glycoside, protects cells against harmful effects, including free radicals, due to its antioxidant, anti-inflammatory, and antiapoptotic properties (33). This enhances rutin's potential to support body weight gain in Al-exposed rats, stimulating anabolic processes and suppressing tissue breakdown.

Organ injury causes membrane damage or necrosis. As demonstrated by our findings, a significant elevation of hepatic enzymes (AST and ALT), GGT, and bilirubin concentration in the serum of Al-exposed rats indicates liver dysfunction (34). This is caused by changes in the permeability of hepatocyte membranes resulting from free radical damage, inflammation, and cellular injury (35). Rutin administration significantly lowered the hepatotoxic effects of Al, as evidenced by reductions in the serum AST, ALT, GGT, and bilirubin, supporting rutin hepatoprotective ability as reported in previous studies (36,37). The current investigation also discovered a significant increase in urea and creatinine levels in Al-exposed rats. This shows the kidney's inability to effectively filter the metabolic waste products, suggesting extensive disruption of renal structure and function (38). The administration of rutin restored urea and creatinine concentration to levels similar to those of the control group, which agrees with the findings of Kucukler et al. (39).

ROS serve as signaling molecules that regulate and maintain homeostasis at physiological concentrations, including hepatorenal homeostasis. Excessive generation of ROS causes oxidative stress by triggering phospholipid peroxidation (LPO), impairing membrane and organelle functions, and eventually leading to death (40). In this study, elevated ROS and MDA concentrations, as well as a reduction in nitric oxide concentration following $AlCl_3$ induction, agree with previous studies [41, 42]. This suggests that Al induces hepatorenal toxicity by enhancing free radical-mediated tissue and cellular damage and potentiates iron-mediated lipid peroxidation (43, 44). Rutin administration improved oxidative stress by stabilizing ROS, MDA, and nitric oxide concentrations to levels close to those of the control group, thereby preventing Al hepatorenal toxicity. This result is plausible as rutin acts as an ROS scavenger by donating hydrogen atoms to superoxide anions and hydroxyl and peroxyl radicals (45). It is also a master redox switch through Nrf2 activation and iNOS upregulation (46, 47).

Besides accompanying MDA accumulation as discussed, the present study also observed reduced GSH as well as repressed activities of SOD, catalase, GST, and GPx in the hepatic and renal tissues of Al-exposed animals. The roles of these antioxidant biomarkers in maintaining tissue redox-homeostasis cannot be overemphasized. SOD converts superoxide radicals into hydroxyl peroxide, which is further converted by catalase into water [48, 49]. GPx oxidizes GSH into GSSG, which is then reduced to GSH by glutathione reductase [49]. The decline in the levels of these antioxidants following Al-exposure suggested they were overwhelmed by Al-induced oxidative stress.

Although a reduced renal catalase antioxidant activity was observed, rutin was effective in reversing the Al-toxicity effects on both renal and hepatic tissues by increasing GSH concentration and enhancing SOD, catalase, GST, and GPx activities. This finding aligns with previous studies, suggesting rutin's ability to directly scavenge the generated ROS, such as hydroxyl radicals, H_2O_2, and superoxide anions, thereby maintaining the body's redox balance [50].

The histological examination of kidney and liver sections revealed distorted cytoarchitecture and cellular degeneration in Al-exposed rats, which agrees with the findings of Samir et al. (42). These alterations might be due to severe cell membrane damage, increased permeability of the plasma membrane, and vascular alteration-induced hemorrhage [51]. Rutin administration exhibited protective effects on the kidney and liver, thereby improving the distorted cytoarchitecture and cellular degeneration observed in Al-exposed rats. This can be attributed to the antioxidant potency of rutin, which significantly reduced the oxidative stress and cell damage.

This study establishes the therapeutic potential of rutin against Al-induced toxicity, as it attenuates functional damage in both the kidney and liver tissues. It also serves as a promising antioxidant and anti-inflammatory agent in alleviating oxidative stress and tissue inflammation posed by heavy metals, especially aluminium. This observation suggests that rutin could be recommended as a daily dose for prevention, particularly for individuals at high risk of aluminium exposure. However, the therapeutic use of rutin for the management of hepatorenal damage should be

used with caution, given its potential adverse effects in other clinical conditions.

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Author's Contribution

Conceptualization, O.I.O.; Methodology, O.I.O.; Supervision, O.I.O.; Investigation, A.M.B.; Writing – original draft, A.M.B.; Writing – review & editing, O.I.O. Both authors have read and approved the published version of the manuscript.

Statement of Ethics

This study was conducted in accordance with the ethical guidelines for the care and use of laboratory animals. The protocol was approved under the approval number UNIOSUNHREC 2026/002B by the Health Research Ethics Committee (HREC) of Osun State University, Osogbo, Nigeria.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availability

All data analyzed during this study are included within the published article.

Statement of Generative AI Technologies Use

No generative AI was used.

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ORIGINAL ARTICLE

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PREVALENCE OF NEW-ONSET HYPERGLYCEMIA AND DIABETES IN HOSPITALIZED ADULTS WITH COVID-19

Samar Maher Yassin[1] **Zaid Al-Attar**[2]

[1]Ministry of Health, Alanbar Health Directorate, Iraq [2]Department of Pharmacology, University of Baghdad, Al-Kindy College of Medicine, Baghdad, Iraq

COVID-19 infection may exacerbate glycemia and lead to metabolic problems in people with diabetes. Emerging data suggest that diabetes may also develop during coronavirus infection in individuals with no prior history of the disease. This study aimed to analyze the prevalence of new-onset hyperglycemia and diabetes mellitus (nDM) in COVID-19 patients. A retrospective study was conducted at AL-Kindy Teaching Hospital in Baghdad, Iraq, from August 2021 to January 2022, including a convenience sample of 150 non-diabetic COVID-19 patients. Data were extracted from medical records and included demographic information, disease severity, laboratory findings, presence of comorbidities, and disease outcomes. nDM was defined as a glucose level > 200 mg/dL on two occasions with no previous history of diabetes mellitus (DM). The mean age of participants was 54.81 ± 14.8 years, with a male-to-female ratio of 1.3:1. During hospitalization, 40 (26.7%) patients developed nDM; among them, 17.5% had moderate, 62.5% severe, and 20.0% critical COVID-19 infection (p = 0.370). nDM was associated with non-smoking status (35%, p = 0.026), hypertension (62.5%, p = 0.045), elevated D-dimer levels (3.261 ± 3.197 g/L, p = 0.036), and reduced lymphocyte counts (0.92×10^9 cells/L ± 0.98, p = 0.010). Non-smoking status and higher D-dimer levels were significant predictors of nDM, with odds ratios (95% CI) of 0.418 (0.175–0.997) and 1.2 (1.04–1.38), respectively, but nDM was not associated with worse outcomes. In conclusion, new-onset diabetes was observed in approximately one-fourth of hospitalized COVID-19 patients, but it did not predict adverse clinical outcomes.

Keywords: COVID-19, hyperglycemia, new-onset diabetes, outcome

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Correspondence to:
Zaid Al-Attar
Department of Pharmacology
University of Baghdad
Al-Kindy College of Medicine
Baghdad, Iraq
E-mail: zaidattar@kmc.uobaghdad.edu.iq

INTRODUCTION

It has been established that diabetes and COVID-19 are interconnected. Diabetes increases the risk of severe COVID-19 infection and has also been linked to COVID-19-induced severe acute respiratory syndrome (SARS-CoV-2) (1, 2). COVID-19 is a virus that can cause pneumonia and other respiratory complications. In infected individuals, the virus may affect pancreatic beta cells by exploiting the expression of angiotensin-converting enzyme 2 (ACE2) receptors. This could lead to a decrease in insulin secretion and, as a consequence, either an exacerbation of pre-existing diabetes or the development of new-onset diabetes mellitus (nDM) (3). Insulin resistance, which may be induced by elevated levels of interleukin-6 and tumor necrosis factor-alpha in COVID-19 patients, is likely another contributing factor to the development of diabetes (4, 5). Moreover, evidence suggests that COVID-19 patients with nDM experience worse outcomes compared to those with controlled glycemia or pre-existing diabetes (6, 7). Determining a direct causal relationship is challenging due to the presence of major confounding variables, which remains an unresolved question. Concerns have also been raised regarding glucocorticoid therapy, as it can trigger nDM and potentially exacerbate its negative impact on COVID-19 severity (7). Therefore, our study aimed to analyze the prevalence of new-onset hyperglycemia and diabetes in COVID-19 patients and to investigate its association with patient characteristics, disease severity, and clinical outcomes.

METHODS

This retrospective cohort study was conducted at Al Kindy General Teaching Hospital in Al-Rusafa Health Directorate, Baghdad, Iraq, from August 1, 2021, to January 31, 2022.
The study population included 150 COVID-19 patients admitted to an isolation ward at a tertiary care unit in Al Kindy Hospital in Baghdad between August and October 2021. Eligible participants were adults aged 18 years or older with a confirmed COVID-19 diagnosis by RT-PCR., while those with pre-existing diabetes and pregnant women were excluded from the study. The study was approved by the Ethics Committee of Medical Research at Al-Kindy College of Medicine, Baghdad University, under registration number 3676 on June 14, 2021.

Sampling method and data collection
Data were obtained from the documented records of 150 COVID-19 patients selected using convenience sampling. Information was collected using a questionnaire completed by the researcher during hospital visits. The dataset included demographics (age, gender, weight, and height), disease severity (moderate, severe, or critical), and laboratory results: white blood cell (WBC) count, lymphocyte, neutrophil, and platelet counts, lactate dehydrogenase (LDH), serum ferritin (s.ferritin) and D-dimer, comorbidities, including hypertension, obesity, and smoking, and disease outcomes (recovery or death).
All included patients were hospitalized with moderate, severe, or critical COVID-19 infection and received steroid therapy. Disease severity was classified according to the guidelines of the World Health Organization (WHO), which define the clinical spectrum as follows:
Mild disease - patients exhibit symptoms of COVID-19 without evidence of viral pneumonia or hypoxia;
Moderate disease (pneumonia): patients show signs of pneumonia, including fever, cough, dyspnea, and rapid breathing, but without severe pneumonia. Oxygen saturation (SpO_2) level is of 90% or higher on room air. Chest imaging (ultrasound, radiograph, CT scan) may aid in diagnosis and rule out or confirm pulmonary problems.
Severe disease (severe pneumonia): pneumonia symptoms, including high temperature, cough, shortness of breath, and rapid heart rate, in addition to one of the following – a respiratory rate exceeding 30 breaths per minute, acute respiratory distress, or a SpO_2 level below 90% on room air, contributed to a severe case of pneumonia.
Medical emergency (acute respiratory distress syndrome): onset occurs within seven days of new or worsening respiratory symptoms or a recognized clinical insult, such as pneumonia, and is confirmed by chest imaging using ultrasound, computed tomography (CT), or radiograph) when nodules, lobar or lung collapse, or volume overload cannot adequately explain bilateral opacities.
Critical illness (septic shock): patients may experience sudden, potentially fatal organ failures. Indicators of organ dysfunction include laboratory findings such as coagulopathy, thrombocytopenia, acidosis, elevated lactate, or hyperbilirubinemia, as well as clinical signs including altered mental status, hypoxia, oliguria, weak pulse, and hypotension. Septic shock is a critical illness characterized by persistent low blood pressure (hypotension) despite adequate resuscitation of blood volume; the patient must be given vasopressors to maintain mean arterial pressure (MAP) at 65 mmHg or higher and a serum lactate level of more than 2 mmol/L (8).

New-onset diabetes mellitus (nDM): nDM is defined as two separate random blood sugar readings (RBS) (200 mg/dL or more) on different days during hospitalization, according to Davidson Principles & Practice of Medicine, edition 23 (9).

Statistical analysis

Data entry and statistical analysis were performed using SPSS software version 23 (Statistical Package for the Social Sciences). Continuous variables were expressed as mean (range), while categorical variables were presented as frequencies and percentages. The Mann–Whitney U test was used to compare continuous variables, and the Chi-square or Fisher's exact test was applied for categorical variables, as appropriate. Factors associated with mortality were predicted using multivariate logistic regression analysis, with results reported as odds ratios (ORs) and 95% confidence intervals. A p-value < 0.05 was considered statistically significant.

Data collection, analysis, and interpretation of results, along with drafting and preparing the manuscript, were conducted over an estimated period of six months.

RESULTS

This study included 150 COVID-19 patients with a mean age of 54.81 ± 14.8 years. Of these, 85 (56.7%) were male, and 65 (43.3%) were female. Patients were categorized into age groups as follows: 18–39 years (23, 15.3%), 40–59 years (64, 42.7%), and ≥ 60 years (63, 42.0%). There were 33 (22%) smokers, 73 (48.7%) hypertensive patients, and 79 (52.7%) obese patients (BMI ≥ 30), as shown in Table 1.

As presented in Table 2, 84 (56%) patients had severe disease, while 27 (18%) were critically ill. During hospitalization, 40 (26.7%) patients developed new-onset diabetes mellitus (nDM), defined as blood glucose levels > 200 mg/dL on two separate occasions. Recovery occurred in 102 (68%) patients, whereas 48 (32%) died.

Table 3 compares the demographic and clinical characteristics of patients who developed nDM during the course of the disease with those who did not. Two of the most noteworthy characteristics were the lower prevalence of smoking (35% vs 65%, P = 0.026) and the higher prevalence of hypertension (62.5 vs 37%, P = 0.045). nDM developed in 7 out of 39 patients with moderate illness (17.9%), 25 out of 80 patients (29.8%) with severe illness, and 8 out of 27 patients (29.6%) with critical illness (P = 0.370). Although mortality was higher among patients with nDM (42.5%) compared to those without (28.2%), this difference was not statistically significant.

When laboratory findings were compared between the two groups (Table 4), higher D-dimer levels and lower lymphocyte counts were significantly associated with the development of nDM: p = 0.036 and 0.010, respectively. Multivariate analysis identified non-smoking status and an increase in D-dimer level as predictors of nDM development

Table 1. *Demographic features of patients*

Demographic features		N	%
Gender	Male	85	56.7%
	Female	65	43.3%
Age group	18-39 years	23	15.3%
	40-59 years	64	42.7%
	≥ 60 years	63	42.0%
Smoking	No	117	78.0%
	Yes	33	22.0%
Hypertension	No	77	51.3%
	Yes	73	48.7%
BMI	Normal (18.5-24.9)	39	26%
	Overweight (25-29.9)	32	21.3%
	Obese (≥ 30)	79	52.7%
Total		150	100 %

Table 2. *Clinical consequences of disease*

Clinical consequences of COVID-19 infection		N	%
		65	43.3%
Severity	Moderate	39	26
	Severe	84	56
	Critical	27	18
Newly diagnosed DM	No	110	73.3
	Yes	40	26.7
Outcome	Recovered	102	68
	Death	48	32
Total		150	100

Table 3. *Demographic and clinical distribution of patients according to the new-onset diabetes (two high readings of RBS ≥ 200 mg/dL on different days)*

Variables		New-onset diabetes				p-value
		No		Yes		
		N	%	N	%	
Age group	18-39 years	17	15.5%	6	15.0%	0.446
	40-59 years	50	45.5%	14	35.0%	
	≥ 60 years	43	39.1%	20	50.0%	
Gender	Female	47	42.7%	18	45.0%	0.804
	Male	63	57.3%	22	55.0%	
Smoking	No	91	82.7%	26	65.0%	0.026
	Yes	19	17.3%	14	35.0%	
Hyperten -sion	No	62	56.4%	15	37.5%	0.045
	Yes	48	43.6%	25	62.5%	
BMI	Normal	28	25.5%	11	27.5%	0.665
	Overweight	22	20.0%	10	25.0%	
	Obese	60	54.5%	19	47.5%	
Severity	Moderate	32	29.1%	7	17.5%	0.370
	Severe	59	53.6%	25	62.5%	
	Critical	19	17.3%	8	20.0%	
Disease outcome	Recovered	79	71.8%	23	57.5%	0.115
	Died	31	28.2%	17	42.5%	

during the course of COVID-19 infection, with OR of 0.418 and 1.2, respectively.

Further details are presented in Table 5, while patient outcomes and their associations with demographic and clinical characteristics are summarized in Table 6. Recovered patients were significantly younger than those who died (51 ± 15 vs. 62 ± 13 years, $p < 0.001$). The mortality rate among patients with severe (29.8%) and critical (77.8%) illness was significantly higher compared to those with moderate disease (5.1%). Although the development of DM during the course of the disease was not significantly associated with patient outcome, deceased patients had higher RBS levels (196 ± 66) compared to recovered patients (172 ± 59; $p = 0.007$). Higher levels of D-dimer, LDH, ferritin, urea, creatinine, white blood cell (WBC), neutrophil, and lymphocyte counts, as well as low platelet count, were associated with poorer patient outcomes.

Among these variables, increased patient age, severe and critical disease, and low platelet count significantly predicted poor patient outcome with OR (95% CI) of 1.059 (1.01-1.112), 3.47 (0.597-20.26), 83.7 (9.48-739.54), and 0.993 (0.987-0.999), respectively. Developing hyperglycemia during the course of the disease did not predict poor patient outcome. Infection was also not a significant predictor, with ORs of 0.418 and 1.2, respectively.

DISCUSSION

Previous studies have recognized the role of diabetes mellitus in the inflammatory response and progression of COVID-19. Recent studies have also reported elevated blood glucose levels in patients with COVID-19. Hyperglycemia in COVID-19, whether due to insulin resistance or pre-existing diabetes mellitus, has been associated with adverse effects on both disease course and outcomes. Recent literature suggests that COVID-19 may induce inflammation of pancreatic β-cells, potentially leading to new-onset diabetes mellitus (nDM) (10). This study highlights the prevalence of new-onset hyperglycemia and diabetes in adults admitted to the hospital due to COVID-19.

Disease severity

In the current study, more than half (56%) of COVID-19 cases were classified as severe. These findings are consistent with those of a study conducted in Kirkuk (11), in which 59.2% of cases were also classified as severe. However, a study from Turkey (12) reported a lower proportion (30.3%) of severe COVID-19 cases. The higher proportion of severe cases observed in our study may be attributed to differences in the study population, as only hospitalized patients were included, and the majority of participants were elderly, with a high prevalence of underlying comorbidities such as obesity and hypertension.

The prevalence of hyperglycemia among the studied COVID-19 patients was considerable, with 26.7% of patients developing hyperglycemia. This finding aligns with

Table 4. *Mean distribution of laboratory findings according to the new-onset diabetes (two readings of RBS > 200 mg/dL on different days)*

Lab test	nDM				p-value
	No		Yes		
	Mean	SD	Mean	SD	
D-dimer (g/L)	2.078	2.341	3.261	3.197	0.036
LDH (U/L)	572	204	611	233	0.386
S. ferritin (µ/L)	526.1	220.6	524.7	169.8	0.810
Blood urea (mg/dL)	58.1	32.9	63.5	47.9	0.954
S. creatinine (mg/ dL)	0.95	0.63	1.03	1.14	0.217
WBC count (x 10^9 cell/L)	13.65	6.02	14.29	4.83	0.249
Neutrophil (x 10^9 cell/L)	9.97	4.68	10.44	4.29	0.201
Lymphocyte (x 10^9 cell/L)	1.19	1.05	0.92	0.98	0.010
Platelet count (x 10^9 cell/L)	273.1	92.3	263.8	125.6	0.362

Table 5. *Multivariate analysis of new-onset diabetes in COVID-19 patients*

		OR	95% CI		p-value
Smoking	No	Reference			0.049
	Yes	0.418	0.175	0.997	
Hypertension	No	Reference			0.090
	Yes	1.96	0.899	4.290	
D dimer (g/L)		1.197	1.041	1.376	0.012
Lymphocytes count (x10^9 cell/L)		0.753	0.472	1.201	0.233

the results reported by Li H et al. (7), which showed that 20.75% of patients developed nDM. Similarly, a nationwide retrospective cohort study involving 12,817 non-diabetic patients reported the prevalence of 14% (13). In another study conducted by Zhang W et al. (14) in China, 12.5% of non-diabetic COVID-19 patients exhibited hyperglycemia. Similarly, a nationwide retrospective cohort study involving 12,817 non-diabetic patients reported a prevalence of 14% (13). In another study conducted by Zhang W et al. in China, 12.5% of non-diabetic COVID-19 patients exhibited hyperglycemia (14). Discrepancies in reported prevalence across studies may be attributed to differences in selection criteria and patient populations.

Hyperglycemia in COVID-19 may result from systemic inflammatory response and severe sepsis. In these conditions, elevated cytokine levels constitute an initial response and are associated with hyperglycemia (15, 16). The systemic inflammatory response syndrome has also been described as metabolic stress, which can induce glycogen breakdown, adrenocorticotropic hormone and catecholamine production, insulin resistance, and increased glucagon synthesis, all contributing to hyperglycemia (17, 18). Some studies have proposed that SARS-CoV-2 may directly infect pancreatic β-cells, reducing insulin synthesis and secretion (19, 20). Additionally, excessive cytokine production in COVID-19 contributes to insulin resistance (19), suggesting that hyperglycemia may arise from a combination of impaired insulin secretion and resistance (19, 20).

Stress hyperglycemia, characterized by elevated blood glucose levels, is mediated by elevated levels of cytokines, particularly tumor necrosis factor and interleukin-1, and counter-regulatory hormones (21).

COVID-19 outcome: In this study, approximately two-thirds of patients achieved full recovery, whereas nearly one-third died (68% and 32%, respectively). These rates are higher than those reported in Yemen and Syria, where mortality rates were 19.8% and 7.2%, respectively (22). Discrepancies in mortality rates between these studies may reflect variations in COVID-19 severity, age distribution, and comorbidities in the included cohorts.

The current study showed a negative correlation between smoking and blood sugar level during COVID-19, with non-smokers being significantly more hyperglycemic. Although the relation between smoking and the risk of severe COVID-19 respiratory syndrome has been previously investigated (23, 24), most studies were methodologically limited. Smoking has not been shown to protect against severe COVID-19. To our knowledge, the effect of smoking on the risk of nDM in COVID-19 has not been previously investigated. This makes the current study the first of its kind to investigate the potential impact of smoking on nDM development, despite smoking being a well-known risk factor for diabetes in the general population.

A significant positive correlation was observed between hypertension and nDM development. The relationship between hypertension and diabetes has been well documented in previous studies (25). Hypertension may increase the risk of developing diabetes, particularly type 2 diabetes. Hypertension may increase the risk of developing type 2 diabetes due to shared risk factors

Table 6. *Distribution of patients' outcomes according to demographic, clinical, and lab findings with multivariate analysis of significant variables*

Variables		Disease outcome				p-value	OR (95% CI)	p-value
		Recovered		Death				
		N	%	N	%			
Age	Mean ± SD)	51	±15	62	±13	< 0.001	1.059 (1.01-1.112)	0.023
Gender	Female	44	43.1%	21	43.8%	1		
	Male	58	56.9%	27	56.3%			
Smoking	No	79	77.5%	38	79.2%	0.837		
	Yes	23	22.5%	10	20.8%			
Hypertension	No	57	55.9%	20	41.7%	0.117		
	Yes	45	44.1%	28	58.3%			
BMI	Normal	31	30.4%	8	16.7%	0.159		
	Overweight	19	18.6%	13	27.1%			
	Obese	52	51.0%	27	56.3%			
Severity	Moderate	37	36.3%	2	4.2%	< 0.001	Reference	
	Severe	59	57.8	25	52.1%		3.47 (0.597-20.26)	0.166
	Critical	6	5.9	21	43.8%		83.7 (9.48-739.54)	0.001
Newly diagnosed DM	No	42	41.2%	22	45.8%	0.115		
	Yes	37	36.3%	26	54.2%			
RBS (mg/dL)	Mean (±SD)	172	± 59	196	± 66	0.007	1.007 (0.997-1.016)	0.160
D. dimer (g/L)	Mean (±SD)	131	± 27	138	± 30	< 0.001	0.96 (0.763-1.204)	0.713
LDH (U/L)	Mean (±SD)	172	± 59	196	± 66	< 0.001	1.003 (1.000-1.007)	0.051
S.ferritin (µ/L)	Mean (±SD)	1.949	± 2.610	3.338	± 2.466	< 0.001	1.000 (0.997-1.003)	0.910
B. urea (mg/dL)	Mean (±SD)	539	± 200	676	± 209	< 0.001	1.027 (1.006-1.047)	0.010
S. creatine (mg/dL)	Mean (±SD)	492.6	± 228.0	596.2	± 132.8	0.237		
WBC (x 10^9 cell/L)	Mean (±SD)	49.5	19.0	81.0	54.4	< 0.001	1.084 (0.953-1.233)	0.219.
Neutrophil (x 10^9 cell/L)	Mean (±SD)	0.80	0.27	1.33	1.28	< 0.001	0.985 (0.831-1.167)	0.859
Lymphocyte (x 10^9 cell/L)	Mean (±SD)	12.62	5.06	16.38	6.23	0.040	1.156 (0.648-2.063)	0.623
Platelet (x 10^9 cell/L)	Mean (±SD)	9.08	3.30	12.26	5.97	< 0.001	0.993 (0.987-0.999)	0.018

such as obesity, insulin resistance, and an unhealthy lifestyle (26). Individuals with hypertension often exhibit insulin resistance, which can lead to elevated blood glucose and diabetes (27). COVID-19 is also known to trigger insulin resistance (28), and the presence of hypertension may further increase the risk of developing nDM in these patients.

Disease outcomes were generally more favorable among non-DMD COVID-19 patients, although the difference was not statistically significant. This is consistent with a study from Sudan, which reported a 91% recovery rate among normoglycemic patients (29). Poor prognosis in COVID-19 has been linked to diabetes, whether newly diagnosed or previously established.

Patients with SARS or COVID-19 who also have hyperglycemia or diabetes are at increased risk of severe illness or death (30). Multi-organ failure in severe COVID-19 is associated with a cytokine storm, characterized by markedly elevated inflammatory cytokines (31).

The current study found that D-dimer levels were significantly higher in hyperglycemic patients compared to normoglycemic patients. This is consistent with a study from Wuhan (32), in which D-dimer levels in COVID-19 patients were higher in patients with hyperglycemia than in those with normoglycemia. Other studies have also documented elevated D-dimer levels in patients with both diabetes and COVID-19 (33–36). Several reports indicate that higher COVID-19 severity is associated with higher D-dimer levels (32–35), and severe disease is more common among hyperglycemic and diabetic patients (29–31, 33–35).

Lymphocyte count has shown a significant negative association with nDM development. Previous studies have shown that lymphopenia is associated with severe COVID-19 infection (37, 38) and can serve as a useful predictor of disease severity (39).

Elevated serum urea and low platelet counts were significant predictors of poor disease outcomes, likely reflecting multi-organ failure characteristic of severe COVID-19.

Interpretation of our findings regarding new-onset hyperglycemia should consider pharmacological management. In our cohort, all patients received glucocorticoid therapy, a standard treatment for mitigating inflammatory lung injury in moderate-to-critical COVID-19. Glucocorticoids are known to induce hyperglycemia by promoting hepatic gluconeogenesis and peripheral insulin resistance. Therefore, distinguishing hyperglycemia caused by direct pancreatic β-cell injury from steroid-induced effects is challenging. The observed hyperglycemia likely reflects a combination of steroid-induced insulin resistance and subclinical β-cell dysfunction triggered by SARS-CoV-2. Future studies incorporating control groups not receiving steroids, or closely monitoring steroid dosing, are needed to clarify causation. Furthermore, investigation into the pharmacological management of hyperglycemia, such as insulin therapy, and its impact on patient outcomes, remains an important area for future research.

In conclusion, the present study demonstrates that COVID-19 itself is a risk factor for hyperglycemia and new-onset diabetes mellitus (nDM), especially in patients with severe disease. Clinical and laboratory predictors of severe COVID-19—such as hypertension, elevated D-dimer, lymphopenia, and low platelet count—also appear to contribute to the development of hyperglycemia and/or nDM. Although smoking was associated with a lower risk of nDM in this cohort, this observation requires further investigation.

Study limitations

As the follow-up required a lot of time, we were unable to use prospective studies. There were no patients who did not get steroids for comparison. It was impossible to determine whether the patient had diabetes prior to COVID-19 using a blood test such as the HbA1c.

As a result of the coronavirus pandemic and the loss of patient files' personal information, laboratory results, and follow-up reports, some patient files were missing, which had an impact on the sample size.

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Authors' Contributions

Conceptualization, methodology, investigation, formal analysis, writing – original draft, review and editing: S.M.Y. and Z.A. Both authors have read and approved the published version of the manuscript.

Statement of Ethics

The study was reviewed and approved by the Ethics Committee of Medical Research at Al-Kindy College of Medicine, Baghdad University, approval number 3676, issued on June 14, 2021.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availability

Not applicable.

Statement of Generative AI Technologies Use

No generative AI was used.

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ORIGINAL ARTICLE

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EXPRESSION OF ANOCTAMIN-1 IN HUMAN GASTROINTESTINAL TRACT DURING EMBRYONIC AND FETAL DEVELOPMENT

Vladimir Petrović[1] Aleksandra Veličkov[1] Marko Jović[1] Julija Radenković[2] Braca Kundalić[3] Dušan Miljković[4] Vukota Radovanović[5] Goran Radenković[1]

[1]Department of Histology and Embryology, University of Niš Faculty of Medicine, Niš, Serbia [2]Department of Pathology, University of Niš Faculty of Medicine, Niš, Serbia [3]Department of Anatomy, University of Niš Faculty of Medicine, Niš, Serbia [4]Clinic for Pediatrics, University Clinical Center, Niš, Serbia [5]Clinic for Physical Medicine, University Clinical Center, Niš, Serbia

Anoctamin-1 (ANO1, TMEM16A) is a transmembrane protein belonging to the ANO family that plays a role in the formation of calcium-activated chloride channels (CaCCs). It is involved in the regulation of physiological processes, including muscle contraction, gastrointestinal motility, secretion, and electrical excitability. Recent data also suggest that ANO1 is a specific marker for interstitial cells of Cajal (ICC). The aim of the paper was to examine the spatial and temporal distribution of ANO1 in the human stomach, small intestine, and large intestine during embryo-fetal development as a potential marker for the differentiation of ICC and smooth muscle cells (SMCs). As study material, we used samples from 2 embryos and 21 fetuses. The tissue samples were routinely processed into paraffin blocks, and 5 µm-thick sections were immunostained for ANO1. Our results showed that ANO1 expression appeared during the 8th week of embryonic development and persisted through the fetal stages. Epithelial, endothelial, and ICC cells consistently expressed ANO1 in all examined samples. SMCs showed strong ANO1 expression in the muscularis propria; however, by the 25th week, this immunopositivity was absent from the outer muscle layers in the stomach and large intestine. In conclusion, ANO1 can be considered a reliable marker for tracking the differentiation of SMCs and ICC during embryonic and fetal development.

Keywords: ANO1, TMEM16A, gastrointestinal system, development, interstitial cells of Cajal

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Correspondence to:
Vladimir Petrović
Department of Histology and Embryology
University of Niš Faculty of Medicine
Bulevar dr Zorana Đinđića 81, Niš, Serbia
E-mail: vladimir.petrovic@medfak.ni.ac.rs

INTRODUCTION

Anoctamin 1 (ANO1, TMEM16A) is a plasmalemmal protein with eight transmembrane domains that belongs to the ANO family (ANO1-10). ANO1 is involved in the formation of calcium-activated chloride channels (CaCCs), which facilitate passive transport of chloride ions into the cytoplasm. These channels regulate physiological processes, such as muscle contraction, gastrointestinal motility, exocrine and endocrine secretion, and electrical excitability (1,2). ANO1 is expressed in epithelial cells in many organs, including the salivary glands, lacrimal glands, exocrine pancreas, bronchial tree, intestines, choroid plexus, and retina. It is also present in some types of smooth muscle cells (SMCs), interstitial cells of Cajal (ICC), vascular endothelial cells, and myocardium (3,4). It has been reported that abnormal ANO1 expression may be linked to the pathogenesis of diseases such as cystic fibrosis, hypertension, and gastrointestinal motility disorders (3,5). Additionally, ANO1 overexpression has been observed in many cancers, promoting tumorigenesis by influencing cancer cell proliferation, survival, and migration (6-9).

ANO1 has recently gained attention as a marker of ICC (10,11). These cells, originating from c-kit-positive mesenchymal precursors in the primitive gut, are observed at the end of embryonic development (12,13). They first appear in the esophagus and stomach, and then, following the rostrocaudal pattern of development, in the small and large intestines. Their development is closely associated with the colonization of the digestive tube by neural crest cells, which will eventually give rise to the neurons and glial cells of the myenteric and submucosal plexuses (14). ICC can first be identified by c-kit immunopositivity at the end of embryonic development, and by the 11th week, they surround the ganglia of the myenteric plexus (14-16).

The ICC are regarded as a crucial component of the enteric nervous system, providing the physiological basis for peristaltic movements (17,18). In response to cholinergic stimulation, ICC generate slow, non-oscillatory intestinal contractions that depolarize the ICC-smooth muscle cell network and convert the excitatory message from motoneurons into muscle contractions (1, 19). Spontaneous pacemaker activity generated by ICC and conducted to SMCs enables slow electrical waves and phasic contraction. In addition, ICC serve as stretch receptors and participate in the reflex peristalsis pathway due to the stretching of the digestive tube by food content (20). Available data suggest that ANO1 is expressed in all ICC classes, even those that do not contribute to slow-wave generation, implying that ANO1 may have an alternate function in these cells (21). The lack of differentiation and absence of ICC lie in the pathogenesis of many motility disorders in the gastrointestinal tract. Furthermore, mice lacking ANO1 were reported to have fewer proliferating ICC in culture, suggesting that ANO1 may also be involved in ICC proliferation (11,22). Lower or absent ANO1 expression has been reported in patients with gastrointestinal disorders, including diabetic gastroparesis, suggesting that this protein may play an important role in the pathogenesis of these conditions (23,24).

Current literature provides limited data regarding ANO1 expression patterns in the developing human gut tube. Therefore, this study aimed to examine the spatial and temporal distribution of ANO1 in the stomach and small and large intestines during embryo-fetal development. We evaluated its potential as a marker for distinguishing ICC from SMCs during their differentiation from common precursors.

METHODS

The study material comprised 2 human embryos and 21 human fetuses with gestational ages ranging from 8 to 25 weeks. Both embryos were at the 8th week of gestational age, while fetal samples included one each from the 10th and 14th gestational weeks; two from the 11th week; three each from the 15th, 17th, and 19th weeks; and four samples each from the 22nd and 25th gestational weeks. All specimens were obtained from the Center for Pathology and Pathological Anatomy, University Clinical Center Niš, Serbia, following legal abortions and premature births due to intrauterine fetal deaths. All procedures were conducted in accordance with ethical principles and were approved by the Ethics Committee of the University Clinical Center Niš (number 34794/3, date 1.10.2019).

Both sexes were represented in the sample, and no specimens had gastrointestinal disorders. Gestational ages were estimated by anatomical criteria according to the Carnegie Staging system, as well as by crown-rump length, head circumference, and foot length.

Each specimen was fixed in 10% neutral formalin for 24h and routinely processed into paraffin blocks. Tissue sections of 4 µm were cut using a microtome, mounted on slides, and subjected to both hematoxylin and eosin, as well as immunohistochemical staining. Hematoxylin and eosin staining was used to confirm the normal morphology

of all samples, consistent with their gestational age.

Immunohistochemistry

After deparaffinization in a thermostat and xylene, the tissue slides were rehydrated through decreasing concentrations of ethanol (100% and 96%) and distilled water. Following 30-minute heat-induced antigen retrieval, the tissue peroxidases were blocked with a 3% hydrogen peroxide solution for 10 minutes. The antibodies were applied overnight at 4°C. Staining continued the following day by using a secondary antibody conjugated with horseradish peroxidase for 30 minutes (Real EnVision System for visualization, Dako, catalogue number: K5007). Between the steps, the tissue slides were rinsed in EnVision FLEX wash buffer (pH = 7.4). Diaminobenzidine (DAB) served as the chromogen. Finally, the slides were dehydrated through a series of increasing ethanol concentrations (96%, 100%), cleared in xylene, and mounted with Canada balsam and coverslips.

The following antibodies were used for immunohistochemical staining: rabbit polyclonal antibody against ANO1 (Abcam, ab53212, 1:50), mouse monoclonal antibody against NSE (Dako, M0873, 1:100), and rabbit monoclonal antibody against desmin (Abcam, ab32362, 1:300).

Descriptive analysis

Three sections from each sample were analyzed using an Olympus BX50 light microscope (Olympus, Japan) equipped with a Leica DFC295 digital camera (Leica Microsystems, Germany) at the Department of Histology and Embryology, University of Niš Faculty of Medicine, Niš, Serbia.

RESULTS

Embryonic development

Our results showed that ANO1 was expressed in the stomach and small and large intestines at the 8th week of embryonic development (Figure 1A).

The muscularis propria of the stomach included both a broader inner circular layer and a thinner outer longitudinal muscle layer, which showed strong positivity for desmin. The outer longitudinal layer, consisting of 1–3 rows of cells, surrounded the inner layer along the entire circumference (Figure 2F). Developing myenteric plexuses, containing neuron-specific enolase (NSE)-positive cells, were identified between the two muscle layers (Figure 2D). ANO1-immunopositive cells were observed in both the inner and outer layers of the muscularis propria, while the large oval ganglionic cells in the myenteric plexuses were ANO1-immunonegative (Figure 1B). In the small and large intestines, only the inner muscle layer was present, containing ANO1-positive cells, while myenteric plexuses were absent (Figures 1C, 1D, 1F). ANO1-positive cells in the developing muscularis propria across all examined parts of the digestive tube formed a continuous layer and exhibited a pleomorphic appearance with euchromatic nuclei. Some cells were round and lacked cytoplasmic processes, while others adopted a spindle-shaped phenotype.

ANO1 positivity was also observed in endothelial cells of developing blood vessels in the submucosa, as well as in epithelial cells of the pseudostratified epithelium in the stomach and small and large intestines.

The stomach during fetal development

In the 10th and 11th weeks of development, the wall of the stomach consisted of mucosa, submucosa, muscularis propria, and serosa. The epithelium was pseudostratified; however, it was lower than in the embryonic period and showed signs of differentiation. The epithelial cells displayed ANO1 positivity, and gastric glands were not yet formed (Figures 2A, 2B). Two muscle layers were discernible in the muscularis mucosa, both exhibiting strong desmin and ANO1 immunopositivity (Figure 2B). Elongated ANO1-positive cells surrounded myenteric plexuses, with cells showing strong NSE-positivity; however, they did not show ANO1 expression (Figures 2B, 2E). ANO1-immunopositive endothelial cells were also observed within the blood vessels.

Between the 14th and 20th weeks, all layers of the stomach were fully developed. The epithelium was simple columnar, and the gastric glands began to form. The epithelial ANO1 immunopositivity was evident, and the pattern of ANO1 expression resembled that seen at the start of the fetal developmental period.

In the 25th week of development, epithelial ANO1 immunopositivity was weak or absent from cells in the surface epithelia and gastric glands. The circular muscle layer exhibited strong ANO1 immunopositivity, whereas in the longitudinal layer, ANO1-immunopositive cells were rare. Highly flattened, elongated cells were observed in the longitudinal muscle layer and around the myenteric plexuses, consistently lacking ANO1 immunopositivity. The ANO1-positive cells formed a continuous single-cell layer around the margins of the myenteric plexuses. Endothelial positivity was evident in small blood vessels (Figure 2C).

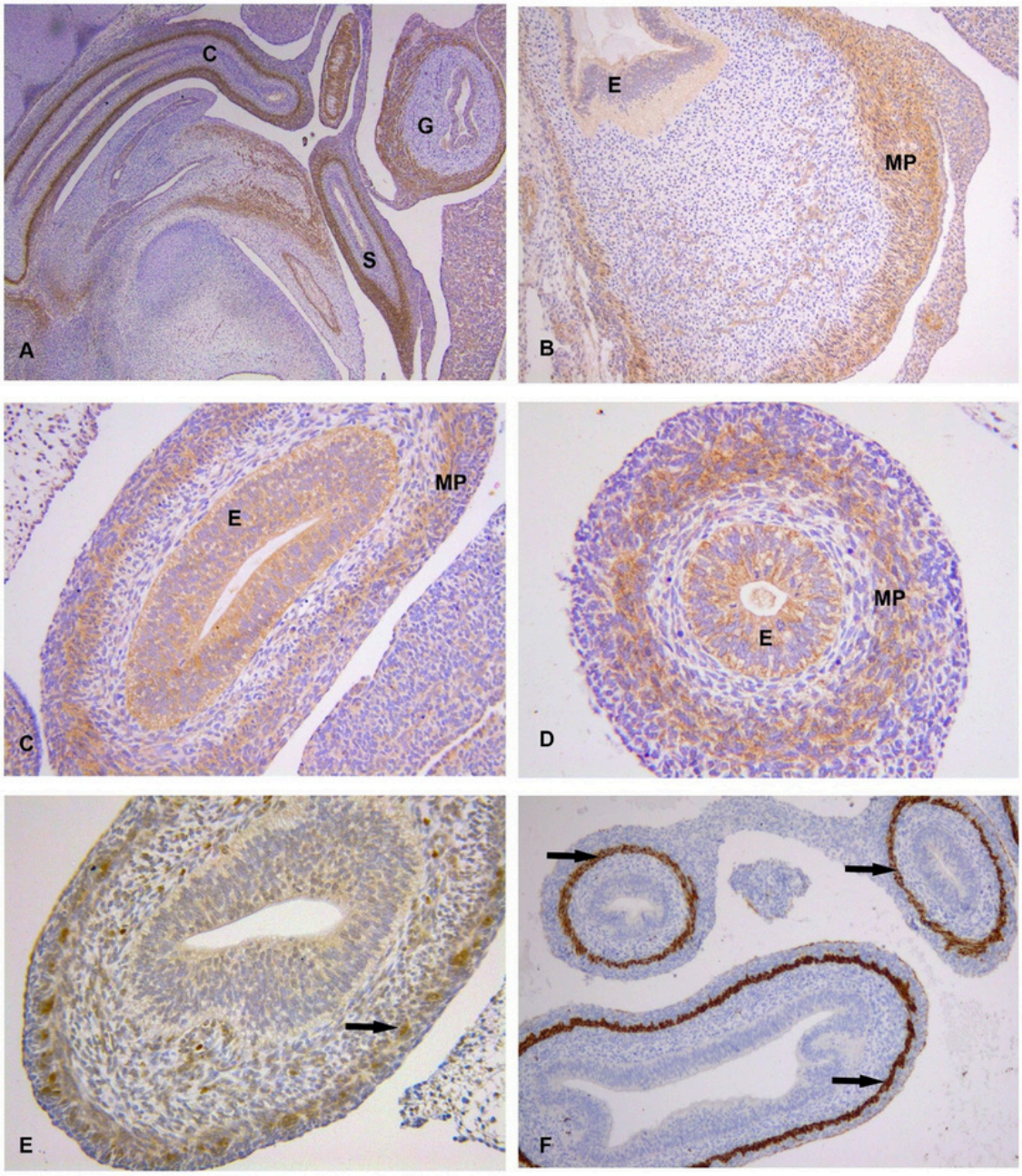

Figure 1. *A) Panoramic view of ANO1 immunopositivity in small and large intestines in late embryonic period at 8th week, x40, G – stomach, S – small intestine, C – large intestine; B) ANO1 immunopositivity in epithelial cells and muscularis propria in stomach at 8th week of development, E – epithelium, MP – muscularis propria, x100; C) ANO1 immunopositivity in epithelial cells and cells of inner layer of muscularis propria in small intestine at 8th week of development, E – epithelium, MP – muscularis propria, x200; D) ANO1 immunopositivity in epithelial cells and cells of the inner layer of muscularis propria in the large intestine at 8th week, E – epithelium, MP – muscularis propria, x200; E) NSE-immunopositivity in ganglionic cells of myenteric plexus in small intestine (black arrow) at 10th week, x200; F) Desmin immunopositivity in SMCs in small and large intestine (black arrows) at 8th week, x125.*

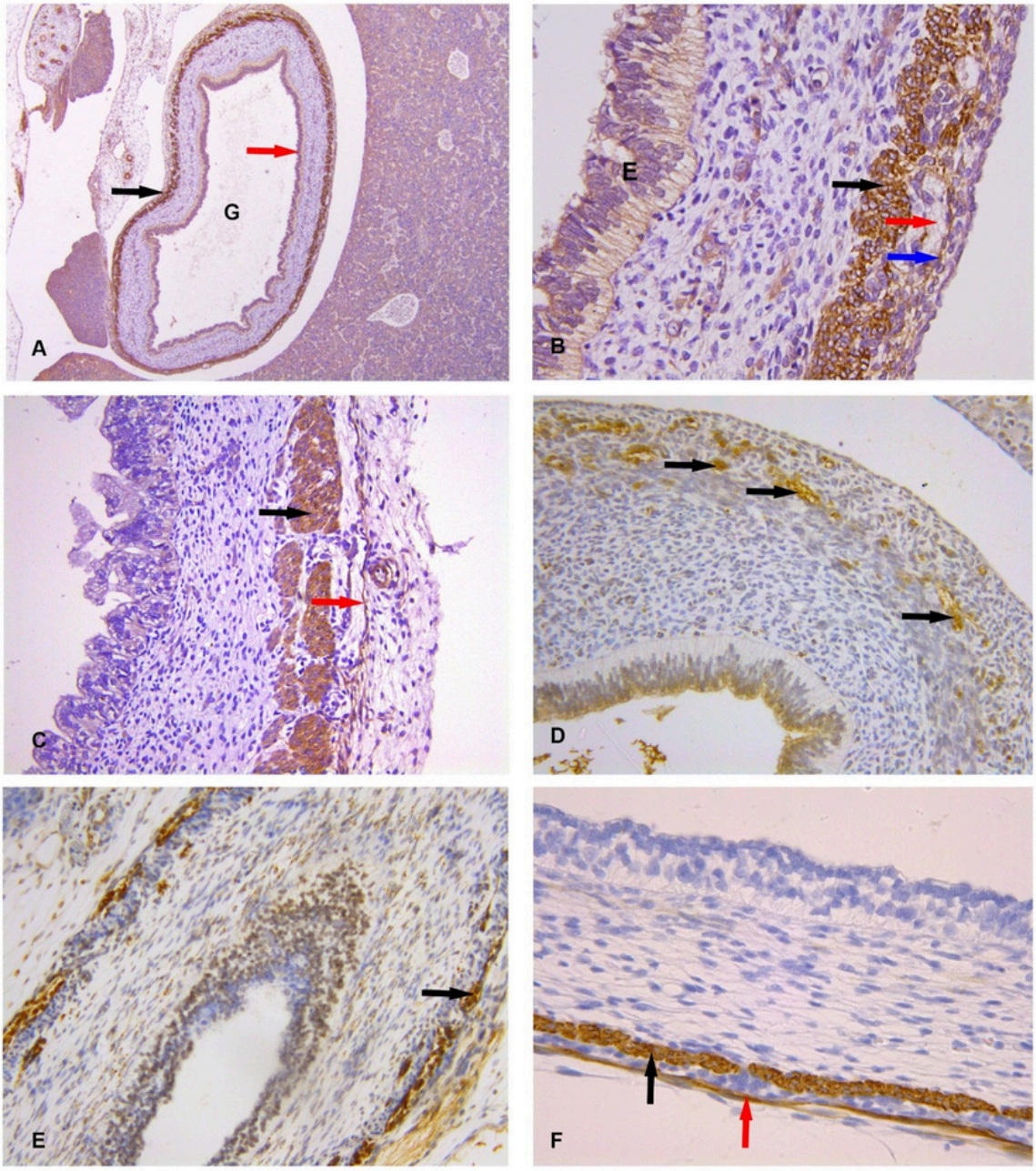

Figure 2. *A) ANO1 immunopositivity in cells of muscularis propria (black arrow) and epithelial cells (red arrow) in the stomach at 10th week of development, x40; B) ANO1 immunopositivity in the stomach in the 10th week of development. ANO1 immunopositivity is observed in epithelial cells (E), endothelial cells of submucosal blood vessels, smooth muscle cells in muscularis propria (black arrow), and elongated cells surrounding the myenteric plexus (blue arrow) (corresponding to ICC). The cells of the myenteric plexus (red arrow) do not show ANO1 immunopositivity, x400; C) ANO1 immunopositivity in the stomach at 22nd week of development. Epithelial cells show low or absent ANO1 expression. Smooth muscle cells of inner muscle layer (black arrow) and elongated cells around the myenteric plexus (corresponding to ICC) (red arrow) show strong ANO1 positivity, which is absent from smooth muscle cells in outer muscle layer, x250; D) NSE immunopositivity in ganglionic cells of myenteric plexus (black arrows) in stomach at 8th week of development, x300; E) NSE immunopositivity in ganglionic cells of myenteric plexus (black arrow) in stomach at 11th week of development, x200; F) Desmin immunopositivity in smooth muscle cells in inner (black arrow) and outer layers (red arrow) of muscularis propria in stomach at 8th week of development, x500.*

Small and large intestine

In the 10th and 11th weeks of development, the primordia of short intestinal villi began to appear in the small intestine, where intestinal glands were also visible. In contrast, they remained absent in the colon (Figure 3A). The thin outer longitudinal muscle layer showed strong desmin positivity observable in both the small intestine and the proximal colon (Figure 3B). Where both muscle layers were present, clearly distinct NSE-immunopositive myenteric plexuses were observed between them (Figure 1E). The epithelium in both the small and large intestines was pseudostratified, though its height was reduced compared to the 8th week, and showed signs of differentiation. Plasmalemmal ANO1 immunopositivity was present across all epithelial cells. ANO1-positive cells were visible within both the circular and longitudinal layers of the muscularis propria. These cells appeared as thin, flattened, elongated structures with bipolar morphology. Flattened, strongly ANO1-positive cells completely enclosed the ANO1-negative myenteric plexuses, forming a continuous belt.

Between the 12th and 20th weeks of development, the intestinal villi were fully formed in the small intestine, and intestinal glands were present in both the small and large intestines. The epithelium consisted of simple columnar cells, which exhibited ANO1 positivity in the apical region and, to a lesser extent, in the lateral areas. In the small intestine, ANO1-immunopositive cells were found within both muscle layers, while in the large intestine, ANO1 immunopositivity was low or absent in the outer muscle layer. Elongated ANO1-positive cells were seen around the myenteric plexuses, whose ganglionic cells lacked ANO1 expression. ANO1-positive cells surrounding the myenteric plexuses were flat and elongated, and formed a complete belt encircling them. Endothelial cells also showed ANO1 immunopositivity in blood vessels (Fig 3C, 3D).

By the 25th week of development, the small and large intestines were fully developed. The muscularis propria comprised a broader circular layer and a distinct, separate outer muscle layer, both of which stained strongly for desmin. The pattern of ANO1 immunopositivity in the muscularis propria resembled that seen at the 20th week of development. In both the small and large intestines, the lamina muscularis mucosae was clearly visible and contained small, stellate, interconnected ANO1-immunopositive cells. These cells were also observed in the intestinal villi, in continuity with the lamina muscularis mucosae, corresponding to the cells of the Brücke muscle (Figure 3E).

DISCUSSION

Our results showed that the stomach and small and large intestines were discernible in histological slides at the end of embryonic development. Their mucosa was lined with pseudostratified epithelium, and the inner circular layer was present in the small and large intestines. In contrast, the thin outer longitudinal muscle layer was observed only in the stomach, where the myenteric plexuses were seen between the two muscle layers. By the 14th week, all histological features of the gastrointestinal tract were developed, including simple columnar epithelium, gastric and intestinal glands, and a well-defined two-layered muscularis propria (15,16,25,26).

ANO1 expression in the stomach and small and large intestines was evident at the end of the embryonic period in epithelial, endothelial, and smooth muscle cells in all examined parts of the gastrointestinal tract. In addition, elongated ANO1-positive cells, corresponding to ICC, were observed surrounding the myenteric plexuses in the stomach. With maturation and development of the outer muscle layer and myenteric plexus in the small and large intestines, ANO1 showed the same pattern of expression in its ICC. Ganglionic cells of the myenteric plexus consistently showed a lack of ANO1 expression in both embryonic and later fetal development. Although there are limited data related to ANO1 expression during the development of the gastrointestinal system, the studies report that c-kit, as a widely accepted marker of ICC, is expressed in the muscle precursors and ICC in the stomach and the proximal part of the duodenum at the end of embryonic development, suggesting that the differentiation of ICC and SMCc begins early during development (26-28). This shared positivity likely results from their common mesenchymal origin. Studies have shown that these two cell types arise from a common c-kit-positive mesenchymal precursor, with c-kit playing a crucial role in directing their differentiation toward SMCs or ICC phenotypes (12,14,29-31). Unlike c-kit-immunopositive cells, which are initially present only in the oesophagus, stomach, and proximal part of the duodenum and appear in other regions only during the 9th and 10th weeks of development, ANO1 positivity was present throughout the gastrointestinal system by the end of embryonic development. (16,27,28,32,33). We therefore suggest that ANO1 may be a potentially earlier or more specific marker for labeling common mesenchymal progenitors compared to c-kit. We observed ANO1 positivity in SMCs and ICC in tissue samples from the 25th week of development,

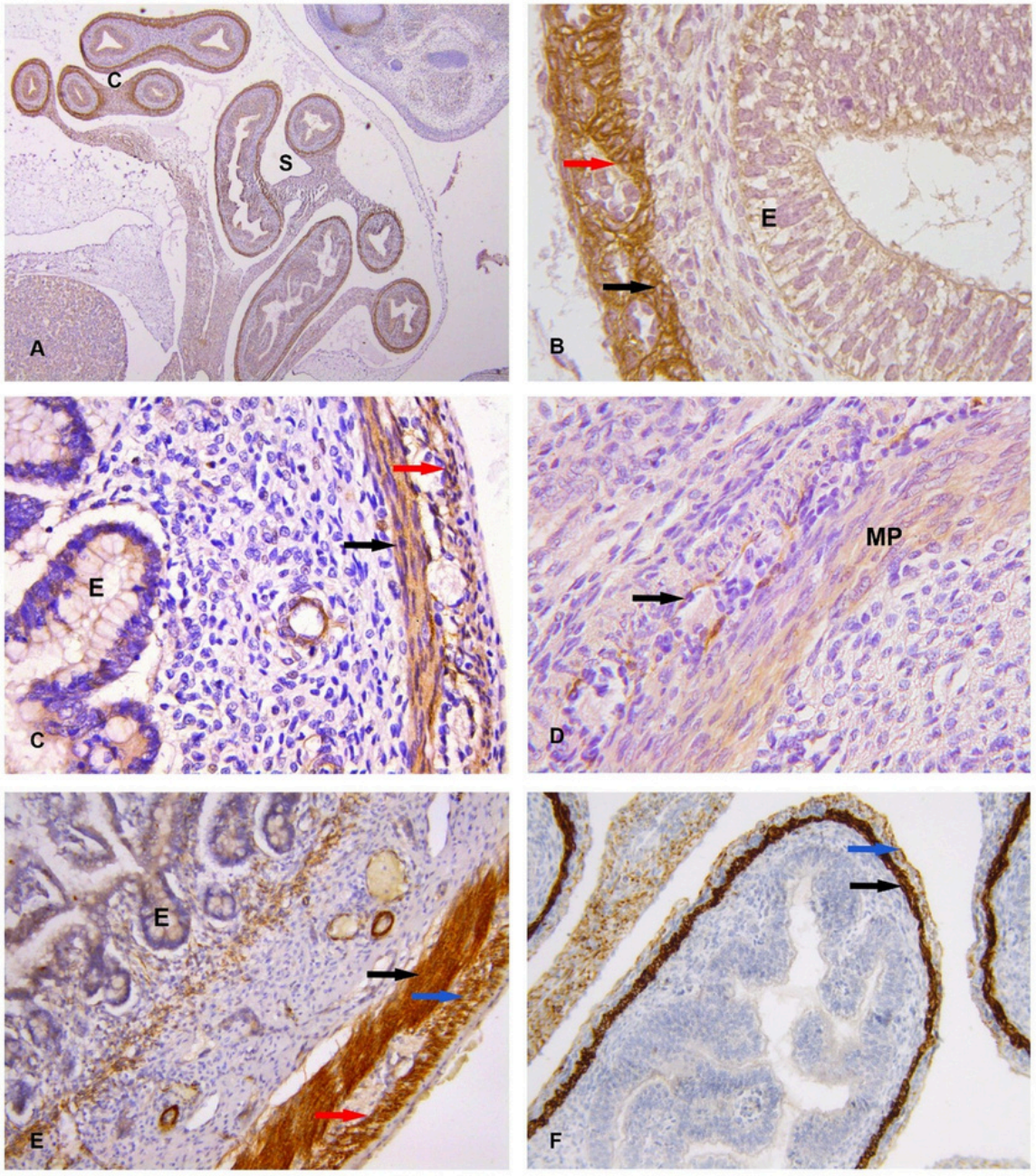

Figure 3. *A) Panoramic view of ANO1 immunopositivity in small and large intestine at 10th week of development, x40, S – small intestine, C – large intestine; B) ANO1 immunopositivity in epithelial cells (E), smooth muscle cells of muscularis propria (black arrow) and cells corresponding to ICC (red arrow) in large intestine at 14th week, x640; C) ANO1 immunopositivity in epithelial cells (E), smooth muscle cells of muscularis propria (black arrow) and cells corresponding to ICC (red arrow) in large intestine at 17th week, x400; D) ANO1 immunopositivity in smooth muscle cells of inner muscle layer (MP) and elongated cells around the myenteric plexus (corresponding to ICC) (black arrow) in large intestine at 19th week, x400; E) ANO1 immunopositivity in epithelial cells (E), smooth muscle cells of muscularis propria (inner layer – black arrow, outer layer – blue arrow) and lamina muscularis musosae, and in cells corresponding to ICC in small intestine (red arrow) in 25th week, x320; F) Desmin immunopositivity in smooth muscle cells of inner (black arrow) and thin, outer muscle layer (blue arrow) in small intestine at 10th week, x200.*

suggesting that these cells maintain ANO1 positivity even beyond the second trimester. In contrast, c-kit expression in SMCs is lost during the early stages of embryonic development. Interestingly, studies on adult human tissue show that ANO1 expression in the gastrointestinal tract is strictly limited to ICC and absent from SMCs (10,11,34). This shift in ANO1 positivity might result from the functional maturation of SMCs and the formation of other types of chloride channels in their plasma membrane (35). Available data also suggest that the deletion or pharmacological inhibition of ANO1 leads to disorders of gastrointestinal motility, resulting from disorganized and reduced contractility of smooth muscle cells (36-39). Currently, experimental efforts are underway to establish stem cell-based therapies for gastrointestinal disorders; however, most studies focus on differentiating ganglionic enteric cells from embryonic or induced pluripotent stem cells, while attempts to differentiate ICC are scarce (40-42). Dave et al. reported that the transplantation of murine ICC stem cells into mice with acute and chronic colitis reduced the severity of symptoms. These cells, whether homing in the colon or studied in vitro, showed the ability to suppress T-cell proliferation (43). Given that ANO1 is expressed in both ICC and their mesenchymal progenitors, it may be used in combination with other markers to identify specific time points during the differentiation of mesenchymal cells into ICC and SMCs, as well as their subsequent maturation.

Endothelial ANO1 positivity was a consistent finding in gastrointestinal tract blood vessels during embryonic and fetal development. Although ANO1 expression has been reported in endothelial cells in the brain, umbilical vein, and heart, we found no data on its expression in endothelial cells during development (44-47). The role of ANO1 in endothelial cells remains incompletely elucidated. Some data suggest that ANO1 promotes vasoconstriction and endothelial dysfunction by generating reactive oxygen species in endothelial cells (44). However, some authors report that activation of ANO1 channels induces vasodilatation and a consequent decrease in blood pressure (48,49). According to Garrud et al., CaCCs activation reduces cytoplasmic chloride ion concentration, thereby activating with-no-lysine kinase (WNK), which in turn stimulates transient receptor potential vanilloid 4 (TRPV4) channels and induces vasodilatation (48).

Our results show that ANO1 is expressed in the epithelial cells of the gastrointestinal tract. This immunopositivity was observed in the pseudostratified epithelium of the embryo and persisted, to a lesser extent, in the simple columnar epithelium of fetal samples. As biological membranes, epithelia play a crucial role in the secretion and absorption of fluids and electrolytes. The expression of ANO1 has been reported in intestinal epithelia, where it is assumed to regulate these processes, given the role of chloride ions in determining the direction of fluid or electrolyte secretion (34,39,50). The lower expression of ANO1 in intestinal epithelium may be explained by the fact that the primary anion channel responsible for chloride secretion in these cells is the cystic fibrosis transmembrane conductance regulator (CFTR) (51). Experimental data have shown an interaction between CFTR and CaCCs signalling pathways, further supported by reports from Benedetto et al. that ANO1 is crucial for the proper membrane function of CFTR (51,52). Furthermore, ANO1 depletion has been shown to reduce calcium-dependent chloride secretion in the small and large intestines of mice, resulting in mild mucosal oedema (50).

In conclusion, ANO1 is considered a reliable marker for monitoring the differentiation of SMCs and ICC during embryonic and fetal development. While ANO1 expression persists in ICC throughout the studied period, SMCs in the outer muscle layer of the stomach and large intestine lose ANO1 positivity by the 25th week of development. Given its early emergence during embryonic development, ANO1 may serve as a valuable biomarker for future studies investigating the differentiation of mesenchymal progenitors into SMCs and ICC lineages, as well as their subsequent maturation and in vitro cultivation.

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Authors' Contribution

Conceptualization, V.P. and G.R.; Formal Analysis, V.P.; Writing – original draft, V.P.; Writing – review & editing, A.V., M.J. and G.R.; Data curation, J.R. and G.R.; Methodology, B.K., D.M. and V.R.; Supervision, G.R. All authors have read and approved the published version of the manuscript.

Statement of Ethics

The study was reviewed and approved by the Ethics Committee of the University Clinical Center Niš, approval number 34794/3, issued on October 1, 2019.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availability

All data analyzed during this study are included within the published article.

Statement of Generative AI Technologies Use

No generative AI was used.

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ORIGINAL ARTICLE

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PEDIATRIC INJURIES RELATED TO CHILD MALTREATMENT

Maja Zečević[1] Dragoljub Živanović[1,2] Ana Bosak Versić[3] Patricija Jakljević[3] Tamara Adamović[1] Dragana Lazarević[4] Vesna Marjanović[2,5] Jelena Lilić[5]

[1]Clinic for Pediatric Surgery, University Clinical Center Niš, Niš, Serbia [2]Department of Surgery and Anesthesiology and Reanimatology, University of Niš Faculty of Medicine, Niš, Serbia [3]Department of Pediatric Surgery, Clinical Hospital Centre Rijeka, Rijeka, Croatia [4]Department of Pediatrics, University of Niš Faculty of Medicine, Niš, Serbia; Clinic of Pediatrics, University Clinical Center Niš, Niš, Serbia [5]Clinic for Anesthesiology and Intensive Therapy, University Clinical Center Niš, Niš, Serbia

Violence against children is a complex socio-medical phenomenon with potential lifetime consequences. Therefore, it is very important to recognize the first signs of violence, as medical staff are quite often the only witnesses of child maltreatment. The aim of the study was to determine the types of abuse and neglect in the pediatric population, which children are most susceptible to, the kinds and severity of injuries, and the required management. A retrospective study was conducted on all patients admitted to two different hospitals due to suspicion of abuse and neglect, with the involvement of a social worker, over a six-year period. This study included 473 patients, whose average age was 8.03 ± 6.01 years; the majority of children were aged 0 to 3 years (35.0%), and 67.2% were boys. Of them, 82.4% were hospitalized because of injuries and other medical conditions caused by neglect. A total of 17.6% were hospitalized due to suspected abuse: peer violence was present in 59.3% of the cases, 22.1% of the children were abused by their parents, and the least number of patients were abused by unknown persons—18.6%. Minor injuries were present in 55.39% of the cases. In total, 232 children underwent surgical treatment due to injuries from abuse or neglect. The majority of children were neglected, not abused, and among the abused children, peer violence was predominant. The most susceptible to neglect and abuse were the youngest members of the pediatric population.

Keywords: child neglect, abuse, pediatric surgery

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Correspondence to:
Tamara Adamović
Clinic for Pediatric Surgery, University Clinical Center Niš
Bulevar dr Zorana Djinjića 48, Niš, Serbia
E-mail: tamara.pesic91@gmail.com

INTRODUCTION

Violence against children is a complex socio-medical phenomenon, based on the interaction of several factors, where many more hidden forms last a long time compared to severe, easily noticeable cases (1). Child maltreatment is the abuse and neglect that occurs to children under 18 years of age. It includes all types of physical and/or emotional ill-treatment, sexual abuse, neglect, neglect, and commercial or other exploitation, which results in actual or potential harm to the child's health, survival, development, or dignity in the context of a relationship of responsibility, trust, or power (2). In the USA, at some point in their life, one in four children experience some form of violence or neglect (3). In the Balkan countries, almost 70% of children were exposed to some form of psychological or physical violence during adolescence, more than 8% of children experienced sexual abuse at least once, 38% of children witnessed violence between adults in the household, and a quarter of children said they had been neglected at least once (4). Maltreatment in childhood is linked with the development of depressive and bipolar disorders later in life, even with an increased rate of suicidal ideation and suicide (5,6). Therefore, it is very important to recognize the first signs of violence; to educate medical staff to react because, very often, we are the only witnesses of child maltreatment and the only advocates for those children.

The aim of the study was to determine the types of abuse and neglect that are most present in the paediatric population, which children are the most susceptible to them, the types of the commonest injuries, and how severe injuries are that require management. Also, an additional aim was to define whether there was a change in the number of patients and days of hospitalization during the COVID-19 pandemic compared to other periods.

METHODS

This was a retrospective study of all patients admitted at two different hospitals due to suspicion of abuse and neglect over a six-year period (2015-2020). The first hospital is the Clinic for Paediatric Surgery, University Clinical Center Niš, Serbia, which covers a region of about 1,500,000 people, and the other is Clinical Hospital Center Rijeka, Pediatric Surgery Department in Croatia, with the region of approximately 450,000 inhabitants, but with extreme enlargement of the population during the summer period as it is a tourist destination.

A social worker was engaged in all cases, which was an inclusion criterion for the study. Patients from outpatient clinics were excluded from the study. Medical documentation was retrospectively reviewed, and the following data were first collected and entered into a Microsoft Excel® (Microsoft Office, Microsoft Corporation, Redmond, WA, USA) spreadsheet database: age, gender, place of residence, type of abuse or neglect, type of injury, management of injuries, and duration of hospitalization. The names of the patients and all other information were exclusively available to the researchers, with the aim to ensure the patient's anonymity. The hypothesis was tested at the $p<0.05$ level of significance. The data were analysed statistically: the Kruskal-Wallis test was used for comparison of the numerical value, while the Chi-squared test and Fisher test were used to compare the categorical features between the groups. All statistical analyses were performed using the R program (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

This study included 473 patients hospitalized in both centers (200 children from Niš and 273 from Rijeka); 318 of them were boys, and 155 were girls. There is a significant difference between the number of patients from Rijeka and Niš; 83.1% vs. 64.5% ($p < 0.001$). The distribution of hospitalized children per year is shown in Figure 1.

The gender and age distribution of abused and neglected children was as follows: aged 0-3 years: n = 166 (92 boys and 74 girls), aged 4-8 years: n = 81 (58 boys and 23 girls), aged 8-13 years: n = 74 (54 boys and 20girls), aged 13-18 years: n = 129 (99 boys and 30 girls). The average age for both genders was 8.03 ± 6.01 (minimum age—1.5months, maximum age—18 years), and the majority of abused and neglected children were aged 0-3 years (35.0%), with the

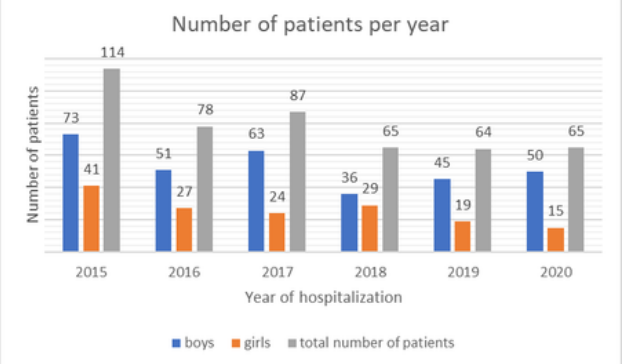

Figure 1. *The average hospitalization rate of children per year*

predominance of male gender in 67.2% of cases. Patients from the University Clinical Center Niš were significantly older—9.1 ± 6.16 compared to patients from the Clinical Hospital Center Rijeka—7.24 ± 5.77 ($p < 0.001$).

The majority of patients (75.1%) were from urban areas (n = 356; boys n = 243, girls n = 113). A total of n = 117 patients were from rural areas (boys n = 76, girls n = 41), while n = 41 children were from the Roma population (boys n = 27, girls n = 14). No patients from the Roma population were recorded at Clinical Hospital Center Rijeka; all were treated at University Clinical Center Niš. The total number of days of hospitalization (pediatric surgery departments and intensive care units) for all cases during the study was n = 2,643 days. The average duration of hospitalization per year is represented in Table 1. Minimal hospitalization was 1 day, and the maximal length of hospitalization was 61days for a neglected two-year-old girl who was severely burned by the open fire with 35% of her body surface covered with second and third-degree burns, including the burns in the throat and respiratory complications. There was no significant difference in the length of hospitalization when comparing the years before and during the COVID-19 pandemic in both centers ($p = 0,073$), or within each center (Niš—$p = 0$ .505, Rijeka—$p = 0.688$). However, the length of hospitalization was significantly longer in Niš compared to Rijeka ($p < 0.001$).

Minor injuries were present in the majority of patients, 262 of them (170 boys and 92 girls), and severe injuries were detected in 212 children (149 boys and 63 girls).Less severe injuries were more frequent in Rijeka than in Niš (59.7% vs 46.6% ($p = 0.007$), while sequelae after injuries were statistically more common in Niš (26.7% vs 3.0%, $p < 0.001$). Two hundred and thirty-two children underwent surgical treatment due to injuries from abuse or neglect—160 were boys and 72 were girls, in both centers. A statistically significantly larger percentage of patients underwent surgical treatment in Niš (66.7% vs 35.5%, $p <$ 0,001). No lethal outcomes due to physical abuse or neglect were reported in this study.

The total number of abused children was 87, and of whom 12 children were not only abused but also severely neglected (7 boys and 5 girls).Peer violence was present in 59.3%, 22.1% of children were abused by their parents, and the least number of patients were abused by unknown persons, 18.6%. The number of abused children divided by age group and gender is given in Table 2.

In the first group, comprising children aged from birth to 3 years of age, there were 9 cases of physical and one case of sexual abuse of a three-year-old girl; all abusers were parents or guardians. In the second group, with children aged from 44 to 7 years, there were four physical and one sexual abuse cases of a four-year-old boy who was forced to take off his clothes and was touched in the genital region by another boy. All abusers were peers, except in one case, where it was a parent. In the third group, aged 8 to 12 years, 9 children were physically abused, and all abusers were peers. In this age group were two cases of sexual abuse: one of a twelve-year-old girl abused by an unknown adult, and the other of an eight-year-old boy abused by his parent. In the fourth group, aged 13 to 18 years, all cases were of physical abuse made by peers in 38 cases, unknown persons in 13, and parents in 7 cases.

In this study, 82.4% of children (n = 390; 255 boys and 135 girls) were hospitalized in both centers because of injuries and other medical conditions caused by neglect. Demographic data are presented in Table 3. Neglect was more present in Rijeka compared to Niš (88.1% vs. 79.7%, $p = 0.019$).

Of the neglected children, 390 of them were injured (255 boys and 135 girls). Isolated injuries were present in 195 patients, and the most common were burns, seen in 93 patients. More than one body region was affected in 53 children, and the largest burn was 35% of the body surface, of the second and third degree. Frostbites (chil-

Table 1. *The average number of days of hospitalization per year*

Year of hospitalization	Both centers in total	Niš	Rijeka
2015	5.27 ± 9.10 2.0 (0.0 – 65.0)	6.54 ± 7.91 2.0 (1.0 - 27.0)	4.86 ± 9.46 2.0 (0.0 - 65.0)
2016	5.67 ± 7.27 3.0 (1.0 - 38.0)	7.88 ± 9.92 3.5 (1.0 - 38.0)	4.56 ± 5.29 3.0 (1.0 - 24.0)
2017	6.56 ± 8.29 3.0 (1.0 - 61.0)	7.39 ± 6.28 5.0 (1.0 - 23.0)	5.83 ± 9.76 3.0 (1.0 - 61.0)
2018	6.00 ± 6.32 5.0 (0.0 - 33.0)	6.41 ± 5.22 6.0 (0.00 - 30.0)	5.14 ± 8.25 2.0 (1.0 - 33.0)
2019	4.73 ± 5.88 3.0 (1.0 - 37.0)	5.79 ± 6.81 4.0 (1.0 - 37.0)	3.61 ± 4.49 2.0 (1.0 - 24.0)
2020	5.17 ± 5.83 3.0 (1.0 - 33.0)	5.57 ± 6.39 3.5 (1.0 - 33.0)	4.86 ± 5.43 2.0 (1.0 - 21.0)
p	0.073	0.505	0.688

Table 2. *The number of abused children divides by age group and gender*

Age groups in years	Total number	Boys	Girls
0-3 years of age	10	6	4
4-7 years of age	5	5	0
8-12 years of age	13	10	3
13-18 years of age	59	45	14

Table 3. *Demographic data of neglected children*

Age groups in years	Total number	Boys	Girls
0-3 years of age	162	89	73
4-7 years of age	74	51	23
8-12 years of age	61	44	17
13-18 years of age	93	71	22

blains) were found in 3 children. In the second place was a head injury (n = 71); 17 injuries were caused by a fall from a bicycle without a helmet, 47 were consequences from the fall from some height bed, chair, arms, tree, window, balcony, etc.), 3 were simple falls, 3 were caused by traffic accident without using a seatbelt or safety seat, and one was caused by a dog. Fractures of the upper extremities were present in 10 children, and in the lower extremities in 11 children; there was only one hip luxation. Semi-amputations of fingers, amputation, and tendon section were found in 7.2, and 7 children, respectively. Simple cuts were seen in 42 patients. Cuts, semi-amputations, amputations, and tendon sections were caused by glass and different tools and machines (knives, axes, saws, circular saws, chainsaws, and other agricultural and machines present in the household). Multiple injuries were detected in 183 children; the most common were multiple contusions seen in 46 children. Twelve children consumed alcoholic drinks, and two mixed alcohol with drugs (diazepam and bromazepam).

During these six years, 56 children were in some way medically neglected. Those were children with previously diagnosed illnesses without regular check-ups or treatment, unvaccinated children, those without medical insurance, which is mandatory and free of charge in both countries, and children whose parents disagreed with further diagnostic and therapeutic procedures and took their children out of hospitals despite medical advice.

In this study, 12 children attempted suicide—4 boys and 8 girls aged from 14 to 17 years. In four cases, there were simple cuts on wrists made by razors or glass. One girl stabbed herself on a chest with a knife, making a partial pneumothorax that did not require thoracal drainage. Three children had deeper cuts with multiple tendon sections, and in one of them, there was also injury to n. medianus and ulnar artery. Jumping from a height was present in three cases, resulting in tibial and L3 vertebral fracture in one patient, and fracture of L1 vertebra and calf in the second case. The third child had the most severe injuries, vertebral fractures from Th6-Th12, serial rib fracture left from VII-X rib and right from IV-VI rib as a result of a 20m high jump. One child who was previously treated for anorexia, when he lost 38kg in a few months, attempted suicide by ingestion of hydrochloric acid. Following surgical treatment, all children were transferred to mental health institutions for further management. There was no significant difference in the number of suicidal attempts between these two centers (p = 0.375).

DISCUSSION

At least, 850 children aged under 15 years die from child maltreatment annually in the WHO European Region, as maltreatment is common but not known to agencies (7). The maltreatment of children has been divided into four major categories: neglect, physical abuse, psychological or emotional abuse, and sexual abuse (8).

This paper provides data on the prevalence of children's exposure to different forms of abuse and neglect over the past six years in two centers with pediatric surgery hospitals/departments in the following countries: Serbia and Croatia. Unlike the majority of studies that could be found in the literature, this study was not based on questionnaires, but on physical evidence of abuse or neglect that required hospitalization and medical help. In the BECAN study, the rates for physical violence and contact sexual violence in Serbia were 46.48% and 3.7%, and in Croatia, 45.54% and 3.26%, respectively. In the same study, no differences could be observed between sexes across these two countries related to lifetime physical and sexual violence exposure, and for experiences of feelings of neglect, differences between males and females could be observed with higher lifetime prevalence among females: Croatia (40.6% vs 29.8%) and Serbia (34.6% vs 23.4%). On the contrary, we found that 67.2% of hospitalized children were boys; however, our study included not only school children but also preschool children (4).

One of the most common forms of child maltreatment is neglect, which could often be associated with other forms

of abuse. Childhood is characterized by progressive physical, emotional, cognitive and social development, and satisfaction of basic needs such as adequate nutrition, hygiene, emotional support, health care, and safe living conditions are necessary for growth and development (9). There is a little evidence base to guide through medical neglect management and research; therefore, medical neglect literature is scarce even though this topic became more significant lately, as medical neglect is associated with significant morbidity and mortality. In this study, 11.8% of children were medically neglected, and most of them had previously been diagnosed with untreated medical conditions. Fortin et al. stated in their research that 91% had a chronic illness; however, our research included only patients hospitalized due to surgical not pediatric conditions, and this could be the reason why this percentage is quite lower in this study. Although the doctor's responsibility is to the child, the management should also involve identifying the cause that led to the medical neglect (10,11).

Peer violence is a complex social problem due to its diversity, and it ranges from 15% to 50% depending on the development of the country (12). Almost 60% of abused children in this paper were victims of peer violence, which indicates how huge this problem is, as it has been proven that youth involved in peer and dating violence as aggressors and victims are at greatest risk for negative sequelae (13).

Diagnosing sexual abuse in children may be challenging, as physical findings are present only in 10% of girls who have been sexually abused and seldom in boys on medical examinations. The reason is that genital area traumas heal quickly in cases when it does occur; therefore, "sexual abuse by history" is the most common medical diagnosis arising from such evaluations. There is no complete agreement on findings and guidelines for interpretation among physicians of suspected child sexual abuse, which makes a firm diagnosis even harder. Commonly, an immediate examination is done if the last instance of penetration occurred within 72 hours (14,15).

Childhood sexual abuse can have far-reaching consequences, and in this study, 4 children aged 3 to 12 years underwent different kinds of sexual abuse. The importance of recognizing it and providing professional mental health help is well emphasized in the paper published by Baytuncaet al., as suicide attempts were significantly more frequent in sexually abused children, especially in adolescent girls, and even 10 times higher among children living in broken families (16,17).

Suicide is the third leading cause of death among adolescents and young people aged 15–35 years, and the second leading cause of death for youth aged 11 to 15. Self-harm is one of the strongest predictors of death by suicide in adolescence. Domestic and peer violence, lack of support, and child maltreatment are known factors that increase the likelihood of self-harm behaviour in adolescents (18). In this research, 2.5% of children aged 14-17 years have attempted suicide. Eight of them were 17 years old, and there were more females compared to males, 8 vs. 4. Steinhof et al. reported that self-injuries were more frequent in females than males, as one in three females and one in five males have self-injured at least once between ages 13 and 20, which correlates with our findings (19,20).

Overall, the findings of this study documented the pediatric population most at risk of abuse and neglect, the types and severity of injuries, and the need for hospitalization due to children's exposure to various forms of maltreatment in the participating centers. Early diagnosis of child neglect or abuse is essential, and care should include not only acute medical treatment but also longer-term, multidisciplinary treatment.

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Authors' contribution

Conceptualization: Z.M., Z.D., and A.B.V.; Methodology, Z.D., A.B.V., T.A., J.L., and D.L.; Investigation, Z.M., A.B.V., T.A., J.L., and D.L.; Data curation, A.B.V., T.A., J.L., and D.L.; Formal analysis, V.M.; Validation, V.M.; Project administration, Z.D.; Supervision, Z.D., V.M., and A.B.V.; Writing – original draft, Z.M.; Writing – review & editing, Z.M., A.B.V., T.A., J.L., D.L., and V.M. All authors have read and approved the published version of the manuscript.

Statement of Ethics

Ethics approval was not required as the retrospective study was performed based on medical documentation without revealing any personal details.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availability

Not applicable. All relevant data are provided in the results section.

Statement of Generative AI Use

No AI was used for writing this manuscript.

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ORIGINAL ARTICLE

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ASSESSMENT OF ORTHODONTIC TREATMENT NEED AMONG SERBIAN CHILDREN AND ADULTS: APPLICATION OF THE INDEX OF ORTHODONTIC TREATMENT NEED

Andjelka Cvijić[1] Iva Jakovljević[2] Aleksandra Arnaut[3] Roberta Marković[4] Tatjana Čutović[5]

[1]Department of Clinical Dentistry, University of Bergen, Bergen, Norway [2]Department of Dentistry, University of Kragujevac Faculty of Medical Sciences, Kragujevac, Serbia [3]Department of Dentistry, University of Kragujevac Faculty of Medical Sciences, Kragujevac, Serbia [4]University of Niš Faculty of Medicine, Niš, Serbia [5]University of Defence, Faculty of Medicine of the Military Medical Academy, Belgrade, Serbia

Malocclusions are common oral health issues that require precise assessment of severity and the need for orthodontic treatment. The Index of Orthodontic Treatment Need (IOTN) is a reliable tool for objective evaluation of the necessity of orthodontic therapy. This study aimed to determine the need for orthodontic treatment among children and adolescents in Serbia using the IOTN index, and to compare participants' subjective perception of dental aesthetics with evaluations provided by orthodontic professionals. The prevalence of malocclusion was also recorded. The study included 211 participants aged 9 to 25 years, all military health insurance beneficiaries who had not previously undergone orthodontic treatment. Examinations were conducted by a dentist and supervised by an orthodontic specialist at the Military Medical Academy. Assessments were performed according to IOTN guidelines, incorporating both the Dental Health Component (DHC) and Aesthetic Component (AC), and data were statistically analyzed. Findings indicated that 63% of participants had a clear need for orthodontic treatment based on the DHC component, while 28% were borderline cases. According to the AC component, 32.2% of participants self-reported a need for treatment, whereas therapists indicated a slightly lower percentage (27%). The most common orthodontic irregularity was contact point displacement, while increased overjet and deep bite were most frequently in need of treatment. Children and adolescents in Serbia exhibit a high demand for orthodontic treatment. The IOTN index has proven effective for prioritizing treatment needs. The study results emphasize the importance of integrating this index into clinical practice to optimize resource allocation and improve treatment efficiency.

Keywords: orthodontic treatment, malocclusion, IOTN, treatment need assessment, orthodontic anomalies

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Correspondence to:
Iva Jakovljević
Department of Dentistry
University of Kragujevac Faculty of Medical Sciences
Svetozara Markovića 69, Kragujevac, Serbia
E-mail: iva_obradovic@yahoo.com

INTRODUCTION

Malocclusions are among the most prevalent conditions in oral health, requiring careful evaluation and appropriate treatment. In addition to functional disorders, malocclusions can lead to aesthetic concerns, diminished quality of life, and cause psychosocial consequences, particularly in children and adolescents (1,2). Given that not all patients with malocclusions require the same type or urgency of treatment, the development of standardized assessment tools has become crucial.

The Index of Orthodontic Treatment Need (IOTN) was developed by Brook and Shaw in 1989 as a system for assessing and quantifying the need for orthodontic treatment. Due to its simplicity, reliability, and objectivity, this index quickly became an internationally recognized tool (3).

IOTN consists of two main components:

1. Dental Health Component (DHC)—A clinical component that focuses on the functional and biological aspects of malocclusion. DHC classifies patients into five categories based on the severity of malocclusion, with the following scores: 1 (no treatment needed), 2 (mild anomaly, treatment not required), 3 (borderline case, treatment may or may not be necessary), 4 (treatment required), and 5 (urgent need for treatment) (4). The scores are assigned based on the most severe orthodontic irregularity and are detailed in Table 1 (5).
2. Aesthetic Component (AC)—This component of the IOTN index assesses the aesthetic aspect of malocclusion. It is based on a series of standardized photographs representing different degrees of dental irregularities, ranked from 1 (almost ideal appearance) to 10 (advanced esthetic disharmony) (Figure 1). Patients who did not match any of the images remained unclassified (score 0) (6). This visual representation facilitates communication between patients and orthodontists in decision-making regarding treatment initiation or prioritization, as it provides insights not only into the functional but also the aesthetic significance of orthodontic therapy (7).

A modification of the index was proposed in 1993, reducing the DHC scale from five to three categories (scores 1-2: no treatment needed; score 3: borderline; scores 4-5: treatment required). Similarly, the AC component was reduced from ten to three categories (scores 1-4: no treatment needed; scores 5-7: moderate treatment needed; scores 8-10: treatment required). This simplification aimed at improving the identification of individuals requiring orthodontic therapy (8).

The IOTN index is internationally recognized and is most commonly used in epidemiological studies. It not only assesses the need for treatment but also determines treatment priorities, which is particularly important in healthcare systems with limited resources. The DHC component provides objective clinical data, while the AC component incorporates subjective aspects and patient perception, ensuring a holistic approach. However, its aesthetic component remains subjective and may lead to variability in assessment, especially among less experienced evaluators. Another limitation is the extensive training required for clinicians to become proficient in using the index (4).

Studies conducted in different countries have demonstrated a wide range of malocclusion prevalence and treatment needs based on the IOTN index. In Serbia, a study conducted in Niš on a sample of 190 participants aged 11–14 years found that 27.4% of participants had a high need for treatment according to the DHC component, whereas only 15.3% of children rated their need for treatment as urgent based on the AC component (9).

In Bosnia and Herzegovina, a study on a sample of 295 students aged 12–14 years revealed a high treatment need according to the DHC component (53.6%) but a low need based on the AC component (3.7%) (10). A study conducted in Belgrade found that, according to the AC component, only 0.63% of participants subjectively perceived a need for treatment, while the therapist's evaluation indicated a significantly higher percentage—7.59% (7).

Studies in other countries have yielded varying results. A study in southern Italy, on a sample of 703 children aged 12 years, reported that 27.3% of children had an urgent need for treatment according to the DHC component (11). In Sweden, the percentage of children requiring treatment was 32.4%, while in the United Kingdom, this number was slightly higher—39.5% (4). Differences in results across populations can be attributed to various socio-demographic and cultural factors, as well as different approaches to malocclusion assessment. These studies highlight the importance of interpreting IOTN results in local contexts and emphasize the need for research combining both objective and subjective evaluation methods.

The aim of this study was to assess the prevalence and severity of malocclusion among children and adolescents in Serbia using the IOTN index. Particular attention was given to comparing the DHC and AC components and too evaluating the subjective patients' and clinicians' subjective perception of treatment needs.

	Overjet	Reverse overbite	Crossbite	Contact point displacement	Open bite	Overbite	Angle class (molars)	Hypodontia	Eruption disturbance	Craniofacial abnormalities	Ankylosis/ retained primary teeth
1				1 mm							
2	3.5 - 6 mm, competent lips	0-1 mm	< 1 mm	1 - 2 mm	1 - 2 mm	< 3.5 mm increased overbite without gingival contact	Class II or III without any dysfunction				
3	3.5 - 6 mm, incompetent lips	1 - 3.5 mm	1 – 2 mm	2 - 4 mm	2 – 4 mm	> 3.5 mm increased overbite without gingival contact					
4	6 - 9 mm	> 3.5 mm no speech and masticatory dysfunction, 1 – 3 mm with dysfunction	> 2 mm crossbite	> 4 mm	> 4 mm	Increased overbite with gingival or palatal contact		Orthodontic treatment indicated for space closure or space gaining	Partially erupted, tilted, or impacted tooth, hyperdontia		
5	> 9 mm	> 3.5 mm with speech and masticatory dysfunction						Hypodontia of at least 1 tooth per quadrant, prosthetic treatment required	Impacted tooth	Definitive treatment required	Definitive treatment required

Table 1. *Criteria table for the IOTN index, adapted from the Orthodontic Handbook by Stjepan Spalj*

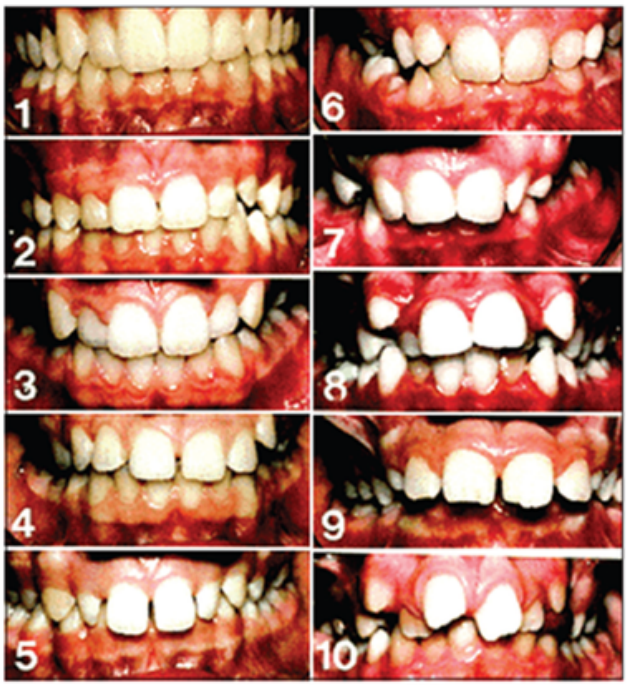

Figure 1. *Photographs illustrating the AC component of the IOTN index: Scores 1-4—treatment needed, 5-7—moderate treatment need, and 8-10—treatment required.*

METHODS

This study examined the need for orthodontic treatment in patients with mixed and permanent dentition using the Index of Orthodontic Treatment Need (IOTN). The sample consisted of 211 participants aged 9 to 25 years, including 112 females and 99 males. Participants were selected at the Department of Orthodontics, Military Medical Academy, between January 2017 and January 2018. All participants were beneficiaries of military health insurance from various cities across Serbia. None had previously undergone orthodontic treatment. Patients with cognitive impairments or chronic diseases were excluded from the study.

The clinical examinations were conducted in the morning under natural daylight at the Department of Orthodontics. Each participant was examined for 15 minutes by a dentist undergoing an academic specialization in Orthodontics, under the supervision of an orthodontic specialist. The examination followed the World Health Organization (WHO) guidelines and was performed using sterile gloves, a dental mirror, a millimeter ruler, and a photographic reference of the AC component of the index.

Participants' personal data were collected directly from them. The DHC score was determined first by the examiner, followed by the AC score. Subsequently, participants were shown the AC component photograph

depicting ten different degrees of dental aesthetics and asked to identify which image best represented their perception of their own dentition.

The following parameters were assessed in the study: molar relationship, overjet and overbite dimensions, presence of anterior crossbite, tooth position discrepancies, including misalignment, rotations, and dislocations, presence of open bite and crossbite, anomalies in tooth number such as hypodontia and hyperdontia, difficulties in tooth eruption, and the presence of craniofacial anomalies.

The collected data were statistically analyzed, including descriptive statistical parameters such as mean values and 95% confidence intervals for both the DHC and AC components of the IOTN index.

RESULTS

A total of 211 participants, aged 9 to 25 years, were included in the study, with 53.1% female and 46.9% male participants. The average age of the participants was 9 years, accounting for slightly more than a quarter of the total sample.

According to the DHC component of the IOTN index, 63% of participants (60.7% female and 65.7% male) demonstrated a clear need for orthodontic treatment, while 28% fell into the borderline category (26.8% female and 29.3% male) (Table 2).

Based on therapists' assessments, the AC component indicated that 27% of participants (25.9% female and 28.3% male) had an urgent need for treatment. However, according to participants' self-perception, this percentage was slightly higher, reaching 32.2% (30.4% female and 34.3% male), suggesting a stricter self-assessment of the aesthetic need for treatment (Tables 3 and 4).

Figure 2 illustrates the relationship between therapists' and participants' AC component scores, showing general agreement between the two assessments, except for scores 8-10, where participants tended to rate their aesthetic need for treatment more strictly.

The DHC component was found to be stricter than the AC component, according to both therapists' and participants' evaluations, with a significantly higher number of patients requiring treatment based on the DHC component (Figures 4 and 5).

The types and prevalence of malocclusions, as well as the need for treatment across the sample, are presented in Figure 5. Analysis of horizontal overjet indicated that treatment was necessary in 18.4% of cases, while anterior crossbite required treatment in 2.3% of cases.

Displacement of contact points was the most common anomaly, observed in 34.1% of participants, though only 4.3% required treatment. Examination of anterior and posterior open bite revealed that treatment was necessary for 2.8% of participants, while crossbite was diagnosed in 11.8% of participants, of whom 10% required treatment.

For vertical overbite anomalies, 10.9% of participants required treatment. Participants with missing or supernumerary teeth, craniofacial anomalies, delayed eruption, impacted teeth, or partially erupted teeth required orthodontic treatment in all cases.

Table 2. *Distribution of subjects by DHC* component of IOTN** index*

DHC		1	2	3	4	5	Σ
Full sample	n	1	18	59	108	25	211
	%	0.5	8.5	28.0	51.2	11.8	100.0
	Confidence interval	0.0-1.4%	4.8-12.3%	21.9-34.0%	44.4-57.9%	7.5-16.2%	
Female	n	0	14	30	53	15	112
	%	0.0	12.5	26.8	47.3	13.4	100.0
	Confidence interval	0.0-0.0%	6.4-18.6%	18.6-35.0%	38.1-56.6%	7.1-19.7%	
Male	n	1	4	29	55	10	99
	%	1.0	4.0	29.3	55.6	10.1	100.0
	Confidence interval	0.0-3.0%	0.2-7.9%	20.3-38.3%	45.8-65.3%	4.2-16.0%	

*DHC – Dental Health Component **IOTN -Index of Orthodontic Treatment Need

Table 3. *Distribution of subjects by AC* component of IOTN** index, graded by therapist*

AC (T)		0	1-2	3-4	5-7	8-10	Σ
Full sample	n	13	25	44	72	57	211
	%	6.2	11.8	20.9	34.1	27.0	100.0
	Confidence interval	2.9-9.4%	7.5-16.2%	15.4-26.3%	27.7-40.5%	21.0-33.0%	
Female	n	7	16	23	37	29	112
	%	6.3	14.3	20.5	33.0	25.9	100.0
	Confidence interval	1.8-10.7%	7.8-20.8%	13.1-28.0%	24.3-41.7%	17.8-34.0%	
Male	n	6	9	21	35	28	99
	%	6.1	9.1	21.2	35.4	28.3	100.0
	Confidence interval	1.4-10.8%	3.4-14.8%	13.2-29.3%	25.9-44.8%	19.4-37.2%	

*AC – Aesthetic Component **IOTN – Index of Orthodontic Treatment Need

Table 4. *Distribution of subjects by DHC * component of IOTN** index, graded by subjects*

AC (S)		0	1-2	3-4	5-7	8-10	Σ
Full sample	n	15	19	42	67	68	211
	%	7.1	9.0	19.9	31.8	32.2	100.0
	Confidence interval	3.6-10.6%	5.1-12.9%	14.5-25.3%	25.5-38.0%	25.9-38.5%	
Female	n	7	11	22	38	34	112
	%	6.3	9.8	19.6	33.9	30.4	100.0
	Confidence interval	1.8-10.7%	4.3-15.3%	12.3-27.0%	25.2-42.7%	21.8-38.9%	
Male	n	8	8	20	29	34	99
	%	8.1	8.1	20.2	29.3	34.3	100.0
	Confidence interval	2.7-13.4%	2.7-13.4%	12.3-28.1%	20.3-38.3%	25.0-43.7%	

*AC – Aesthetic Component **IOTN – Index of Orthodontic Treatment Need

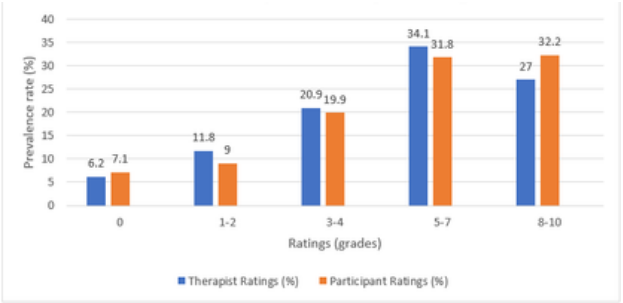

Figure 2. *Agreement between therapists' and subjects' Aesthetic component (AC) ratings for full sample*

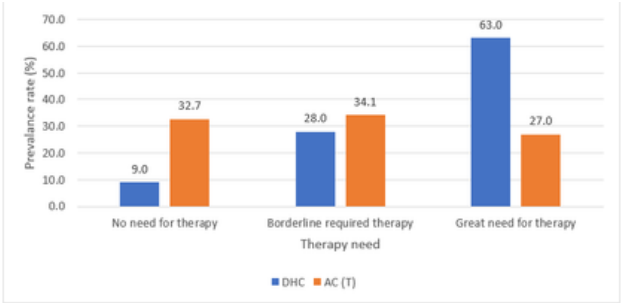

Figure 3. *Comparison between Dental Health Component (DHC)* grades (No need for therapy—1-2, borderline required therapy—3, great need for therapy—4-5) and Aesthetic Component (AC) grades by therapist** of Index of Orthodontic Treatment Need (IOTN)*** (No need for therapy—1-4, borderline required therapy—5-7, great need for therapy— 8-10).*

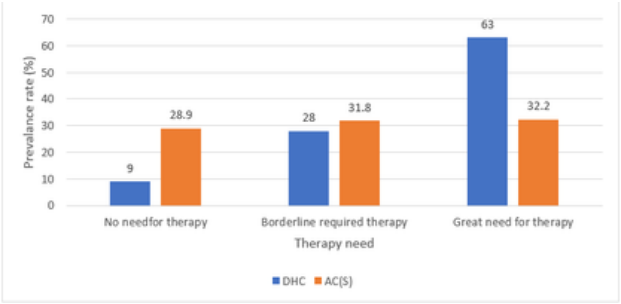

Figure 4. *Comparison of Dental Health Component (DHC)* grades (No need for therapy—1-2, borderline required therapy—3, great need for therapy—4-5) and Aesthetic Component (AC) grades by subjects** of Index of Orthodontic Need (IOTN)***(No need for therapy— 1-4, borderline required therapy—5-7, great need for therapy— 8-10)*

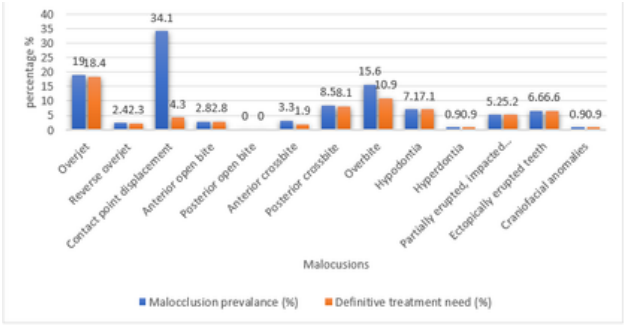

Figure 5. *Analysis of malocclusion prevalence within the sample and the proportion of subjects with a definitive treatment need*

DISCUSSION

The evaluation of the need for orthodontic treatment is a key step in planning and providing dental services, particularly in settings with limited resources (3). The results of this study, indicating that 63% of participants required orthodontic treatment based on the DHC component of the IOTN index, provide significant insight into the prevalence of malocclusions in the Serbian population. These findings differ substantially from a 2005 study conducted in Serbia, where the need for orthodontic therapy was identified in only 27.4% of participants (9). This percentage is higher compared to similar studies conducted in Italy (24.4%), France (21.3%), and the United Kingdom (35%) (11-13). These differences may be attributed to the broader age range (9–25 years) included in this study, whereas most previous studies focused on younger age groups.

Similar research highlights the relationship between the defined need for orthodontic treatment and its actual application in various European countries. A study conducted in Perugia, Italy, found that 27.55% of patients had a DHC score of 4 or 5, which is considerably lower than the 63% recorded in this study in Serbia. However, 72% of patients who received orthodontic treatment did not belong to this priority group, suggesting a need for better resource allocation toward patients with higher health risks (14). In contrast, in the United Kingdom, 35% of participants were identified as needing treatment, but it was actually administered to only 8% of them (13).

Recent studies further support the importance of using the IOTN index in assessing orthodontic treatment needs. Al-Hummayani and Taibah (2018) found that 24.3% of young adults in Saudi Arabia had a severe or extreme need for treatment, with the most common malocclusions being crowding (48.8%) and increased overjet (21.8%) (15). Similarly, Bhagyalakshmi et al. examined the perception of aesthetic need for orthodontic treatment and recorded a high level of agreement between children, parents, and orthodontists, underscoring the significance of subjective factors in treatment decision-making (16). Additionally, Kadu et al. observed in a study of schoolchildren in southwestern Maharashtra that most participants had aesthetic scores indicating a need for treatment (17). These results further support the role of the IOTN index as a standardized tool for assessing orthodontic needs across different demographic groups (16).

In the present study, the most frequently recorded malocclusion was contact point displacement (34.1%), likely due to lack of space, which can be explained by jaw-to-tooth size discrepancies, early loss of primary teeth, migration of first permanent molars, and their rotation (18). Increased overjet (18.4%) and increased overbite (10.9%) were also identified as common issues, which may lead to serious health consequences, highlighting the importance of early intervention during the mixed dentition phase, when treatment is most effective and least complex (19).

A crucial aspect of this study is the discrepancy between treatment need assessment based on the DHC and AC components of the IOTN index. While 63% of participants required treatment based on the DHC component, the AC component results varied: 27% according to therapists and 32.2% to participants' self-assessments. These findings suggest that more patients may require orthodontic treatment than indicated by either their own or their therapist's subjective assessment. The discrepancies in the

AC component highlight subjective factors in the perception of dental aesthetics, as patients are often more critical of their own appearance (20). Similar findings have been reported in Bosnia and Herzegovina, where there was significant agreement between children and dentists regarding the need for orthodontic treatment based on the AC component of the IOTN index; however, children were frequently more critical of their own appearance (10).

The results emphasize the importance of using both components of the IOTN index in clinical decision-making, especially in borderline cases with a DHC score of 3, where the aesthetic component plays a crucial role (21). All participants with an AC score ≥ 5 should be considered for treatment, further confirming the importance of a combined assessment (4).

This study provides valuable insights into the prevalence and nature of malocclusions among young individuals in Serbia. However, further research is needed to evaluate the functional, psychosocial, and subjective factors that may influence the need for orthodontic treatment. The integration of the IOTN index into routine orthodontic practice could enhance diagnostic objectivity and facilitate optimal resource allocation, particularly in healthcare systems with limited access to these services (22,23).

The results of this study confirm that the IOTN index is a reliable tool for assessing the need for orthodontic treatment. According to the DHC component, treatment was required for 63% of participants, whereas the AC component showed variability in assessments (27% according to therapists and 32.2% according to participants), highlighting differences in subjective perception.

The most common malocclusions were contact point displacement (34.1%), increased overjet (18.4%), and increased overbite (10.9%). These findings emphasize the importance of a combined evaluation of DHC and AC components in treatment decision-making.

The application of the IOTN index in daily orthodontic practice could improve diagnostic objectivity and allow for more efficient resource allocation in orthodontic treatment.

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Author's contribution

Conceptualization, methodology, & investigation: A.C., I.J., A.A., R.M., and T.Č.; Writing – original draft, review, & editing: A.C., I.J., A.A., R.M., and T.Č.; All authors have read and approved the published version of the manuscript.

Statement of Ethics

Ethical approval was not required for this study, as it did not involve biomedical interventions, experiments on humans or animals, or clinical trials.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availability

Not applicable.

Statement of Generative AI Use

No generative AI was used.

Conflicts of interest

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

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ORAL HEALTH IN CHILDREN WITH TYPE 1 DIABETES MELLITUS IN RELATION TO METABOLIC CONTROL

Mirjana Djuričković **Aleksa Djuričković**

Department for Dental Medicine, University of Montenegro Faculty of Medicine, Podgorica, Montenegro

Diabetes mellitus is one of the most common chronic diseases in childhood. The aim of this study was to evaluate the oral health of children with type 1 diabetes in relation to the level of glycemic control. Eighty-seven children aged 10 to 15 participated in the study and were divided into two groups based on the value of glycosylated hemoglobin (HbA1c): 34 children with good metabolic control (HbA1c < 7.5%) and 53 children with poor metabolic control (HbA1c > 7.5%). Oral health was assessed using the index of carious, extracted, and filled permanent teeth (DMFT), plaque index (PI), and gingival index (GI). The stimulated salivary flow rate, buffer capacity of saliva, and the level of presence of *Streptococcus mutans* (*S. mutans*) and *Lactobacillus* in saliva were measured. Participants completed a questionnaire about oral hygiene habits when visiting the dentist. The t-test and chi-square test were used, with a significance level set at $p < 0.05$. Children with poor metabolic control had significantly more extracted teeth ($p = 0.002$), higher PI ($p = 0.002$), higher GI ($p = 0.001$), and a higher risk of *S. mutans* and *Lactobacillus* ($p < 0.005$). No significant differences were found in overall DMFT scores, salivary flow, saliva buffer capacity, oral hygiene habits, dental visits, and socioeconomic status ($p > 0.05$). Poor metabolic control in children with type 1 diabetes is associated with poorer oral health, lower levels of oral hygiene, increased risk of caries and periodontal disease due to the presence of pathogenic bacteria.

Keywords: children, type 1 diabetes mellitus, oral health, metabolic control, saliva

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Correspondence to:
Mirjana Djuričković
Department for Dental Medicine
University of Montenegro
Faculty of Medicine
E-mail: miradjurickovic@gmail.com

INTRODUCTION

Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disease that most frequently affects children and adolescents, and is characterized by the selective destruction of pancreatic β-cells, resulting in absolute insulin deficiency. Insulin is a key hormone in glucose metabolism regulatory processes, and its absence leads to chronic hyperglycemia, which, without appropriate therapy, can lead to serious consequences for a child's health (1,2). In addition to the increased risk of acute complications such as diabetic ketoacidosis, long-term hyperglycemia is associated with the development of microvascular and macrovascular complications, including damage to the kidneys, eyes, nervous and cardiovascular systems (3).

The cornerstone of treatment for type 1 diabetes is lifelong insulin therapy, aimed at maintaining optimal glycemic control and preventing complications. In addition to insulin therapy, patients and their parents must be educated about the importance of self-monitoring, proper nutrition, and regular physical activity. When managed appropriately, therapy can significantly reduce the risk of systemic and oral complications. Despite the availability of various therapeutic options, many patients struggle to achieve satisfactory glycemic control, highlighting the need for further research and the development of preventive strategies targeting oral health (1–4).

In addition to systemic complications, type 1 diabetes significantly affects oral health. Oral complications of type 1 diabetes include xerostomia, periodontal disease (gingivitis and periodontitis), dental abscesses, tooth loss, soft tissue lesions, and burning mouth syndrome (1,2). Numerous studies have linked poor glycemic control to an increased incidence of caries, gingivitis, and periodontal disease (5-7). Mechanisms include decreased salivation, decreased salivary buffering capacity, increased glucose in oral fluids, and a favorable environment for the growth of cariogenic bacteria such as *Streptococcus mutans* and *Lactobacillus*. Children with T1D are thus more prone to dental plaque formation, inflammatory changes in the gingiva, and progression of periodontal destruction, especially in cases of poor metabolic regulation (elevated glycated hemoglobin -HbA1c), with hyperglycemia contributing to the creation of a favorable environment for the growth of pathogenic bacteria (8-12). Periodontal disease, recognized as the sixth clinical complication of diabetes, is also more common in children with T1DM, with vascular changes in periodontal tissues resulting from microvascular damage characteristic of diabetes (13,14). With increasing glycated hemoglobin (HbA1c), the risk of gingivitis and periodontitis increases significantly, and is clinically manifested by increased plaque indices, gingival bleeding, and inflammatory changes (9).

Regular and proper oral hygiene, along with frequent preventive dental examinations, plays a key role in preventing the development of caries and periodontal disease in children with type 1 diabetes. Interestingly, studies have also shown a feedback loop: adequate oral health can contribute to better metabolic control, confirming the importance of dental care in a multidisciplinary approach to T1D treatment (8–10).

Given the growing incidence of T1DM (1,2) in the pediatric population and the association between metabolic control and oral health (8-12), it is necessary to integrate dental assessment and care into the comprehensive treatment of this disease.

The aim of this paper was to evaluate the state of oral health in children with type 1 diabetes in relation to the level of glycemic control.

METHODS

An epidemiological case-control study was conducted on 87 children with type 1 diabetes mellitus (47 boys and 40 girls), aged 10–15 years, who were treated at the Institute for Children's Diseases, Clinical Center of Montenegro. The study was conducted in accordance with the Declaration of Helsinki and adhered to the principles of good clinical practice. The research protocol was approved by the Ethics Committee of the Clinical Center of Montenegro (No. 03-5/23), and the study was conducted from June 2017 to December 2018. A qualified and calibrated dental team was responsible for the clinical measurements. Intra-examiner reliability was rated as excellent, with a kappa coefficient value of 0.94. Children were included in the study only after their parents provided signed informed consent and after they received a detailed explanation of the study's aims and procedures.

Subjects were divided into two groups according to their level of metabolic control. Data on glycosylated hemoglobin (HbA1c) values from the previous three months were taken from medical records. According to the criteria of the American Diabetes Association, good metabolic control was defined as an HbA1c value < 7.5% (58 mmol/mol), while values>7.5% were considered an indicator of poor metabolic control (15). Accordingly, two groups were formed: a group with good metabolic control

(HbA1c < 7.5%; n = 34) and a group with poor metabolic control (HbA1c >7.5%; n = 53). General exclusion criteria for all participants were the need for antibiotic prophylaxis and refusal of children to cooperate. All of the participants and their families lived in the same geographical area.
All dental examinations were performed in the Dental Clinic of the Faculty of Medicine, University of Montenegro in Podgorica, after regular endocrinological check-ups, in the presence of parents.

Dental examination
The presence of caries on permanent teeth was recorded following the DMFT index (number of decayed, extracted, and filled teeth), in accordance with the standards of the World Health Organization (WHO) (16). Caries was diagnosed by visual inspection using standard dental instruments. Lesions with cavities were registered as caries, while initial changes without cavitation were registered as healthy teeth.
Oral hygiene was assessed using the Plaque Index (PI) according to Silness and Löe (17). The assessment was performed on four surfaces of each tooth: mesiobuccal, distobuccal, mesiolingual, and distolingual.
For clinical assessment of the gingival condition, the Gingival Index (GI), proposed by Loe and Silness (17), was employed. The condition of the gingiva was assessed from the vestibular, oral, mesial, and distal surfaces of each present tooth. The total gingival index was calculated by summing up the scores from all surfaces of all teeth and then dividing the obtained sum by four. This value was then divided by the number of examined teeth. Individuals with a gingival index of 0.1–1.0 were classified as having mild gingival inflammation, while those with an index of 1.1–2.0 were classified as having moderate inflammation. If the mean value of the gingival index was 2.1–3.0, the inflammation of the gingiva was assessed as severe, that is, very pronounced.

Saliva sample collection
Children had not received antibiotic therapy for at least 15 days prior to sampling and were not undergoing dental treatment. Stimulated saliva samples were collected at least two hours after the last meal (between 10:00 and 11:00 am). Saliva secretion was stimulated by chewing a paraffin tablet, and samples were collected in graduated containers for five minutes, excluding foam bubbles. The saliva rate flow was expressed in mL/min.

Buffer capacity and saliva volume were determined using the Dentobuff Strip system (Orion Diagnostica, Espoo, Finland). Scoring, or results of saliva buffer capacity, was performed as follows:
0—blue color, the test strip immediately turned this color; it indicated very high buffer capacity (pH > 6);
1—blue color, the test strip changed color within five minutes; it indicated a high buffer capacity (pH = 6);
2—green color, it represented medium buffer capacity (pH = 4.5-5.5);
3—yellow color, represented a low buffer capacity (pH = 4, or less than 4).
The blue color of the test strip, i.e., the values 0 and 1, indicated a high buffer capacity (18).

Microbiological analysis
The presence of *Streptococcus mutans* (SM) and *Lactobacillus* (LB) was determined by the Dentocult SM Strip mutans and Dentocult LB System (Orion Diagnostica, Espoo, Finland) CRT bacterial test on saliva pre-stimulated by chewing paraffin. The number of bacteria was recorded as colony-forming units per milliliter (CFU/mL) of saliva. According to the manufacturer's scorecard, the bacterial colony count for *Streptococcus mutans* was scored as follows: Class 0: < 10,000 CFU/ml; Class 1: < 100,000 CFU/ml; Class 2: 100,000-1,000,000 CFU/ml; Class 3: > 1,000,000 CFU/ml. The *Lactobacillus* colony count was scored as follows: Class 0 (0–10^3 CFU/mL); Class 1 (10^4 CFU/mL); Class 2 (10^5 CFU/mL); Class 3 (10^6 CFU/mL). Findings indicating low risk of caries were classes 0 and 1, while findings indicating high risk of caries development were classes 2 and 3.

Questionnaire
The parental questionnaire consisted of two parts. The first part included questions about socioeconomic data (municipality, school, class, gender, date of birth, parents' education and employment, parents' marital status, number of children in the household, family income).
The second part was about children and included questions about their oral hygiene habits (tooth brushing frequency and use of fluoride toothpaste). Each question had two options related to participants' tooth-brushing habits (twice a day or more; once a day) and frequency of dental visits (every 6 months; once a year or less).
An assessment of socioeconomic status was also conducted. It was classified as low, moderate, or high,

based on household income, with nationally defined thresholds according to Eurostat (19). After the examination, each child was trained in proper tooth brushing.

Statistical analysis

Statistical data processing was performed using SPSS 19 (SPSS Inc, Chicago, Illinois, USA). Descriptive and analytical statistics methods were used to describe the results. Descriptive statistical methods used were: mean value, standard deviation, and percentages. Differences in individual parameters between the examined groups were tested using Student's t-test and Chi-square test (χ^2 test). P-values less than 0.05 were considered statistically significant.

RESULTS

This study was conducted on 87 subjects with type 1 diabetes, of whom 34 had well-controlled metabolic disease control (HbA1c < 7.5%), and 53 had poor metabolic control (HbA1c > 7.5%). The average age of children with poor metabolic control was 11.4 ± 1.53, while the average age of children with good metabolic control was 10.7 ± 1.42 (p = 0.283). In the total sample, 10.34% (n = 9) of children had all permanent teeth healthy. The percentage of children with all healthy permanent teeth in the children group with well-controlled diabetes was 6.8%, while in the group with poorly controlled diabetes, it was 4.5%. Statistical analysis did not show significant differences in the values of this index (χ^2, p > 0.05).

The results show that subjects with poor metabolic control (HbA1c > 7.5%) had significantly more extracted teeth (t-test, p < 0.05), dental plaque (t-test, p < 0.05), and gingivitis (t-test, p < 0.001) compared to subjects with good metabolic control (HbA1c < 7.5%) (Table 1).

However, the average DMFT index values, the number of carious and repaired teeth, and the speed of stimulated saliva flow and buffer capacity did not show significant differences between the two groups of children with diabetes (t-test, p > 0.05) (Table 1).

The differences in comparisons in stimulated salivary flow rate, salivary buffering capacity, DMFT index, DMF components, plaque index, and gingival index between groups based on metabolic control are shown in Table 1.

Analysis of the gingival condition in children with good and poor metabolic control (Table 2) showed significant differences in the distribution of gingival inflammation between the groups. In the group of children with HbA1c < 7.5%, most children had moderate inflammation (73.5%), while only 2.9% had severe inflammation. In contrast, the group of children with HbA1c > 7.5% showed a significantly higher percentage of respondents with severe gingival inflammation (28.3%).

The Chi-square test confirmed a statistically significant difference in the distribution of gingival inflammation between the tested groups (χ^2 test, p < 0.05). These results indicate a significantly more pronounced gingival inflammation in children with poor metabolic control (Table 2).

There was a significant difference in SM (χ^2 test, p < 0.05) between groups regarding caries risk. It is evident from the findings that a group of children with poor metabolic control had a predisposition to a high risk of caries (60.4%). However, there were no significant differences between the two groups for SM colonies (divided into classes) (χ^2 test, p > 0.05) (Table 3).

There was a significant difference in LB (χ^2 test, p < 0.05) between groups regarding caries risk. It is evident from the findings that a group of children with poor metabolic control had a predisposition to a high risk of caries (64.2%).

Table 1. *The values of DMFT components, DMFT index, plaque index, gingival index, stimulated salivary flow rate, and buffer capacity saliva in the study groups*

Groups	HbA1c < 7.5% (N = 34)	HbA1c > 7.5% (N = 53)	
Variable	Mean ± sd	Mean ± sd	p (t-test)
D	1.41 ± 1.48	1.52 ± 1.65	0.775
M	0.11 ± 0.32	0.26 ± 0.61	0.002
F	2.26 ± 1.62	2.55 ± 1.58	0.434
DMFT	3.88 ± 1.78	4.30 ± 1.68	0.457
Plaque index (PI)	1.03 ± 0.57	1.27 ± 0.49	0.002
Gingival index (GI)	1.00 ± 0.61	1.36 ± 0.46	0.001
Salivary flow rate (ml/min)	1.00 ± 0.11	0.98 ± 0.18	0.802
Buffer capacity saliva	1.14 ± 0.74	1.32 ± 0.76	0.280

HbA1c < 7.5%—children with type 1 diabetes mellitus with good metabolic control of glycated hemoglobin
HbA1c > 7.5%—children with type 1 diabetes mellitus with poor metabolic control of glycated hemoglobin
N = sample size; SD = standard deviation; p = p level
DMFT— decayed (D), missing (M), filled (F), teeth (T)

Table 2. *Gingival index in the study groups*

Gingival index (GI)	Group			
	HbA1c < 7.5%		HbA1c > 7.5%	
	N	%	N	%
Normal gingiva	2	5.8	3	5.6
Mild inflammation	6	17.6	8	15.1
Moderate inflammation	25	73.5	27	50.9
Severe inflammation	1	2.9	15	28.3
p (Chi test)	Chi = 9.44; p = 0.024			

Table 3. *General and specific distribution of Streptococcus mutans between groups*

Parameters	Groups				p (Chi test)
	HbA1c < 7.5%		HbA1c > 7.5%		
Streptococcus mutans (SM)	N	%	N	%	
Class 0 (<10^3 CFU/mL)	3	8,8	3	5,6	Chi = 6.60 p > 0.05
Class 1 (<10^4 CFU/mL)	20	58,8	18	33,9	
Class 2 (10^4–10^5 CFU/mL)	10	29,4	30	56,6	
Class 3 (>10^5 CFU/mL)	1	2,9	2	3,7	
SM values in CFU/mL saliva (Caries risk test for SM)					
Low (0 and 1)	23	67.6	21	39.6	Chi=5.43 p < 0.05
High (2 and 3)	11	32.4	32	60.4	
Total	34	100.0	53	100.0	

However, there were no significant differences between the two groups for LB colonies (divided into classes) (χ^2 test, p > 0.05) (Table 4).

Regarding tooth brushing habits and visits to the dentist, the study did not show a significant difference between the examined groups (Table 5). Most children from both groups brushed their teeth only once a day, and visited the dentist only when necessary (χ^2 test, p > 0.05). Family socioeconomic status was similar in both groups (χ^2 test, p > 0.05). The oral hygiene habits and socioeconomic status of the studied groups are shown in Table 5.

Table 4. *General and specific distribution of Lactobacillus between groups*

Parameters	Groups				p (Chi test)
	HbA1c < 7.5%		HbA1c > 7.5%		
Lactobacillus (LB)	N	%	N	%	
Class 0 (0–10^3 CFU/mL)	5	14.7	3	5.6	Chi = 7.31 p > 0.05
Class 1 (<10^4 CFU/mL)	17	50.0	16	30.2	
Class 2 (10^5 CFU/mL)	11	32.4	32	60.4	
Class 3 (10^6 CFU/mL)	1	2.9	2	3.8	
LB values in CFU/mL saliva (Caries risk test for LB)					
Low (0 and 1)	22	64.7	19	35.8	Chi = 5.81 p < 0.05
High (2 and 3)	12	35.3	34	64.2	
Total	34	100.0	53	100.0	

Table 5. *Oral hygiene habits, dental visits, and socioeconomic status of the examined patients*

Parameters	HbA1c < 7.5%	HbA1c > 7.5%	p (Chi test)
Daily brushing			
1 daily	22	33	n.s.
≥ 2 daily	12	20	n.s.
Using fluoridated toothpaste	34	53	
Dental visits			
Once every 6 months	10	13	n.s.
≥ once a year	24	40	n.s.
Socioeconomic status			
Low	7	11	n.s.
Medium/high	27	42	n.s.

DISCUSSION

This study evaluated the oral health status of schoolchildren with type 1 diabetes mellitus in Montenegro in relation to metabolic control. To our knowledge, this is the first study of its kind conducted in Montenegro.

The results of this study show that children with good metabolic control had lower DMFT values compared with children with poor glycemic control; however, this difference was not statistically significant.

The DMFT components were similar in both groups. Specifically, filled teeth predominated in both groups, followed by untreated caries, with extractions representing the smallest proportion. No statistically significant differences were observed when analyzing the caries and filling components separately. However, significantly more extracted teeth were recorded in children with uncontrolled diabetes compared to children with good metabolic control. Previous studies conducted in Montenegro, involving both healthy children and those with diabetes, reported similar dental caries prevalence in diabetic children (10,12).

Diabetes mellitus can increase susceptibility to dental caries. Numerous studies have investigated the influence of metabolic control on dental caries (20-27). Hyperglycemia is associated with reduced salivary secretion and elevated glucose concentrations in saliva and gingival crevicular fluid. Elevated HbA1c levels and periods of hyperglycemia may increase caries risk in individuals with poorly controlled diabetes mellitus (28-31). Additionally, these individuals are more susceptible to infections such as dental abscesses and tooth loss, often resulting from progressive caries (5,32-34). Some studies, however, have found no correlation between caries and HbA1c levels (35,36). It is important to emphasize that caries risk is influenced not only by metabolic control but also by factors such as fluoride exposure, oral hygiene practices, diet, salivary flow, overall health, and socioeconomic status. Habits and behaviors of patients and their parents are also of great importance. Poor glycemic control may reflect a negligent attitude toward overall health, which can also manifest as inadequate oral hygiene and care (23). In our study, children with poor metabolic control had significantly more missing teeth, likely resulting from progressive, untreated caries. This finding suggests a late diagnosis and possible inadequate or delayed dental interventions, highlighting the need for more frequent preventive examinations and timely treatment in this vulnerable population.

The results also showed that patients with poor metabolic control had significantly higher Plaque Index (PI) and Gingival Index (GI) values compared to those with good metabolic control. Similarly, children with poor metabolic control exhibited significantly more pronounced gingival inflammation. A key etiological factor contributing to increased plaque accumulation on tooth surfaces is inadequate or ineffective oral hygiene. Additionally, children in this age group often lack the habit of maintaining regular and proper daily oral care. Most studies conducted in children with diabetes have reported high plaque and gingival index values (9,10,12,37,38), which aligns with our findings.

Diabetes increases the risk of both gingivitis and periodontitis. Poor glycemic control is frequently associated with a higher incidence of gingivitis, as shown in our previous research. (9,37,38,39,40). Specifically, elevated glucose levels in the gingival crevicular fluid and blood of poorly controlled diabetic patients can alter the microbial environment, leading to qualitative changes in the bacterial composition that contribute to periodontal disease (41–43). Disturbed glucose metabolism in diabetics is directly correlated with the degree of gingival inflammation (44). Increased gingival bleeding associated with hyperglycemia may be explained by immunological alterations and reduced immune response. However, some authors have not observed a clear association between gingival inflammation and levels of metabolic control in diabetic patients (29,30).

Patients who have well-controlled diabetes and a high level of oral hygiene, who follow the usual periodontal maintenance procedures as well as a very strict schedule of control examinations at the dentist, have the same risk for periodontal alteration as non-diabetic people (45).

In our study, the average stimulated saliva flow was similar in both groups. Although children with poor metabolic control showed a lower salivary buffer capacity, the difference was not statistically significant. Similar findings have been reported in other studies (8,9).

Streptococcus mutans (SM) is the primary microorganism responsible for dental caries in humans. In our study, salivary levels of SM and *Lactobacillus* (LB) were significantly higher in children with elevated HbA1c values. Similar findings have been reported in previous studies (9, 20, 29, 46). Elevated glucose concentrations in saliva alter the biofilm structure, facilitating faster colonization of SM and LB in individuals with poor metabolic control. Combined with reduced saliva flow, lower buffering capacity, and inadequate oral hygiene, this can increase the risk of caries in children with poorly controlled diabetes (8,20,29,46–48), consistent with our results. However, some authors do not observe this association, noting that caries is a multiphasic, multicausal infectious disease largely influenced by nutrition (8, 22, 36, 39, 47, 49).

Proper oral hygiene habits, such as regular tooth brushing and routine dental checkups, are key components in preventing oral diseases, including dental caries and gingivitis. However, the results of this study indicate a

worrying pattern: most children with type 1 diabetes brush their teeth only once a day, and visits to the dentist are infrequent, once a year or less.

These results are consistent with findings from other studies reporting inadequate oral hygiene routine among children with type 1 diabetes (9, 12, 50, 30). In some exceptions, some studies have shown better oral hygiene, with a higher percentage of children with T1DM brushing their teeth two to three times daily (51, 52), suggesting that several factors—such as education, socioeconomic status, parental support, and the health care system—may influence the formation of healthy habits.

Furthermore, the frequency of dental examinations in our study was also low, which has been previously confirmed by numerous studies (9, 12, 24). Such a pattern of behavior can be partly explained by the fact that attention in children with diabetes is primarily focused on controlling the underlying disease, while oral health is often put on the back burner. On the other hand, there are also works that show a more positive trend, stating that children with diabetes regularly visit the dentist (53, 54), which may be the result of a better integrated healthcare approach in certain environments.

These contradictory data in the literature indicate the need for standardized education and the inclusion of dental prevention as an integral part of care for children with diabetes (55). By introducing systematic dental examinations and targeted preventive programs within diabetes clinics, both the oral and general health conditions of these patients could be improved.

Although this study has some limitations, including a relatively small sample size, particularly few children with well-controlled diabetes, its findings are valuable. For the first time, they provide insight into the oral health of children with type 1 diabetes mellitus in relation to metabolic control in Montenegro, highlighting the importance of additional parameters, such as saliva analysis, in oral risk assessment. Furthermore, our results open the way for future large-scale research to develop targeted preventive programs and integrate dental care into the routine care of children with chronic diseases.

The findings of this study show that children with poor metabolic control of type 1 diabetes show a tendency towards reduced salivary flow, lower buffering capacity, increased plaque accumulation, more pronounced gingivitis, and a higher risk of caries. These changes in the oral cavity are associated with an increase in the number of pathogenic bacteria, which further impairs oral health.

The high average DMFT index in both groups indicates a lack of effective preventive measures and an inadequate curative dental policy in Montenegro.

These findings suggest the need to develop and implement an organized preventive plan, including education for children with diabetes and their parents, as well as the introduction of individualized prophylactic measures in routine dental practice.

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Author's contribution

Conceptualization and investigation: M.DJ. and A.DJ.; Writing – original draft, review, & editing: M.DJ. and A.DJ. Both authors have read and approved the published version of the manuscript.

Statement of Ethics

The study protocol was reviewed and approved by the Ethics Committee of the Clinical Center of Montenegro (No. 03-5/23). Written informed consent was obtained from the parents of all patients for the publication of this study.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availability

Not applicable.

Statement of Generative AI Use

No generative AI was used.

Conflicts of interest

The authors declare no conflict of interest.

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AFMN BIOMEDICINE

CASE REPORT

18F-FLUORODEOXYGLUCOSE POSITRON EMISSION TOMOGRAPHY WITH COMPUTED TOMOGRAPHY IN THE DIAGNOSIS OF LOEFFLER'S ENDOCARDITIS: A CASE REPORT

Milica Kotur[1] **Nikola Pantić**[1] **Lenka Grujičić**[1] **Isidora Grozdić Milojević**[1,2] **Dragana Šobić Šaranović**[1,2] **Vera Artiko**[1,2] **Strahinja Odalović**[1,2]

[1]Center for Nuclear Medicine with PET, University Clinical Center of Serbia, Belgrade, Serbia [2]University of Belgrade Faculty of Medicine, Belgrade, Serbia

Loeffler's endocarditis (LE) is a rare form of inflammatory cardiomyopathy. The condition arises from diffuse eosinophilic infiltration of the endomyocardium, followed by eosinophil degranulation and progressive tissue fibrosis. The aim of this case report is to present a rare form of restrictive cardiomyopathy, LE, in a young female patient, and to highlight the importance of fluorodeoxyglucose positron emission tomography combined with computed tomography (FDG PET/CT) as a valuable diagnostic tool for accurate diagnosis, assessment of cardiac involvement, and determination of disease extent. We present the case of a female patient who underwent FDG PET/CT imaging due to suspected LE, following the detection of eosinophilia and a series of prior diagnostic procedures. FDG PET/CT contributed to the timely diagnosis and precise localization of inflammatory lesions in the cardiac walls. Based on the presented case, we conclude that FDG PET/CT imaging can serve as a helpful tool in the diagnosis and evaluation of LE due to its unique capability to visualize metabolic activity in tissues, a feature often beyond the reach of conventional diagnostic methods. This modality enables accurate identification of inflammatory lesions in the endocardium and myocardium, which are characteristic of LE.

Keywords: hypereosinophilic syndrome, eosinophilic infiltration, Loeffler's endocarditis, FDG PET/CT

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Correspondence to:
Milica Kotur
University Clinical Center of Serbia, Belgrade, Serbia
Center for Nuclear Medicine with PET
Pasterova 2, Belgrade, Serbia
E-mail: koturmilica99@gmail.com

INTRODUCTION

Loeffler's endocarditis (LE) is a rare form of inflammatory cardiomyopathy, first described by Swiss physician Wilhelm Loeffler in 1936 (1, 2). The condition arises from diffuse eosinophilic infiltration of the endomyocardium, followed by eosinophil degranulation and progressive tissue fibrosis. This pathological process leads to impaired diastolic function and classifies LE as a subtype of restrictive cardiomyopathy (2). LE is estimated to occur in approximately 60% of patients with hypereosinophilic syndrome (HES), a disorder characterized by persistent overproduction of eosinophils (3, 4). In the differential diagnosis of eosinophilia-associated cardiac involvement, other potential etiologies should be considered, including eosinophilic granulomatosis with polyangiitis (formerly Churg–Strauss syndrome), early-stage giant cell myocarditis, drug-induced hypersensitivity reactions, and parasitic infections (5).

In addition to peripheral blood analysis, where hypereosinophilia represents a primary diagnostic criterion, other key diagnostic modalities include echocardiography, chest computed tomography (CT), cardiac magnetic resonance imaging (CMRI), bone marrow biopsy, and endomyocardial biopsy (6). Fluorodeoxyglucose positron emission tomography combined with computed tomography (FDG PET/CT) enables accurate detection of inflamed myocardial tissue during the earliest stages of LE, thereby facilitating timely therapeutic intervention and potentially preventing irreversible cardiac damage (7). This modality is particularly useful when CMRI is contraindicated, when standard diagnostic methods yield inconclusive results, or when evaluating the extent of systemic involvement in HES. However, it should be noted that while FDG PET/CT can reliably identify inflamed areas, it lacks the ability to differentiate the specific cellular components of the infiltrate (8).

The aim of this case report is to present a rare form of restrictive cardiomyopathy, LE, in a young female patient, and to highlight the importance of FDG PET/CT as a valuable diagnostic tool for accurate diagnosis, assessment of cardiac involvement, and determination of disease extent.

CASE REPORT

A 21-year-old female patient was referred to the Center for Nuclear Medicine with PET at the University Clinical Center of Serbia for FDG PET/CT imaging, with suspected LE. Anamnestic data revealed that approximately nine months prior, she experienced an intermittent urticarial skin rash, treated with antihistamines and corticosteroids. Laboratory tests demonstrated marked eosinophilia (7.96 × 10^9/L, 62%), elevated C-reactive protein (CRP) levels (90.9 mg/L), and significantly increased N-terminal pro B-type natriuretic peptide (NT-proBNP) concentrations, ranging from 2,911 pg/mL to 11,333 pg/mL over five days. Flow cytometry immunophenotyping revealed an increased eosinophil count (52% of total leukocytes) alongside a decreased absolute CD4+ T lymphocyte count (250 cells/μL), without evidence of atypical T lymphocytes. Histopathological analysis of bone marrow biopsy suggested hypereosinophilia of reactive or neoplastic origin. Chest CT imaging showed no pathological abnormalities.

Two weeks prior to FDG PET/CT imaging, the patient was evaluated by a cardiologist due to severe chest pain, dyspnea, and elevated body temperature. Echocardiography revealed thickening of the left ventricular wall with heterogeneous echogenicity and minimal pericardial effusion. CMRI, performed ten days before FDG PET/CT, demonstrated reduced left ventricular systolic function (ejection fraction 49%) and an intracavitary thickened endocardial layer exhibiting edema and granulation along all walls except the septum, with thickness ranging from 14 to 21 mm. The presence of mural thrombosis and a thrombus at the apex of the left ventricle, along with the involvement of the posterior mitral leaflet and both papillary muscles by thrombotic changes, further indicated cardiac complications. The extent of myocardial fibrosis was estimated at approximately 21%. These findings were consistent with the diagnosis of LE.

FDG PET/CT imaging was performed using the Discovery PET/CT Elite scanner (GE Healthcare). Prior to the procedure, the patient provided informed consent for the study. The patient fasted for six hours before receiving an intravenous injection of FDG at a dose of 195 MBq. Scanning commenced 82 minutes post-injection. CT images were acquired from the skull vertex to the mid-thigh, followed by PET data acquisition over the same region. All PET/CT images were reconstructed and analyzed using Volume Viewer software on the AW 4.5 Workstation (GE Healthcare). FDG uptake was quantitatively evaluated by calculating the maximum standardized uptake value (SUVmax) of the radiotracer.

FDG PET/CT imaging revealed cardiomegaly. The left

ventricular walls, including the interventricular septum, showed diffusely increased FDG uptake (SUVmax 15.5) (Figures 1 and 2), with focal wall thickening observed in the upper lateral segment corresponding to the papillary muscle region (Figure 3). Mild to moderate FDG uptake was also noted in the walls of the left atrium and right ventricle (SUVmax 9 in the right ventricle; SUVmax 7 in the left atrium) (Figure 4). No structural abnormalities were detected on the low-dose CT component of the PET/CT scan; thus, interpretation was based on subjective visual assessment and SUVmax values. Based on the observed pattern of FDG distribution and uptake in the cardiac walls, wall thickness was estimated to reach up to 20 mm in the left ventricle and up to 7 mm in the left atrium.

The conclusion of the FDG PET/CT scan is that the diffuse and intense radiotracer uptake in the walls of the left ventricle, and, to a lesser extent, in the right ventricle and left atrium, primarily reflects inflammatory changes associated with the underlying disease.

DISCUSSION

This study presents a rare case of HES-associated LE in a 21-year-old female patient. The patient had previously undergone CMRI, which revealed significant findings consistent with LE, corroborating the results of the FDG PET/CT examinations.

HES is a rare hematological disorder, and LE accounts for approximately 50% of all HES cases (4, 9). The presented case is noteworthy for several reasons. LE often occurs in younger populations, as demonstrated here, where it can cause permanent myocardial damage and increase morbidity and mortality. Furthermore, this case underscores the importance of utilizing both invasive and non-invasive diagnostic methods in the diagnosis of LE and assessment of disease extent.

Langwieser et al. (10) reported a case of a 73-year-old female patient with LE who underwent PET/MRI imaging following the detection of eosinophilia in the blood. Similar to our case, the patient presented with dyspnea, and laboratory tests revealed elevated cardiac enzyme levels three weeks prior to the PET/MRI examination. The MRI component of the hybrid PET/MRI system demonstrated late gadolinium enhancement (LGE) lesions in the endocardium of the apical regions of both left and right ventricles, along with masses, likely thrombi, also located in the apical regions of both ventricles, which did not exhibit LGE. Concurrently, the FDG PET/CT examination showed significant FDG uptake in the LGE areas and apical masses, indicating the presence of active inflammatory tissue.

This case parallels ours, in which PET and MRI scans, although performed as separate modalities rather than a hybrid system, revealed characteristic findings that, in conjunction with clinical presentation, laboratory data, and other information, supported the diagnosis of LE.

Khalid et al. (11) reported a case of an 83-year-old female patient with asthma treated with corticosteroids who presented with worsening dyspnea. Laboratory findings demonstrated leukocytosis, eosinophilia, and elevated levels of troponin T, NT-proBNP, and CRP, findings that correlate with those in our study. Transthoracic echocardiography revealed an ejection fraction (EF) of 35%. CMRI identified LGE in the epicardium of the left ventricle. The FDG PET/CT scan, however, did not reveal any hypermetabolic lesions consistent with LE. Despite this, the diagnosis of LE was subsequently confirmed, and high-dose steroid therapy was initiated, resulting in significant clinical improvement.

Compared to our case, there is a similarity in the presenting symptoms and laboratory findings. However, the patient described by Khalid et al. exhibited a significantly reduced EF, which may be attributed to her advanced age, making it difficult to determine if her EF was preserved prior to the onset of HES. Furthermore, the FDG PET/CT results differ from ours, as hypermetabolic lesions indicative of LE were detected in our patient but not in theirs.

Chen P. et al. (12) reported a case of a 34-year-old patient who underwent FDG PET/CT imaging due to suspected LE. The patient initially presented with high fever and severe cough, and medical history revealed bronchial asthma diagnosed more than ten years earlier. Laboratory findings demonstrated marked peripheral eosinophilia, along with elevated troponin T, NT-proBNP, and C-reactive protein (CRP) levels. Echocardiography showed thickening of the mid to apical segments of the left ventricular wall, with the most pronounced changes in the apex, raising suspicion of eosinophilic endocarditis. FDG PET/CT imaging revealed diffusely increased FDG uptake in the left ventricular wall, further supporting the diagnosis of LE.

Unlike the patient in our case, this patient predominantly exhibited respiratory symptoms. Nonetheless, similar to our case, the integration of clinical presentation, laboratory results, echocardiography, and FDG PET/CT findings was essential in confirming the diagnosis of LE.

Chen et al. (12) also reported a case of a 46-year-old patient with liver cirrhosis who presented with abdominal

pain. The medical history revealed a previous episode of malaria. Laboratory tests demonstrated elevated NT-proBNP levels, eosinophilia, and increased total IgE, IgG, and IgG4 antibody concentrations. Transthoracic echocardiography showed dilated cardiac chambers, reduced right ventricular systolic function, and the presence of mural thrombi. Further serological analyses revealed elevated PR3-Ig (cANCA) antibody levels, suggestive of ANCA-associated vasculitis. CMRI demonstrated subendocardial late gadolinium enhancement (LGE) in both ventricles, consistent with findings characteristic of LE. Subsequently, FDG PET/CT identified multiple hypermetabolic lymph nodes in various anatomical regions, along with an enlarged cardiac silhouette. As a final diagnostic procedure, a biopsy of the right axillary lymph node was performed, with histopathology confirming lymphoid hyperplasia with abundant plasma cells and eosinophilic infiltration.

This case was considerably more complex than ours, primarily due to the presence of multiple comorbidities. In both cases, CMRI and echocardiography revealed pathological cardiac changes indicative of LE. However, unlike our patient, FDG PET/CT did not show significant cardiac abnormalities, serving mainly to detect hypermetabolic lymphadenopathy. Nonetheless, when combined with other diagnostic modalities, FDG PET/CT played a crucial role in establishing the diagnosis of ANCA-associated vasculitis and LE.

Based on this and other reported cases used for comparison, it is evident that FDG PET/CT holds significant diagnostic value in the evaluation of LE. However, it is important to emphasize that this imaging modality cannot differentiate whether myocardial infiltration is mediated by eosinophils or other inflammatory cells, which limits the sensitivity and specificity of FDG PET/CT in this context.

Based on the presented case, we conclude that FDG PET/CT imaging can serve as a helpful tool in the diagnosis and evaluation of LE, due to its unique capability to visualize metabolic activity in tissues, a feature often beyond the reach of conventional diagnostic methods. This modality enables accurate identification of inflammatory lesions in the endocardium and myocardium, which are characteristic of LE. When integrated with clinical presentation, laboratory findings, and other imaging techniques, FDG PET/CT substantially contributes to establishing a precise and timely diagnosis of LE.

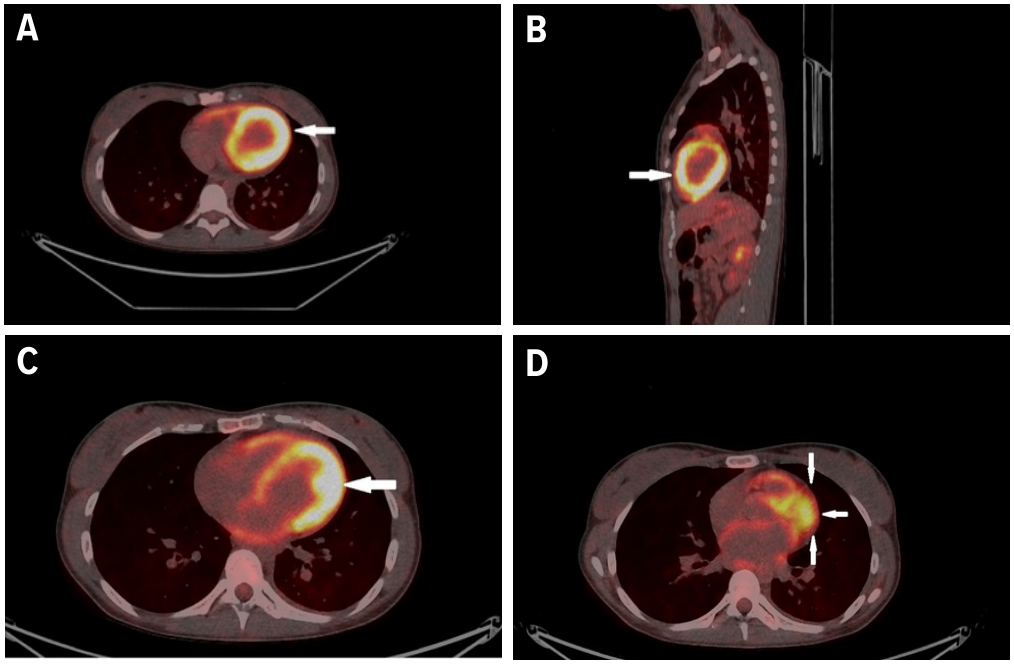

Figure 1. *(A) The PET/CT image of the transversal section shows intense accumulation of FDG in the walls of the left ventricle and the septum (white arrow). (B) The PET/CT image of the sagittal section shows diffuse intense accumulation of FDG in the walls of the left ventricle (white arrow). (C) The PET/CT image of the transversal section shows focal left ventricular thickening, most likely corresponding to papillary muscle and less likely mural thrombus seen on CMRI septum (white arrow). (D) PET/CT image of the transversal section shows intense accumulation of FDG in the walls of the left atrium (white arrows).*

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Authors' Contribution

Conceptualization & investigation: M.K., N.P., L.G., I.G.M., D.Š.Š., V.A., and S.O.; Writing – original draft, review, & editing: M.K., N.P., L.G., I.G.M., D.Š.Š., V.A., and S.O. All authors have read and approved the published version of the manuscript.

Statement of Ethics

Written informed consent was obtained from the patient for publication of this case report and any accompanying images. All procedures performed were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availability

All relevant data supporting the findings of this case report are included within the article. Additional information is available from the author upon reasonable request, in accordance with patient confidentiality requirements.

Statement of Generative AI Use

No generative AI was used.

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AFMN BIOMEDICINE

CASE REPORT

NIZON-ISIDOR SYNDROME, A RARE GENETIC DISEASE WITH LATE DIAGNOSIS PRESENTING WITH AUTISM SPECTRUM DISORDER : A CASE REPORT

Mehmet Semih Demirtaş[1] **Yakup Köylü**[2]

[1]Department of Pediatrics, Division of Social Pediatrics, Aksaray University Faculty of Medicine, Aksaray, Turkey [2]Department of Pediatrics, Aksaray Training and Research Hospital, Aksaray, Turkey

Nizon-Isidor syndrome (NIZIDS) is a rare genetic disorder associated with mutations in the MED12L gene and is usually characterized by a distinctive facial appearance, neurodevelopmental delay, autism spectrum disorders (ASD), and chronic gastrointestinal symptoms (GIS). In this case report, we emphasize the importance of early evaluation of genetic tests and diseases with Nizon-Isidor syndrome in our patient, who had characteristic facial findings, ongoing GI symptoms since birth, diagnosed with ASD at the age of 2, and diagnosed with Nizon-Isidor at a late stage (age 4) in an 8-year-old girl. In particular, it has been demonstrated once again that early genetic testing in patients with similar clinical features plays a critical role in reaching the correct diagnosis and treatment planning. These findings indicate the importance of a multidisciplinary approach in the recognition of rare syndroms of genetic origin.

Keywords: Nizon-Isidir syndrome, autism spectrum disorder, neurodevelopmental delay, genetic diseases

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Correspondence to:
Mehmet Semih Demirtaş
Department of Pediatrics
Aksaray University Faculty of Medicine
Aksaray, Turkey
E-mail: md.semihdemirtas@gmail.com

INTRODUCTION

Nizon-Isidor syndrome (NIZIDS), associated with mutations in the MED12L gene, is a rare genetic disease first reported by Nizon Isidor et al. (1) in 2019. This syndrome usually presents with prominent facial dysmorphia, neurodevelopmental delay, and gastrointestinal symptoms (GIS) (1). Genetic diseases are the etiological factor in a significant portion of autism spectrum disorder (ASD). ASD, which is a polygenic disorder, is also associated with monogenic syndromes and chromosomal anomalies. In particular, clues such as neurodevelopmental delay, characteristic facial findings, and chronic GIS symptoms that persist from birth reveal the importance of early referral to genetic testing in these children (2, 3). In this case report, the importance of early genetic diagnosis is discussed through the case of a pediatric patient diagnosed with NIZIDS.

CASE REPORT

An 8-year-old girl was admitted to our clinic with a diagnosis of ASD and characteristic facial findings for cognitive training. Physical examination revealed a height of 130 cm (50-75 percentile) and a weight of 30 kg (75-90 percentile). Hypertelorism, a bulging nasal tip, a flat nasal bridge, an everted lower lip, an enlarged earlobe, an inward squint in the right eye, and fullness in the upper eyelids were found (Figure 1). Her medical history included GIS such as chronic vomiting and diarrhea that had persisted since birth. Neurodevelopmental delay was noted at the age of 1.5 years, and she was diagnosed with ASD at the age of 2 years. Her family history was not significant in terms of genetic diseases. As a result of genetic tests performed at the age of 4 years, the diagnosis of Nizon-Isidor syndrome with a mutation in the MED12L (NM_053002.5:c.4898A>T, p.(Asp1633Val), Chr3:g. 151,385,106>T) gene was confirmed.

DISCUSSION

There is very limited information about NIZIDS syndrome, which was first reported in the literature in 2019. It is possible to discuss three main points from this case. First, it is necessary to draw attention to the relationship between ASD and genetic diseases. Genetic factors are essential for understanding the clinical presentation of ASD. Genetic disorders such as fragile X syndrome, Rett syndrome, and tuberous sclerosis are the main diseases associated with ASD (2) (Table 1). Considering that rare genetic disorders, such as Nizon-Isidor syndrome, are also involved in the etiology of ASD, early genetic testing in children with ASD may positively affect the diagnosis and treatment processes.

Secondly, characteristic facial findings provide a critical guide in the differential diagnosis of genetic diseases (4). The distinctive dysmorphic features present in NIZIDS, such as hypertelorism, a flat nasal bridge, and a thin upper lip, provide important clues for guiding the genetic testing. Clinical observations suggest that such dysmorphic findings should be evaluated together with neurodevelopmental and other systemic symptoms (4).

Finally, the role of the genetic diagnostic process and MED12L gene mutations in Nizon-Isidor syndrome should be emphasized. Genetic confirmation not only provides a definitive diagnosis but also guides patient management in the long term (3, 5). This case demonstrates once again that early application of genetic testing in children with ASD and GIS symptoms is critical to reaching a diagnosis.

In conclusion, rare genetic diseases such as NIZIDS may be overlooked due to clinical variability. However, as seen in this case, genetic testing in patients with ASD, neurodevelopmental delay, and characteristic facial findings may guide clinicians in the diagnostic process. This approach will allow for the creation of an appropriate treatment and counselling plan for both the patient and the family at an early stage.

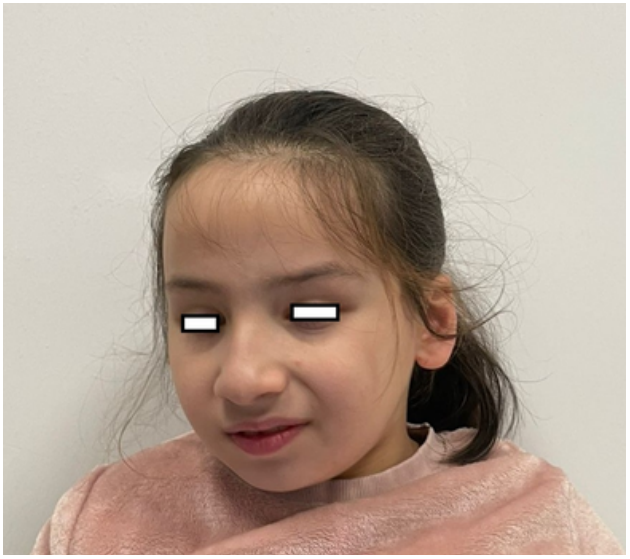

Figure 1. *Characteristic facial and head findings of the patient** *Hypertelorism, a protruding nasal tip, a flat nasal root, an everted lower lip, enlarged earlobes, right-sided esotropia, and fullness of the upper eyelids are observed.

Table 1. *Important known genetic diseases that progress to autism spectrum disorder*

Disease	Affected gene	Location description
Fragile X syndrome	FMR1	Associated with mental retardation and ASD-like behaviors.
Rett syndrome	MECP2	Usually seen in girls; neurodevelopmental delay and ASD features.
Tuberous sclerosis complex	TSC1, TSC2	Tumor formation in the brain, skin and other organs; ASD prevalence is high.
Angelman syndrome	UBE3A	Associated with mental retardation, speech difficulties, and ASD-like features.
15q11-13 duplication syndrome	15q11-13	Associated with ASD, epilepsy, and developmental delays.
Phelan-McDermid syndrome	SHANK3	Difficulties with social interaction and symptoms of ASD are common.
Smith-Magenis syndrome	17p11.2	Associated with sleep disorders, learning disabilities, and ASD features
CHARGE syndrome	CHD7	Sensory-motor deficits and ASD-like behaviors may be seen
Neurofibromatosis type 1	NF1	Neurofibromas are associated with learning disabilities and a predisposition to ASD.
PTEN hamartoma tumor syndrome	PTEN	Macrocephaly, tumor risks, and ASD symptoms are common.
Nizon-Isidor syndrome	MED12L	Prominent facial dysmorphia, neurodevelopmental delay, and gastrointestinal symptoms (GIS).
Williams syndrome	7q11.23	Associated with social extremism and some behaviors that may be on the ASD spectrum.
Prader-Willi syndrome	15q11-q13	Eating disorders, developmental delays, and ASD features may be seen.
Down syndrome	Trisomy 21	Mental retardation may be associated with an increased prevalence of ASD.
Dup15q syndrome	15q11.2-q13	Duplication associated with epilepsy and distinct ASD features.
Klinefelter syndrome	XXY	Chromosome social interaction difficulties and ASD-like features may be seen.
DiGeorge syndrome	22q11.2	Deletion is associated with mental retardation, psychiatric disorders, and ASD symptoms
Sotos syndrome	NSD1	Developmental delays, macrocephaly, and increased prevalence of ASD may be seen.
SYNGAP1-associated encephalopathy patients	SYNGAP1	Seizures, mental retardation, and ASD symptoms are common
Joubert syndrome	AHI1, PHP1, TMEM67	Associated with brain developmental disorders and ASD-like behaviors.
ADNP syndrome	ADNP	Associated with developmental delay, language delay, and autistic features.

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Author's contribution

Conceptualization, M.S.D.; Investigation, M.S.D.; Resources, Y.K.; Writing - original draft preparation: Y.K. and M.S.D.; Writing - review & editing, M.S.D.; Visualization: M.S.D. and Y.K.; Supervision: M.S.D. Both authors have read and approved the published version of the manuscript.

Statement of Ethics

The study was conducted in accordance with the Declaration of Helsinki. Complete written informed consent was obtained from the patient's parents for the publication of this study and accompanying images.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availability

Not applicable.

Statement of Generative AI Use

No generative AI was used.

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