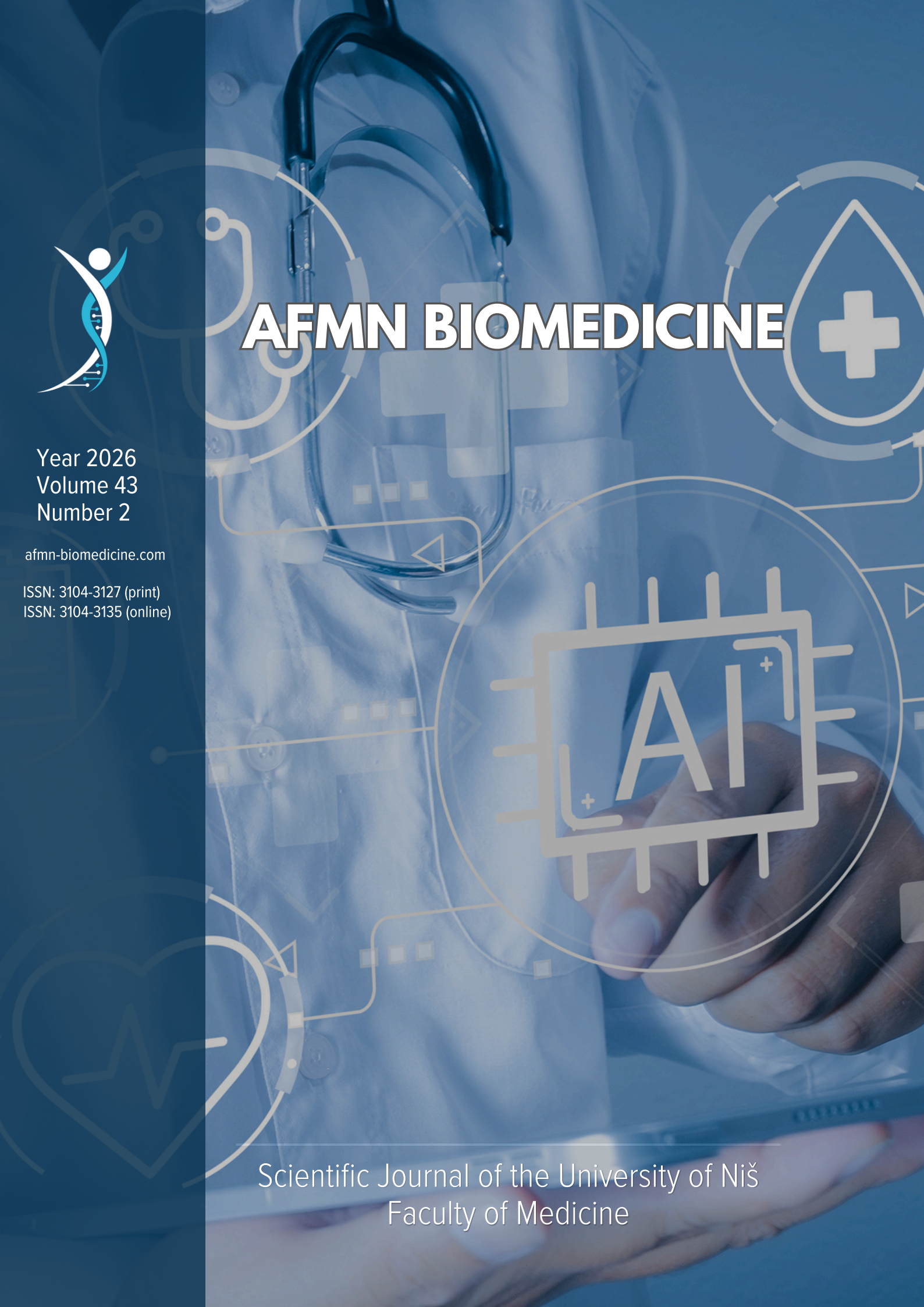

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REVIEW ARTICLE

REGULATION OF APPLIED ARTIFICIAL INTELLIGENCE IN BIOMEDICAL ENGINEERING AS A HIGH-RISK ARTIFICIAL INTELLIGENCE SYSTEM IN THE EU AI ACT

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Artificial intelligence (AI) represents a global phenomenon changing all spheres of human life. Biomedical engineering is no exception, as many AI systems are applied to biomedical engineering inventions. The European Union has enacted the new EU AI Act, one of the world's first laws on AI. The main topic of this research is to examine what changes this new regulation brings to AI development in the field of biomedical engineering. An AI system applied in biomedical engineering is often considered a high-risk AI system, which means that AI developers are bound by a set of requirements and obligations to achieve a trustworthy, human-centric AI system. The authors analyze the impact and appropriateness of these requirements for developing AI systems in biomedical engineering using the legal dogmatic method, as well as by analyzing the secondary sources in the literature. The authors aim to present the current situation in AI regulation and make suggestions for further development.

Keywords: artificial intelligence, AI law, AI in biomedical engineering, EU AI Act, AI development

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INTRODUCTION

In mid-2024, the European Union introduced a new Regulation laying down harmonized rules on artificial intelligence and amending Regulations (EC) No 300/2008, (EU) No 167/2013, (EU) No 168/2013, (EU) 2018/858, (EU) 2018/1139 and (EU) 2019/2144 and Directives 2014/90/EU, (EU) 2016/797 and (EU) 2020/1828 (hereinafter EU AI Act), representing one of the first comprehensive regulatory frameworks for AI globally. The primary objective of the EU AI Act is to protect citizens by ensuring that only human-centric and trustworthy AI is deployed within the EU (1,2). The Act primarily addresses high-risk AI systems, dedicating most of its provisions to their regulation. As we will see, AI systems incorporated into medical devices are classified as high-risk, imposing substantial obligations on developers and specific requirements for medical devices. It is therefore essential to examine these requirements and the associated burdens on EU-based AI developers, particularly given the competitive landscape with Chinese and American counterparts (3). Key questions include how these obligations affect the development of biomedical engineering and whether alternative regulatory approaches could better support AI innovation in this field.

To address these questions, this study examines the EU AI Act, related documents, and relevant literature. The analysis is structured as follows: first, potential applications of AI in biomedical engineering are surveyed to examine whether they would qualify as high-risk AI. Next, the principal requirements for high-risk AI under the EU AI Act are analyzed through the lens of ethical AI principles. Finally, the study provides an assessment and critique of these requirements, with particular attention to the necessity and significance of developing AI applications in biomedical engineering.

AI APPLIED IN BIOMEDICAL ENGINEERING AS A HIGH-RISK AI SYSTEM

AI in biomedical engineering

Biomedical engineering encompasses various subfields that advance healthcare and improve human well-being (4). To determine whether AI systems used in biomedical engineering qualify as high-risk, we first need to analyze the types of AI used. There are three main areas in which AI is applied.

The first area is experimentation and drug development. Experimentation, although based on extensive knowledge, still relies on a trial-and-error method. Consequently, many materials are wasted in the search for the right substance. AI systems enable predictive modeling for drug testing, and recurrent neural networks are already used to predict chemical reactions in order to develop the desired drug or substance (5). For example, INS018_055, the first anti-fibrotic small-molecule inhibitor with promising anti-tumor relevance, was developed using the AlphaFold AI program from DeepMind, the drug discovery platform *Pharma.AI*, and the generative chemistry platform *Chemistry 42* (6). In this way, AI systems drastically reduce the cost and time of research and development. This means that the production price of the medications might be lower (7). Also, in the field of DNA research, predictions are useful for planning nucleic acid sequence assembly, cell culture, protein structure prediction, and related tasks (8).

The second area of application is diagnostics. Convolutional neural networks (7) are already applied in CT and MRI scanning, electroencephalography (EEG), and other fields of imaging diagnostics used for cancer, cardiovascular disorders, and neurological conditions (4). Great accomplishments have been achieved in the diagnosis of hepatocellular carcinoma, myelofibrosis, prostate cancer, and hypersensitivity pneumonitis (5). In many cases, the success rate of AI systems exceeded that of humans. This means these systems will be of great assistance once they are integrated into physicians' everyday work. The use of AI systems in diagnostics, alongside human control, will most likely minimize the risk of error. This is because machines have strong analytical capabilities that can identify key indicators, while human controllers have real-world experience that can prevent potential false diagnoses by the machine. It is fair to say that some of these systems are already in use, although not declared as AI (9). Another application is precision medicine. With AI-powered devices, parameters such as heart rate, blood pressure, and pulse can be continuously monitored. This has immense potential for early diagnostics and appropriate therapy initiation (4). Programs such as *xRapid-lab, xRapid-Malaria, PCR.AI*, and *PIVOT* enable faster, more accurate detection of diseases under a microscope (7).

Finally, the third area of AI application in biomedical engineering is prosthetics and healing. AI systems are integrated into implants and exoskeletons, which could drastically improve users' quality of life. The fact that AI has “learning” capabilities could prove useful, as it can learn user patterns to help guide movement (7).

Biomedical AI as a high-risk AI system

The EU AI Act (10) adopts a so-called risk-based approach, meaning that all AI systems are classified into four groups

(or, by some accounts, three groups) (11): forbidden AI practices, high-risk AI systems, low-risk AI systems, and/or minimum-risk AI systems. There is no official, legally binding definition of high-risk AI systems in the EU AI Act. However, recital 7 provides that, in order to ensure a consistent and high level of protection of public interests in the fields of health, safety, and fundamental rights, common rules for high-risk AI systems should be established. This sentence, although not an official definition, contains important factors for classifying a system as a high-risk AI system (namely, danger to health, safety, and fundamental rights).

On the other hand, Article 6 of the EU AI Act contains the actual classification criteria for high-risk AI systems, dividing them into two groups. The first group comprises AI systems applied in specific areas of human life, which make them so risk-prone that they cannot be excluded as high-risk AI under any circumstances. AI systems are considered to fall in this group if two conditions are cumulatively fulfilled: a) the AI system is intended to be used as a safety component of a product, or the AI system is itself a product, covered by the Union harmonization legislation listed in Annex I; and b) the product whose safety component pursuant to point (a) is the AI system, or the AI system itself as a product, is required to undergo a third-party conformity assessment prior to being placed on the market or put into service pursuant to the Union harmonization legislation listed in Annex I.

Annex I provides a compilation of EU legislative acts regulating various products and services subject to special conditions for internal market placement, including toys, machinery, etc. The other group consists of AI systems that are enumerated in Annex III of the regulation. In certain cases, some of the AI systems from this group can be treated as a low-risk AI system if they do not pose a significant risk of harm to the health, safety, or fundamental rights of natural persons, including by not materially influencing the outcome of decision-making, and if conditions from Article 6, paragraphs 4-8 are met.

The list contained in Annex I of the EU AI act enlists, among others, Regulation (EU) 2017/745 of the European Parliament and of the Council of 5 April 2017 on medical devices, amending Directive 2001/83/EC, Regulation (EC) No 178/2002 and Regulation (EC) No 1223/2009 and repealing Council Directives 90/385/EEC and 93/42/EEC (hereinafter MDR) and Regulation (EU) 2017/746 of the European Parliament and of the Council of 5 April 2017 on *in vitro* diagnostic medical devices and repealing Directive 98/79/EC and Commission Decision 2010/227/EU (hereinafter IVDR). The MDR and IVDR are the regulations governing the field of medical devices. In the MDR (to which the IVDR also refers for this definition), it is explicitly defined that a "medical device means any instrument, apparatus, appliance, software, implant, reagent, material or other article intended by the manufacturer to be used, alone or in combination, for human beings for one or more of the following specific medical purposes…investigation, replacement or modification of the anatomy or of a physiological or pathological process or state…" This simplifies the classification, since it is clear that AI systems applied in biomedical devices are high-risk if they are intended to serve as a safety component of a product, or if the AI system itself is a product of biomedical engineering. Since these medical devices undergo third-party conformity assessment, AI systems used in biomedical engineering must meet both requirements to be classified as high-risk AI. Since they fall into the first group, there is no possibility for these systems to be exempted from the high-risk AI systems categorization (11–13). However, it is possible that some systems not regarded as high-risk because they do not fall within the definition of a medical device may still pose significant risks in practice.

The question that remains open is whether every system applied to a biomedical engineering product can be categorized as AI. The EU AI Act defines an AI system as a machine-based system that is designed to operate with varying levels of autonomy and that may exhibit adaptiveness after deployment and that, for explicit or implicit objectives, infers, from the input it receives, how to generate outputs such as predictions, content, recommendations, or decisions that can influence physical or virtual environments (10). The main challenge will be to determine the level of autonomy required for such systems to be considered AI (14). However, most systems applied in medical devices are likely to qualify as AI systems, and hence as high-risk AI systems under the EU AI Act. This means there is a set of requirements these systems must comply with, which we discuss in the following section.

PRINCIPLES AND REQUIREMENTS FOR HIGH-RISK AI SYSTEMS UNDER THE EU AI ACT

Requirements for high-risk AI systems are set out in Recital 1 in order to achieve human-centric, trustworthy AI, which is the ultimate goal of the EU AI Act. These requirements were derived from seven ethical principles developed by the High-Level Expert Group on Artificial Intelligence in its Ethics Guidelines for Trustworthy AI (HLEG Ethics Guidelines), which were subsequently incorporated into Recital 27 of the EU AI Act. Since they form the core of the high-risk AI

regulation, as well as the EU AI Act as a whole, we will further analyze the requirements for high-risk AI systems through these principles:

Human agency and oversight

This principle means that AI systems must be developed and used as tools that serve people, respect human dignity and personal autonomy, and function in a way that can be appropriately controlled and overseen by humans (10). It is a continuation of the "human-in-the-loop" principle already applied in the GDPR (15). The purpose of this principle is to promote safe and trustworthy AI, as it is believed that only AI controlled by humans can be considered human-centric and trustworthy (16). It aims to guarantee that AI will never gain supremacy over the human race, i.e., that "black-box" systems cannot replace human decision-making, which would not be in accordance with European values and fundamental rights (15). Due to its importance, it is placed before other principles. This principle also means that humans must be involved in AI decision-making. Given the present distrust in AI systems, the final decision must always be made by humans, which is also important for the accountability principle (16). The principles operationalized in Article 14 of the EU AI Act require human oversight. This requirement aims to prevent or minimize risks to health, safety, or fundamental rights, which is the general rationale for all high-risk AI system requirements. Repeating this aim in the human oversight requirement underscores its importance and the fact that it is the main protection against AI misuse. This requirement is to be achieved through two types of measures: those built into AI systems by the provider before they are placed on the market, and those implemented by the deployer (10). Built-in measures could, for example, be technical measures to facilitate the interpretation of outputs, while measures implemented by deployers could include guidelines and processes deployed within their organizations (17). It is required that a natural person assigned to the oversight of AI is able to: understand the capacities and limitations of the system, remain aware of tendencies toward relying or over-relying on the output produced, correctly interpret the output, decide not to use the high-risk AI system or to disregard, override or reverse its output, and intervene in the operation of the high-risk AI system or interrupt the system through a 'stop' button (10). According to these sub-requirements, it is essentially requested that the human controller has significant control over the AI system, which was deemed paramount in theory (18). This is achieved only when a controller understands the AI system's functioning, which is particularly important given the "black box" problem in AI, i.e., the fact that humans lack insight into the AI "thinking" process and do not know how AI creates its output (15,18,19). It is also important that a human can override a machine's decision and have a "killer switch" – a stop button that can at any moment abort the AI system process in case of emergency. In this way, it is believed that humans will retain sufficient control to prevent excessive AI system autonomy. However, the level of human responsibility and training is only provided in principle, and these aspects should be regulated in more detail (18). Interpreted literally, this principle would establish human dominance over the AI. Humans clearly hold a higher position. However, humans are not inherently superior to machines, since, as is well known, we also make mistakes. Therefore, we believe that humans should not be merely the controllers of the machine; human oversight cannot be a panacea for all the risks AI poses. The relationship between humans and machines should be one of mutual cooperation, in which each party performs the task it is best at and is controlled by the other to achieve a minimum level of error. Acknowledging that neither humans nor machines are perfect, in our opinion, is the path that could lead us to further scientific and technical development. On the other hand, rigid demands for human dominance over machines could have a chilling, or even negative, effect on AI development (15,16). Hence, these rules should be interpreted so that human oversight serves as a fallback mechanism in emergencies (20).

Technical robustness and safety

This principle means that AI systems must be developed and used in a way that allows robustness in the case of problems, provides resilience against attempts to alter the use or performance of the AI system so as to allow unlawful use by third parties, and minimizes unintended harm (10). The quality of an AI system must be at the highest level to minimize the potential dangers of misuse and backfiring. Through the requirements of accuracy, robustness, and cybersecurity, it is provided that high-risk AI systems shall be designed and developed to achieve an appropriate level of accuracy, robustness, and cybersecurity, and to perform consistently in those respects throughout their lifecycle. In theory, accuracy, robustness, and cybersecurity are three distinct requirements, different in nature, which makes interpretation much harder. It is also somewhat difficult to measure the "appropriate" level for all three requirements, given their distinct natures. Accuracy is not even defined in the EU AI Act, but it should not be regarded identically to

the accuracy principle in the GDPR; this does not mean that high-risk AI systems must never give false outputs, but rather that the system provides an appropriate level of correctness given the circumstances (20). Regarding robustness, it is provided that high-risk AI systems shall be as resilient as possible to errors, faults, or inconsistencies that may occur within the system or its environment. Some technical and organizational measures are enumerated, such as backup or fail-safe plans. Furthermore, systems that continue to learn must be developed in a way that eliminates or reduces the risk of biased outputs influencing input for future operations (feedback loops), which should be duly addressed (10). Robustness should be understood as resilience to every type of adversity, excluding cyberattacks (17). Finally, high-risk AI systems shall be resilient against attempts by unauthorized third parties to alter their use, outputs, or performance by exploiting system vulnerabilities, which guarantees cybersecurity (13). This requirement creates an obligation of consistent performance (20), which is also operationalized, among other things, by providing a risk management system. This system operates as a continuous iterative process planned and run throughout the entire lifecycle of a high-risk AI system, requiring regular systematic review and updating in the prescribed four steps (10). A risk management system is also designed to embrace the transparency principle, as risk management documentation allows us to partially observe the AI's work process (21).

Privacy and data governance

This principle means that AI systems must be developed and used in accordance with privacy and data protection rules while processing data that meets high standards in terms of quality and integrity (10). To protect human rights, it is necessary that personal data be protected in accordance with the GDPR. Furthermore, the faultless development and operation of an AI system depend heavily on providing it with adequate data. Hence, this requirement is not limited to personal data protection (20) but also prescribes that all other data must come from reliable sources and be processed in a way that does not discriminate against anyone. This is necessary because early applications of AI systems showed they were prone to bias and extremely unreliable when trained on inadequate datasets. It is provided that training, validation, and testing data sets shall be subject to data governance and management practices appropriate for the intended purpose of the high-risk AI system, and that these testing datasets shall be relevant, sufficiently representative, and, to the maximum extent possible, free of errors and complete in view of the intended purpose (10). This basically means that the data input into a high-risk AI system must also be of high quality (20), in accordance with its intended purpose, and should be assessed for reasonableness and proportionality (17). Finally, regarding personal data protection, it is explicitly stated that special categories of personal data may be processed only in accordance with the GDPR and specific conditions (10). Unlike other data, personal data must meet a higher level of accuracy and quality under the GDPR. In one survey conducted among AI developers, this requirement was labeled as the hardest to implement. In their opinion, the most difficult task is to keep track of the data AI uses as input and to monitor for eventual biases and exposed personal data (22).

Transparency

This principle means that AI systems must be developed and used in a way that allows appropriate traceability and explainability while making humans aware that they are communicating or interacting with an AI system. It also requires duly informing deployers of the capabilities and limitations of that AI system and affected persons about their rights (10). Transparency is considered the greatest challenge with AI systems; therefore, most regulatory requirements are derived from this principle (1). An AI system's operation must be sufficiently transparent to enable deployers to interpret its output and use it appropriately. Furthermore, systems must include instructions for use containing concise, complete, correct, and clear information that is relevant, accessible, and comprehensible to deployers. The opacity and complexity of AI systems (i.e., the "black box" problem) are regarded as the highest risks concerning AI applications. Consequently, it is essential that the information necessary to understand the system is provided prior to placing it on the market (30). The minimum information required for these instructions is set out in Article 13, Paragraph 3 of the EU AI Act. These requirements for the instructions for use are essential given that these systems are highly sophisticated devices. The completeness of the instructions must also be measured to assess compliance with the EU AI Act (17). Yet, the most critical operational requirement is record-keeping. High-risk AI systems must technically support the automatic recording of events (logs) throughout their lifetime. This is of paramount importance due to the "black box" problem. To ensure a level of traceability of the functioning of a high-risk AI system that is appropriate to the intended purpose of the system, logging capabilities must enable the recording of

risk-relevant events (10). This requirement, as well as its importance, was recognized even at the level of HLEG Ethics Guidelines (20). It is, however, limited by the minimization principle of personal data protection (17). Finally, this principle is traced in the notification obligation, which dictates that the provider of an AI system is obliged to inform the market and the AI authority of any significant risks.

Diversity, non-discrimination, and fairness

This principle means that AI systems must be developed and used in a way that includes diverse actors and promotes equal access, gender equality, and cultural diversity, while avoiding discriminatory impacts and unfair biases that are prohibited by Union or national law (10). Highlighting this principle was necessary due to the recurrent biases shown in AI systems' operations. Sometimes biases are "inherited" from input data, whereas at other times, there is no clear explanation for them. This principle is operationalized through human oversight and technical robustness requirements. On the one hand, it is necessary that a human can check and remove unfair biases if and when they occur, and on the other hand, it is necessary that the system be built in a manner where these biases and discriminations do not occur, or only occur in a minimal number of cases (18). This principle also means that the use of AI systems should be user-centric and available to the widest possible range of users, without prejudice based on race, ethnicity, language, religion, skin color, sexual orientation, disability, social status, or other factors (23).

Social and environmental well-being

This principle means that AI systems must be developed and used in a sustainable and environmentally friendly manner as well as in a way that benefits all human beings while monitoring and assessing the long-term impacts on the individual, society, and democracy (10). AI systems have already changed the way we live. However, their future effects could be even more drastic and dangerous for society. First, it is necessary that AI systems do not harm the environment. Using AI systems is technically demanding and can require significant energy consumption and the creation of waste. Therefore, the technical robustness requirement and other obligations require that AI systems be environmentally friendly. Second, these systems can enhance or deteriorate social skills. Systems must be built in such a way as to prevent destruction or dereliction of social life and generally to protect the physical and mental well-being of their users (23). Therefore, this principle is also operationalized through the technical robustness requirement.

Accountability

Accountability is not explicitly mentioned in Recital 27 as one of the principles, yet it was enumerated in the HLEG Ethics Guidelines. Moreover, several norms make it clear that this principle is implemented throughout the regulation. It means that, under the EU AI Act, developers, deployers, and other entities must keep records and documents to demonstrate compliance. With this in mind, alongside the EU AI Act, two international standards for risk management and compliance have been published: ISO/IEC 42001 "Information technology-Artificial intelligence-Management system" and ISO/IEC 23894 "Artificial intelligence-Guidance on risk management". According to these standards, as well as the EU AI Act itself, compliance proceedings have to be conducted (21). The compliance requirement provides that high-risk AI systems shall comply with the requirements, taking into account their intended purpose and the generally accepted state of the art in AI and AI-related technologies. The risk management system referred to in Article 9 shall be taken into account when ensuring compliance with those requirements. Also, the technical documentation requirement operationalizes this principle. The technical documentation shall be drawn up in such a way as to demonstrate that the high-risk AI system complies with the requirements set out in the EU AI Act and to provide national competent authorities and notified bodies with the necessary information in a clear and comprehensive form to assess the compliance of the AI system with those requirements (10). However, technical documentation should not be developed in isolation but rather in an iterative process as part of the risk management system, so these two requirements are very closely linked (20). Technical documentation will be comprehensive, especially in the field of medicine, as the MDR and IVDR already require extensive documentation (12). Finally, the accountability principle is also evident in the quality management obligation and document-keeping obligation. Notably, the quality management system obligation will be somewhat easier for developers in the medical field, as they already have a quality management system mandated by the MDR and IVDR, and the EU AI Act quality management system can be integrated into the existing one (12).

ASSESSMENT OF THE REGULATORY FRAMEWORK OF THE EU AI ACT

The EU AI Act's regulatory framework is both praised and criticized. On the one hand, it should be pointed out that the EU AI Act is one of the world's first comprehensive, legally binding pieces of legislation on AI (12). It is also important that the adopted regulatory framework is grounded in moral principles to place AI applications under the rule of law and morality. In this way, there is a clear attempt to prevent the use of AI that would be harmful or even dangerous to humanity (24).

However, the EU AI Act is often criticized for its approach to regulating AI development. First of all, the framework applied in the EU AI Act is essentially modeled after the GDPR (12,25). It is questionable whether data protection methods are appropriate for AI regulation. The fact is that AI is a far more complex phenomenon with a greater impact than simple data processing (14). Autonomous risk management and compliance procedures are necessary for personal data protection, as they are the only way for data processing by companies and governments to become transparent. Although transparency is important in AI, the issue here is very different. The challenge with AI transparency lies in the "black-box" problem: we do not know how AI reaches a conclusion or makes a decision, because the rules of human logic do not always apply to it. Hence, the activity log requirement is very important, as it helps illuminate how AI is "thinking," which, in turn, makes allocation of responsibility easier. However, it is questionable how much of this process can actually be revealed and how much of it can be meaningfully interpreted by humans. On the other hand, it is not clear why compliance proceedings are important for transparency in the AI industry. A quality management system, alongside extensive documentation, does not guarantee that the ultimate product will be flawless, nor will it solve the "black-box" problem. Furthermore, these requirements are quite expensive. According to some studies, they could cost between 193,000 and 330,000 euros (11). Although achieving this requirement is not considered the most challenging, a quality management system is the most expensive component for developers (22). Theoretical literature indicates that the documentation required for compliance often fails to meet the technical detail requirements of authorities, and that standardization is needed because information provided by different developers varies widely (1). Further, the subjects of these obligations are not only large corporations and government bodies but also startups and small and medium-sized enterprises (SMEs), which often lack the manpower or funds to engage in complex compliance proceedings. Special regulatory measures are indeed in place to support startups and SMEs. However, these measures do not exempt them from compliance proceedings; they merely make the process somewhat less expensive (13). To protect the interests of AI system developers and remain in the race for AI development, the so-called “regulatory sandbox” method was introduced in the EU AI Act. Sandboxes are usually found in the financial sector. When new products are to be introduced, providers are allowed for some time to test them on the market in a controlled environment without being subjected to full regulatory enforcement. This way, a regulatory body can also learn about the product, and the developer can test it in real life. Therefore, it is important to implement them properly (26). Experts praised sandboxes as a good solution for AI development (2). However, sandboxes under the EU AI Act are provided only as an obligation for Member States, since the Union does not have authority over all fields of AI application in which sandboxes could be applied. This is the first flaw of the system, since there are no detailed rules for sandboxes, and control by the Union can only be exercised through reports. Second, sandboxes primarily address compliance obligations that can be burdensome during the development phase. Furthermore, a developer is still obliged to submit an exit report for activities performed in a sandbox. Third, sandboxes do not exclude the developer's liability, and competent authorities in member states could still stop work in the sandbox at any time. It is clear that the advantages provided by sandboxes are very limited and offer only uncertain guarantees to developers, who, in return, must reveal their trade secrets. Consequently, most developers will likely not participate in sandboxes (26). In the field of biomedical engineering, it is even questionable whether it would be rational to approve sandboxes, as health and safety could be at risk. The exception would be sandboxes for data governance obligations under the EU AI Act. This is the main reason the EU AI Act is often discussed in theory as being more accommodating to large AI companies (24,26). Conformity assessments are common for physical products and therefore include sampling, testing, inspection, and evaluation, which are challenging for intangible software (25). There are also problems with the risk-based approach. The risk-based approach, as well as the risk management system, rests on making compromises, since some levels of risk can be accepted and others cannot. However, for fundamental human rights, especially in health and safety, it is hard to argue that there

are acceptable risks (14). Finally, there is no clear criterion on why some systems are considered high-risk, while others are not (27). It is pointed out that classification is not based on empirical evidence and that research that was conducted prior to the EU AI Act adoption may be questionable, suggesting the classification was possibly conducted on a political basis (14,28). Bearing all this in mind, it is safe to say that the enumeration method for defining high-risk AI systems is quite inflexible and not future-proof, although that was one of the primary goals of adopting the EU AI Act (11,29).

In scientific areas such as biomedical engineering, the EU AI Act could introduce further complications. First of all, systems covered by Annex I cannot be arbitrarily added or removed, which means that AI systems classified as medical devices will automatically be considered high-risk, regardless of their specific clinical impact. This could discourage developers in this field; even when they develop a system that does not pose a high risk to health, safety, and fundamental rights, they must still comply with all the aforementioned obligations. Second, providers of biomedical services and equipment already have strict compliance obligations under the MDR and IVDR, which are now expanded by the EU AI Act. It is indeed provided that these conformity assessments should be merged and that requirements under multiple acts should be applied only once (10). However, it is argued in theory—because of this horizontal approach to regulation—that there are many inconsistencies across different regulations. This is especially true for the MDR. There are many different definitions of the same term in the EU AI Act and the MDR. Many requirements overlap, and there are obvious differences in their risk-based approaches: the MDR applies a risk-benefit balancing framework, whereas the EU AI Act does not. In theory, it is also noted that there are inconsistencies between compliance proceedings under the MDR and the EU AI Act, which would make merging the fact sheets more difficult (11–13). Fortunately, Article 43, paragraph 3, of the EU AI Act prescribes that compliance proceedings under the Act should be incorporated into existing proceedings, which should alleviate some pressure from developers. Additionally, the joint guidance document titled "*Interplay between the Medical Devices Regulation (MDR) & In vitro Diagnostic Medical Devices Regulation (IVDR) and the Artificial Intelligence Act (AIA)*", adopted by the European Artificial Intelligence Board (AIB) and the Medical Device Coordination Group (MDCG), provides assistance to medical device developers for implementing EU AI Act rules into existing MDR and IVDR frameworks, resolving some of the issues pointed out in theory (30). There is also hope that the Commission's proposal for the European Biotech Act, adopted in December 2025 (31), will make the obligations of developers clearer and somewhat more manageable.

We believe that the main issue is that a one-size-fits-all approach might not be the best solution, especially in biomedical engineering. In the literature, transparency is considered the most important requirement for achieving a trustworthy AI system (21). Yet, in biomedical engineering, reproducibility is far more important. Reproducibility means that the AI system always gives the same answer to the same question. For example, it is necessary that whenever it analyzes the same CT scan, it provides a consistent diagnostic output. Also, when predicting chemical reactions in experiments, the system must always generate the same prediction for the same inputs.

The reproducibility requirement is not even mentioned in the requirements for a high-risk AI system, although it could be understood as a form of an accuracy requirement. Further, it is very important that the AI system be well-trained and that inputs include a variety of data. When input data comes from a single group (e.g., people of a certain age or from certain areas), AI models can adjust too closely to that group's characteristics, leading to incorrect diagnoses or potentially dangerous applications in medicine. Hence, a variety of input data is a critical requirement. Finally, medical data is multidimensional, which means that to make conclusions in medicine, you need data of different types, such as images, numbers, and texts, which makes the creation of specific AI systems in medicine difficult (32). Therefore, we strongly believe that AI regulation must be tailored to the specific field in which it is applied, with rules specifically designed for that field. As we can see, that is also the intention of the European Commission, which has already adopted the proposal for the European Biotech Act.

The research conducted allows for several conclusions regarding the initial questions. The EU AI Act represents a significant first step in regulating AI, serving as a foundation for the further development and refinement of AI governance. Unlike other emerging fields, AI regulation has not been widely addressed at the national level within EU member states, making the process more challenging due to the lack of prior experience. Consequently, regulatory shortcomings are to be expected, and additional time and experience will be required to develop effective solutions. The EU AI Act provides a valuable starting point within the regulatory hierarchy. While AI is a complex social phenomenon that

cannot be comprehensively governed by a single law, an overarching regulation, similar to the GDPR, may serve as a constitutional framework for this legal domain. As sector-specific AI regulations are developed, the EU AI Act is expected to realize its full potential and resolve current inconsistencies. Limiting the EU AI Act to principled rules would enable the creation of tailored laws for each sector, reflecting the varying levels of risk and application across different fields.

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Author contributions

Conceptualization: N.I. and N.M.; Methodology: S.Dj., N.I., and N.M.; Investigation: S.Dj., N.I., and N.M.; Data curation: S.Dj., N.I., and N.M.; Formal analysis: S.Dj.; Writing – original draft: S.Dj., N.I., and N.M.; Writing – review & editing: S.Dj. and N.I. All authors have read and approved the published version of the manuscript.

Statement of Ethics

Not applicable.

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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AFMN BIOMEDICINE

REVIEW ARTICLE

THERAPEUTIC POTENTIAL OF MEDICINAL PLANTS IN POLYCYSTIC OVARY SYNDROME: A NARRATIVE REVIEW

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Polycystic ovary syndrome (PCOS) is a complex endocrine disorder that affects the reproductive, metabolic, and psychological health of women of reproductive age. Conventional therapies for PCOS primarily focus on symptom management; however, their associated side effects have led many women to explore complementary approaches. This review aimed to evaluate the clinical efficacy and safety of herbal medicines in the management of PCOS based on data from clinical trials and preclinical models. An extensive literature search was conducted using PubMed, ScienceDirect, Scopus, and the Cochrane Library, covering studies published between 1995 and 2025. Inclusion criteria encompassed clinical trials and randomized controlled trials investigating herbal interventions for PCOS, as well as relevant in vivo and in vitro preclinical studies. The main outcomes analyzed were hormonal regulation, insulin sensitivity, ovarian function, and anti-inflammatory effects. Numerous herbs demonstrated therapeutic potential in PCOS management, including *Nigella sativa* L. (Ranunculaceae), *Vitex agnus-castus* L. (Lamiaceae), *Trigonella foenum-graecum* L. (Fabaceae), and *Cinnamomum verum* J.Presl (Lauraceae). These herbs exhibited diverse mechanisms of action, including modulation of insulin signaling pathways, reduction of oxidative stress, hormonal regulation, and anti-androgenic effects. Clinical studies reported improvements in menstrual regularity, insulin resistance, and hirsutism, with generally favorable safety profiles. Herbal medicine appears to be a promising adjunctive or alternative strategy for the management of PCOS. However, while existing evidence supports its efficacy, variability in study designs, dosage regimens, and outcome measures limits definitive conclusions. Future standardized, high-quality clinical trials are needed to confirm these therapeutic benefits and safety profiles.

Keywords: polycystic ovary syndrome, herbal medicine, insulin resistance, phytotherapy, reproductive health, complementary therapy

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INTRODUCTION

Polycystic ovary syndrome (PCOS), originally described as Stein-Leventhal syndrome (2), is an endocrine disorder widespread among women, affecting various aspects of their health (1). Research indicates that PCOS currently affects 11.9% of women globally, according to the Rotterdam diagnostic criteria (3). This common endocrine condition affects women of reproductive age and is characterized by hyperandrogenism, ovulatory dysfunction, and polycystic ovarian morphology (4). Increasing evidence suggests that PCOS is a complex multisystem disorder extending beyond reproductive abnormalities and involving substantial metabolic disturbances and an increased risk of cardiovascular and psychological comorbidities. Changes in the diagnostic criteria greatly affect the reported prevalence of PCOS (5). PCOS incidence in the USA is increasing and is now estimated to approach 18% (6), whereas among Australian women, it ranges from 12% to 21% (7).

The pathophysiology of PCOS is multifactorial and involves hormonal imbalance, insulin resistance, and ovarian dysfunction (8,9). Elevated luteinizing hormone (LH) levels stimulate androgen production in ovarian theca cells, contributing to hyperandrogenism, which, in turn, disrupts normal follicular development, leading to anovulation and the characteristic polycystic ovarian morphology (10). Furthermore, insulin resistance and compensatory hyperinsulinemia play central roles in the pathogenesis of PCOS by further promoting ovarian androgen production and decreasing sex hormone-binding globulin levels, thereby exacerbating hyperandrogenism (11). In addition, chronic low-grade inflammation and oxidative stress have been increasingly implicated in the pathogenesis of PCOS, as they disrupt insulin signaling pathways, enhance ovarian steroidogenesis, and contribute to both metabolic disturbances and reproductive dysfunction (12,13). Hyperandrogenism, as a key feature of PCOS, is detected in approximately 60%–80% of affected women, while insulin resistance occurs in around 50%–70%, representing a central metabolic disturbance that contributes to the increased risk of long-term complications such as type 2 diabetes mellitus, dyslipidemia, hypertension, and cardiovascular disease (14,15).

According to the Rotterdam criteria, four PCOS phenotypes are distinguished based on different combinations of hyperandrogenism, oligo-anovulation, and polycystic ovarian morphology, as summarized in Table 1 (3,16,17).

The primary, first-line approach for all patients with PCOS is modification of lifestyle (18, 19). Clomiphene citrate (CC), letrozole, or estradiol are widely used in patients seeking fertility treatments, whereas oral contraceptives are commonly used for the chronic management of PCOS symptoms (20,21). Metformin is generally not considered for use alone, but is often combined with CC for treating women who are resistant to CC treatment alone (22). Conventional treatments for PCOS, besides lifestyle modifications, frequently involve insulin-sensitizing agents, anti-androgens, and ovulation-inducing medications (19). However, concerns about the potential side effects associated with conventional pharmacological therapies, together with growing interest in natural and complementary treatment approaches, have encouraged many women with PCOS to seek alternative therapeutic options. Among these, herbal medicine has attracted increasing scientific attention in recent years (4,23). As PCOS remains a complex and challenging disorder to manage, this study aimed to provide an overview of herbal medicines that have demonstrated potential therapeutic benefits in the treatment of this condition, with particular emphasis on clinically evaluated herbal drugs (24).

Table 1. *Classification of PCOS phenotypes according to the Rotterdam criteria*

	Phenotype A	Phenotype B	Phenotype C	Phenotype D
Hyperandrogenism	+	+	+	-
Oligo/anovulation	+	+	-	+
Polycystic ovarian morphology	+	-	+	+

Herbal medicine has been used for centuries in various traditional medical systems to treat a wide range of health conditions, including PCOS (4). Several medicinal plants have been investigated for their potential benefits in managing PCOS symptoms and associated metabolic and reproductive disturbances (25). These herbs may exert insulin-sensitizing, anti-androgenic, anti-inflammatory, and ovulation-regulating effects (25). However, the efficacy and safety of herbal medicines for PCOS management remain inconclusive due to the limited number of high-quality clinical trials and the substantial heterogeneity in study designs, interventions, and outcome measures (4).

Several review articles have previously addressed the role of medicinal plants and natural compounds in the management of PCOS (23–25). However, most available reviews focus on individual plants or on general herbal approaches, without systematically discussing underlying mechanisms of action or incorporating the most recent experimental and clinical findings. Furthermore, given the increasing number of studies published in recent years,

there is a need for an updated and comprehensive synthesis of the available evidence. Therefore, this review aimed to provide a comprehensive and up-to-date overview of medicinal plants investigated in the context of PCOS, based on a thorough analysis of recent literature, with particular emphasis on their bioactive compounds, mechanisms of action, and findings derived from experimental and clinical studies. By integrating available data, this review aimed to provide insights into the potential therapeutic benefits, limitations, and future research directions of herbal medicine as a complementary or alternative approach to PCOS treatment.

METHODS

We performed an extensive literature search of English-language studies across electronic databases, including PubMed, ScienceDirect, Scopus, and the Cochrane Library, from 1995 through 2025. Our objective was to identify clinical research on the use of herbal treatments for polycystic ovary syndrome (PCOS). The search terms included “polycystic ovary syndrome” and “PCOS” in the title or abstract, along with “plant”, “medicinal plant”, “herb”, “phytotherapy”, “botanical extract”, “root”, “fruit”, “hydroalcoholic”, “powdered”, “traditional medicine”, “integrative”, and “alternative therapy” in all fields.

Articles were selected based on title and abstract reviews in accordance with specific inclusion and exclusion criteria. Studies were included if they were English-language articles reporting clinical trials, randomized controlled trials (RCTs), and relevant in vivo and in vitro preclinical studies investigating the use of medicinal plants or natural compounds in PCOS. Exclusion criteria included conference abstracts without full-text availability and studies not directly related to the condition.

Ultimately, scientific papers that met the inclusion criteria were selected for in-depth analysis. The types of herbs used, the specific plant parts studied, the year of publication, study design, sample size, and the effects of these herbal treatments on reproductive and metabolic outcomes in patients with PCOS were all carefully extracted from the full-text articles.

RESULTS

Various herbs, such as *Aloe barbadensis* Mill. (Asphodelaceae), *Cinnamomum verum* J.Presl (Lauraceae), *Camellia sinensis* (L.) Kuntze (Theaceae), *Trigonella foenum-graecum L. (Fabaceae), and Mentha spicata L. (Lamiaceae),* have garnered attention for their potential therapeutic applications in the management of PCOS (26). An overview of key medicinal plants and their reported therapeutic actions is summarized in Table 2 for clinical studies and Table 3 for preclinical studies. *A. barbadensis* gel, when administered to rats with PCOS induced by the nonsteroidal aromatase inhibitor letrozole, demonstrated notable effects, including increased HDL cholesterol levels and reduced plasma triglyceride and LDL cholesterol levels (27,28). Studies investigating *C. verum* have highlighted its insulin-sensitizing and anti-inflammatory properties, suggesting a potential role in improving glucose metabolism and restoring estrous cyclicity in PCOS models (29). *C. sinensis*, known for its antioxidant and anti-inflammatory properties, has also been proposed as a beneficial herbal remedy for PCOS (30). *T. foenum-graecum*, another plant under investigation, has shown potential in regulating ovarian function in PCOS by promoting follicle-stimulating hormone (FSH) levels and decreasing luteinizing hormone (LH) levels (31). In addition, recent research has indicated that *M. spicata* may be effective in alleviating mild hirsutism in women with PCOS (32,33). Collectively, these findings suggest a promising role of herbal medicine as a complementary approach to addressing various aspects of PCOS symptoms and associated metabolic disturbances.

Camellia sinensis (L.) Kuntze (Theaceae)

Camellia sinensis has attracted considerable attention as a potential therapeutic agent for PCOS due to its antioxidant and anti-inflammatory properties. Several studies have investigated the effects of *C. sinensis* extract on metabolic parameters, hormone levels, and ovarian function in PCOS. Green tea contains a variety of catechins, among which epigallocatechin gallate (EGCG) is considered the most biologically active and influential constituent of *C. sinensis* (34).

A systematic review summarized findings from four human and four animal primary studies on *C. sinensis* extract in PCOS (35). Human studies reported reductions in body weight and, in some cases, testosterone levels. For instance, in a randomized double-blind clinical trial, overweight and obese women with PCOS aged 20–40 years received either a *C. sinensis* tablet at an unspecified dose or placebo capsules containing wheat flour daily for 12 weeks. This study found that *C. sinensis* supplementation significantly reduced body weight, fasting insulin levels, and free testosterone concentrations compared to the placebo group. These results suggest that

Table 2. *Summary of medicinal plants investigated for their therapeutic effects in polycystic ovary syndrome (PCOS) - clinical studies*

Plant/extract	Patients	Results	Active substance	Dosing	Duration of administration	Author
Camellia sinensis (L.) Kuntze, (Theaceae)	20-40 y.o.PCOS women	↓BW, FI, T	Green tea tablets	500 mg green tea capsules	12 weeks	(36)
Mentha spicata L. (Lamiaceae)	Women with PCOS	↓T, ↑LH,	Spearmint tea	250 ml freshly prepared tea, 2 x daily	30 days	(32)
Mentha spicata L. (Lamiaceae)	Women with hirsutism caused by PCOS	↓FT, ↓TT, ↑LH, ↑FSH, ↓hirsutism	Spearmint tea	tea 2 x daily	30 days	(33)
Crocus sativus L. (Iridaceae)	Obese women with DMT2		Saffron powder	200 mg, 400 mg	12 weeks	(46)
Cinnamomum verum J.Presl, (Lauraceae)	Women with PCOS	↓TC, ↓ LDL, ↑HDL	Cinnamon capsules	500 mg, 3 capsules	8 weeks	(48)
Cinnamomum verum J.Presl, (Lauraceae)	Women with PCOS	↓, WC, ↓BMI, ↓LDL	Cinnamon powder	500 mg cinnamon, 3 x daily	12 weeks	(50)
Trigonella foenum-graecum L. (Fabaceae)	Premenopausal women with PCOS	↓ LH, ↓ FSH, improved ovarian function	Fenugreek seed extract (Furocyst) enriched with ≈40% furostanolic saponins	500 mg capsule, 2 x daily	90 days	(51)
Apium graveolens L. [Apiaceae] *Pimpinella anisum* L., (Apiaceae)	Obese women with PCOS	↓T, ↓LH/FSH ratio, ↑menstrual bleeding episodes	Apigenin (celery) and anethole (anise)	750 mg capsules, 3 x daily for 15 days over 3 menstrual cycles	-	(54)
Nigella sativa L., (Ranunculaceae)	Adolescent girls with PCOS	↓LH, ↓FSH, ↓T, improve menstrual cycle regularity, ↓OV	-	1000mg capsules (*N. sativa*) daily	16 weeks	(72)

BW -body weight; FI- fasting insulin; T- testosterone (F-free, T-total); LH – luteinizing hormone; FSH – follicle-stimulating hormone; DMT2 -diabetes mellitus type 2; TC – total cholesterol; LDL – low-density lipoprotein; HDL – high-density lipoprotein; WC- waist circumference; BMI – body mass index; OV- Ovarian Volume

C. sinensis consumption may contribute to weight loss and improvements in metabolic and hormonal profiles in women with PCOS (36).

Another study examined the effect of a hydroalcoholic extract prepared from dried leaves of *C. sinensis* on reproductive improvement in estradiol valerate-induced PCOS in rats. Following the induction of PCOS, the rats were divided into four groups: a PCOS group and three experimental groups receiving 50, 100, or 200 mg/kg BW of *C. sinensis* extract intraperitoneally for 10 days. The results showed a significant reduction in serum LH levels, body and ovarian weights, and the insulin resistance index in the *C. sinensis* extract-treated groups compared with the PCOS group. Histomorphometric studies further revealed significant improvements in the number of follicles and thickness of the theca layer, indicating a marked improvement in PCOS symptoms (37).

Overall, supplementation with *C. sinensis* extract may reduce serum levels of LH, free testosterone, and 17-β-estradiol, while significantly increasing the serum levels of FSH and progesterone in PCOS. C. sinensis regulates PCOS through its polyphenol content (especially EGCG), which modulates insulin signaling, reduces oxidative stress, and helps decrease androgen production, thereby improving metabolic and reproductive outcomes (35).

Notably, while intraperitoneal administration of high-dose EGCG resulted in reduced serum 17-β-estradiol and LH levels in female rats and reduced serum testosterone levels in male rats, such reductions in female sex hormones were not consistently observed in human subjects (34).

A study by Farhadian et al. (2020) demonstrated that theca layer of ovarian follicles was thicker in the PCOS control group than in the normal control group. Treatment with *C. sinensis* extract significantly decreased the thickness of the theca layer in a dose-dependent manner compared to the PCOS control group. This reduction in theca layer thickness may relate to decreased androgen and steroid hormone secretion, contributing to the amelioration of PCOS symptoms. The thickness of the granulosa layer was, conversely, reduced in the treated groups, further indicating normalization of follicular structure. These changes, along with increased counts of healthy follicles

Table 3. *Summary of medicinal plants investigated for their therapeutic effects in polycystic ovary syndrome (PCOS) - experimental studies*

Plant/extract	Model - animal	Results	Active substance	Dosing	Duration of administration	Author
Camellia sinensis (L.) Kuntze, (Theaceae)	Estradiol valerate-induced PCOS in rats	↓LH, ↓BW, ↓OW, ↓IRI	Hydro-alcoholic green tea extract	50 mg/Kg BW or 100 mg/Kg BW green tea extract intraperitoneally daily	10 days	(37)
Mentha spicata L. (Lamiaceae) *Linum usitatissimum* L. (Linaceae)	Estradiol-valerate induced PCOS in rats	↑P, ↓T, ↓E	Hydro-alcoholic spearmint and flaxseed extracts	40 mg/kg hydroalcoholic extract of spearmint + 200 mg/kg flaxseed extract for 30 days by gavage	30 days (7 weeks induction)	(39)
Mentha aravensis L. (Lamiaceae)	T-enanthate-induced PCOS in rats	↓LH, ↓FSH, ↑antioxidant capacity	Mentha arvensis hydroalcoholic extract	50, 100, and 200 mg/kg of extract powder daily	4 weeks (3 weeks induction)	(42)
Crocus sativus L. (Iridaceae)	T-enanthate-induced PCOS in rats	↓inflammatory markers, ↑insulin sensitivity, ↓LH, ↓E, ↓T, ↑FSH	Saffron petal extract (SPE) and saffron petal anthocyanins (SPA)	SPE [50, 200, and 600 mg/kg/body weight] and SPA [20, 40, and 80 mg/kg/body weight] daily	-	(43)
Vitex negundo L., (Lamiaceae)	Letrozole-induced PCOS rat model	↓LH, ↑FSH, ↑ serum concentrations E & P	Aqueous and hydroalcoholic extracts	200 and 400 mg/kg daily	66 days	(76)
Vitex negundo L., (Lamiaceae)	Letrozole-induced PCOS rat model	↑hormonal profiles, ↓OSM, ↓ BW	Hydro-ethanolic extract of Vitex negundo seed (VNE) (Cinnamic acid, plumbagin, nigundin B)	250 mg/kg daily	-	(77)
Vitex agnus-castus L. (Lamiaceae)	Letrozole-induced PCOS rat model	↑FSH, ↑E, ↑P	Ethanolic extracts	365 mg/kg orally	30 days	(78)
Glycyrrhiza glabra L. (Fabaceae)	Letrozole-induced PCOS rat model	↓LH, ↓T, ↑FSH, ↑E, ↑P	Methanolic extract	3.5 g tablet per day	21 day	(81)
Zingiber officinale Roscoe (Zingiberaceae)	Letrozole-induced PCOS rat model	↓LH, ↓FSH, ↓E, ↓T, ↑P,	6-gingerol and ginger extract	100mg/kg or 200 mg/kg of ginger extract, 200 µg/kg or 400 µg/kg of 6-gingerol	28 days	(84)
Trifolium pratense L. (Fabaceae)	Letrozole-induced PCOS rat model	↓OW, ↓OV, ↓ ST, ↑E, ↓LDL, ↑HDL	Red clover extract	500 mg/kg or 750 mg/kg orally	30 days (21 days model induction)	(87)

BW -body weight; FI- fasting insulin; T- testosterone (F-free, T-total); LH – luteinizing hormone; OW-ovarian weight; IR(I) – insulin resistance (index); P – progesterone; E – estradiol; FSH – follicle-stimulating hormone; DMT2 -diabetes mellitus type 2; TC – total cholesterol; LDL – low-density lipoprotein; HDL – high-density lipoprotein; WC- waist circumference; BMI – body mass index; OV-Ovarian Volume; OSM – oxidative stress markers; STR – serum triglycerides; FBGL- fasting blood glucose level

and corpus luteum numbers, suggest a beneficial effect of C. sinensis extract on ovarian morphology and function in PCOS (38).

Mentha spicata L. and *Mentha arvensis* L. (Lamiaceae)

Mentha spicata has been investigated as a potential treatment for mild hirsutism in women with PCOS, with two studies demonstrating its potential to reduce androgen levels (32,33).

Akdoğan et al. (2007) investigated the effects of *M. spicata* tea prepared from dried leaves on androgen levels in hirsute women. Twenty-one participants (12 with PCOS and 9 with idiopathic hirsutism) consumed *M. spicata* tea twice daily for 5 days during the follicular phase. After treatment, free testosterone levels decreased significantly, whereas LH, FSH, and estradiol levels increased markedly, and total testosterone and dehydroepiandrosterone sulfate (DHEA-S) levels showed no significant change. The authors suggested M. spicata could serve as a mild natural antiandrogen for hirsutism, warranting further research (32).

In a 1-month clinical trial, Grant (33) demonstrated that *M. spicata* tea significantly reduced free and total testosterone levels in women with PCOS-induced hirsutism, while concurrently elevating LH and FSH levels. Subjective assessments of hirsutism, evaluated via the modified Dermatology Life Quality Index (DLQI), showed significant improvement in the treatment group, while objective Ferriman-Gallwey scores did not decrease significantly. Despite only partially meeting the primary clinical endpoints, the study demonstrated significant changes in hormone levels, and the herbal tea was well-tolerated with no reported side effects (33).

In a rat model of estradiol valerate-induced PCOS, treatment with hydroalcoholic extracts prepared from dried leaves of *M. spicata* and ground seeds of *Linum usitatissimum* L. (Linaceae) for 30 days, starting 7 weeks after PCOS induction, led to a significant increase in serum progesterone and significant decreases in testosterone and estradiol levels compared to the untreated PCOS group. Conversely, no significant hormonal differences were found among the treatment, treatment-control, and control groups. Histologically, the treated group showed a significant increase in primary, pre-antral, and antral follicles, a reduction in cystic follicles, increased granulosa layer thickness, and decreased theca layer thickness relative to the PCOS group. These findings suggest that the combined *M. spicata* and *L. usitatissimum* treatment improves endocrine profiles and ovarian morphology in PCOS, possibly through phytoestrogenic effects, inhibition of 5-α reductase, increased sex hormone-binding globulin (SHBG), and modulation of key enzymes such as aromatase, which collectively may help alleviate hyperandrogenic symptoms like hirsutism and acne (39).

A recent preclinical study further supports these findings, demonstrating that *M. spicata* extract and its major constituent, carvone, improved hormonal imbalances, metabolic dysfunction, and ovarian inflammation in a letrozole-induced PCOS rat model. Treatment suppressed overactivation of the sterol regulatory element-binding protein 1 (SREBP1) and Toll-like Receptor 4 (TLR4) pathways, thereby restoring key reproductive and metabolic parameters (40). This suggests an additional mechanistic basis for the potential therapeutic effects of *M. spicata* in PCOS. Sadeghi Ataabadi et al. also showed that M. spicata effectively reduces testosterone levels (41).

In a rat model of PCOS induced by daily subcutaneous injections of testosterone enanthate (250 mg/kg) for 3 weeks, administration of *Mentha arvensis* hydroalcoholic extract resulted in a significant reduction in LH and FSH levels. This effect was most pronounced at the highest dose (200 mg/kg), where hormone levels approached those observed in healthy controls. The extract was prepared from whole, dried, and ground *M. arvensis* plants and processed into a hydroethanolic extract with 80% ethanol. After 4 weeks of treatment with the extract, blood samples were collected for hormone analysis. This highest concentration also notably decreased the number of ovarian cysts compared to the untreated PCOS group. Additionally, there was a significant increase in total antioxidant capacity in the treated PCOS rats. At the molecular level, *M. arvensis* treatment downregulated the expression of *Cyp17* and *Ptgs2* genes in ovarian tissue, which are implicated in androgen production and inflammation. These effects collectively suggest that *M. arvensis* can improve hormonal imbalances, reduce cyst formation, and enhance ovarian antioxidant defenses in PCOS (42).

Crocus sativus L. (Iridaceae)

Crocus sativus, commonly known as saffron, has been investigated for its potential therapeutic effects in PCOS and type 2 diabetes. Several studies have examined its effects on metabolic parameters, hormonal profiles, and ovarian function in women with PCOS (43–47). In a randomized, double-blind, placebo-controlled clinical trial, obese women with type 2 diabetes received either 200 mg/day of saffron powder or a placebo for 12 weeks, alongside aerobic training 3 times a week. The study demonstrated that,

compared with placebo, *C. sativus* supplementation, particularly when combined with exercise, significantly reduced body weight, BMI, body fat percentage, fasting glucose levels, and homeostatic model assessment of insulin resistance (HOMA-IR). Additionally, *C. sativus* intake led to significant decreases in inflammatory markers, including IL-6 and TNF-α, and improved hemostatic parameters, including fibrinogen and homocysteine levels. These findings suggest that *C. sativus* supplementation, particularly in combination with exercise, may improve metabolic, inflammatory, and hemostatic profiles in obese women with type 2 diabetes (46).

A recent systematic review and meta-analysis summarized findings from four human and three animal primary studies investigating the effects of *C. sativus* in PCOS (43). Across human trials, *C. sativus* supplementation was associated with reductions in body weight, BMI, waist circumference, fasting insulin levels, and insulin resistance. In animal models, studies reported improvements in ovarian morphology, reductions in cystic follicles, and decreases in testosterone levels (47).

Moshfegh et al. reported elevated levels of IL-6, TNF-α, IL-1β, IL-18, and CRP in the testosterone enanthate-induced PCOS rat group, consistent with previous findings. Treatment with *C. sativus* petal extract significantly decreased these inflammatory markers, suggesting the anti-inflammatory potential of flavonoids, tannins, and anthocyanins present in the petals. *C. sativus* exerted beneficial effects by modulating metabolic and hormonal pathways, including improving insulin sensitivity through the PI3K/Akt pathway and reducing oxidative stress and inflammation via its antioxidant properties. The study also showed that *C. sativus* extract and *C. sativus* petal anthocyanins decreased LH, estrogen, and testosterone levels, increased FSH levels, and enhanced the activities of antioxidant enzymes (GPx, SOD, CAT, GST, and GSH), collectively contributing to the amelioration of PCOS symptoms in mice (43,44).

C. sativus is widely recognized for its versatile botanical supplement properties. A network pharmacology study explored its potential in managing PCOS by identifying key targets, including AKT1, MAPK1, MAPK3, and STAT3. Subsequent molecular docking simulations suggest that *C. sativus* could potentially address PCOS-related insulin resistance and metabolic disorders (45).

C. sativus and its extracts have shown promising results in improving various aspects of PCOS, including metabolic parameters, hormonal profiles, and ovarian function. However, more research is needed to fully understand the mechanisms of action and establish the optimal dosage and duration of *C. sativus* supplementation for PCOS management (43–45).

Cinnamomum verum J.Presl (Lauraceae)

Cinnamomum verum, commonly known as cinnamon, has been extensively studied for its therapeutic effects in PCOS. Several clinical trials have evaluated its impact on metabolic parameters, hormonal profiles, insulin sensitivity, and ovarian function in women with PCOS (24).

In a clinical trial by Borzoei et al. (2017), 84 women with PCOS were evaluated over 8 weeks to determine the effects of bark powder from Cinnamomum zeylanicum Blume (Lauraceae), which is recognized as a synonym of *C. verum* J.Presl by World Flora Online. Participants who received *C. zeylanicum* capsules containing approximately 500 mg of cinnamon powder showed decreased levels of total cholesterol and low-density lipoprotein (LDL), alongside increased levels of high-density lipoprotein (HDL). Additionally, the *C. zeylanicum* group exhibited a significant increase in total antioxidant capacity, with no significant change in malondialdehyde levels. Although triglyceride levels did not significantly decrease, *C. zeylanicum* supplementation was associated with improved lipid profiles and antioxidant status (48).

Similarly, a study by Kort and Lobo (2014) investigated the effects of cinnamon supplementation on menstrual cyclicity in women with PCOS for 6 months. The results revealed a significant improvement in menstrual cycle regularity, suggesting cinnamon's role in modulating reproductive function. However, no notable changes were observed in insulin resistance markers, serum androgen levels, or body weight (49).

Hajimonfarednejad et al. (2017) found that 12 weeks of cinnamon powder supplementation reduced fasting insulin levels and improved insulin sensitivity in women with PCOS. In this double-blind trial, the treatment group received 500 mg capsules containing pure cinnamon derived from bark, while the parallel control group received capsules containing 50 mg of cinnamon powder and 450 mg of starch. The study also noted reductions in waist circumference, BMI, and LDL cholesterol, highlighting cinnamon's potential in managing both the metabolic and reproductive aspects of PCOS (50). Cinnamon improves insulin sensitivity in women with PCOS by activating the insulin receptor and increasing glucose uptake in muscle cells, while concurrently reducing systemic inflammatory markers, such as TNF-α and IL-6 (49,50).

C. verum has demonstrated promising effects in regulating

menstrual cycles, improving lipid profiles, and enhancing insulin sensitivity in women with PCOS. However, further research is required to understand its long-term effects and precise molecular mechanisms of action.

Trigonella foenum-graecum L. (Fabaceae)

Trigonella foenum-graecum, commonly known as fenugreek, has been widely investigated for its beneficial effects in the management of PCOS. Studies have explored its role in improving ovarian function, regulating menstrual cycles, and enhancing fertility in women with PCOS (24).

Swaroop et al. (2015) evaluated the effects of *T. foenum-graecum* seed extract (Furocyst) in 50 premenopausal women with PCOS over 90 days. The extract was produced using a patent-pending water-ethanol extraction process enriched with approximately 40% furostanolic saponins and administered as 500 mg capsules per day. The results showed a significant reduction in ovarian volume in all participants and a decrease in cyst size in 46% of the women, with complete cyst dissolution in 36% of cases. Additionally, regular menstrual cycles returned in 71% of the women, and 12% successfully conceived during the study. Significant increases in LH and FSH levels were observed compared to baseline. The study highlighted fenugreek's potential to improve ovarian function, regulate hormonal imbalances, and address PCOS-related infertility without significant adverse effects (51).

Similarly, another study by Bashtian et al. (2013) found that hydroalcoholic extracts of *T. foenum-graecum* seeds normalized menstrual cycles in women with oligomenorrhea caused by PCOS. Participants treated with *T. foenum-graecum* showed a significant decrease in polycystic ovaries on ultrasound scans, indicating improved ovarian health. Although the study reported no significant changes in BMI, insulin resistance, or testosterone levels, the positive effects on menstrual regularity and ovarian function were notable (52).

Apium graveolens L. (Apiaceae)

Apium graveolens, commonly known as celery, has been investigated for its regulatory effects on hormonal balance and menstrual cycles, particularly in women with PCOS. In an evaluation using a letrozole-induced PCOS rat model, Khosrowpour et al. (2022) utilized the dried fruits of *A. graveolens* as part of a multi-herbal formulation (53). This plant contains several bioactive compounds, including flavonoids (such as apigenin), essential oils, coumarins, and phenolic acids, which are believed to contribute to its therapeutic potential in reproductive health (53). Among these, apigenin, a flavonoid found abundantly in *A. graveolens*, has attracted attention for its potential to modulate hormone-related pathways. Studies suggest that apigenin may influence estrogen receptor activity, contributing to hormonal balance and improving menstrual regularity. Furthermore, apigenin has also been noted for its anti-inflammatory, antioxidant, and anti-androgenic properties, which can help mitigate symptoms of PCOS, such as hyperandrogenism and menstrual irregularities (53).

In a triple-blind randomized clinical trial, treatment with a combination of *A. graveolens* and *Pimpinella anisum* L. (Apiaceae) demonstrated superior clinical outcomes compared to metformin in obese patients with PCOS. Specifically, the combination of *A. graveolens* and *P. anisum*, administered as 750 mg capsules 3 times daily for 15 days per cycle over three consecutive cycles, achieved a 58.3% improvement in oligomenorrhea, compared to 25.0% in the metformin cohort ($p < 0.01$). The intervention also significantly increased menstrual bleeding episodes ($p = 0.003$) and induced marked reductions in both serum testosterone levels (mean difference: 0.16 vs. −0.02 ng/mL, $p = 0.005$) and the LH/FSH ratio (mean difference: 0.75 vs. −0.08, $p = 0.002$). Furthermore, the herbal therapy exhibited a favorable safety profile with no major adverse events reported. Phytochemical analysis suggests that the synergistic interaction between apigenin from *A. graveolens* and anethole from *P. anisum* may contribute to hormonal regulation by exerting anti-androgenic activity and modulating gonadotropin secretion (54).

Another study by Khodaeifar et al. (2019) explored the hydroalcoholic extract of *A. graveolens* and *C. zeylanicum*, particularly apigenin, in a rat model of PCOS induced with estradiol valerate. The findings indicated that in the therapy groups, blood sugar, insulin, and lipid profiles in plasma decreased significantly, while serum HDL levels were enhanced, and oxidative stress was reduced in treated groups (55).

In addition to apigenin, *A. graveolens* essential oils, such as limonene and selinene, have been identified as contributors to its anti-inflammatory and antispasmodic properties, which could help alleviate menstrual discomfort and improve reproductive health in women with PCOS (53).

Lepidium meyenii Walp. (Brassicaceae)

Lepidium meyenii, commonly referred to as maca, has attracted interest for its potential benefits in managing PCOS. *L. meyenii*, a member of the Brassicaceae family, is traditionally consumed for its purported effects on fertility, energy levels, and hormonal balance (56–58).

Recent studies have highlighted the antioxidant properties of the dried hypocotyl of *L. meyenii* as a key factor in its therapeutic potential for PCOS. According to Alarcón-Yaquetto et al. (2021), oxidative stress is a significant component of PCOS pathology, characterized by an imbalance between ROS and antioxidant defenses. *L. meyenii* has been shown to enhance total antioxidant capacity, which may mitigate the oxidative stress associated with this condition (56).

Recent analyses of *L. meyenii* extracts indicate that its antioxidant activity is primarily attributed to alkaloids and phenolic compounds, with alkaloids showing a somewhat stronger contribution. These findings help clarify the basis of the antioxidant effect of *L. meyenii*, although they are derived from laboratory analyses and do not represent clinical evidence in PCOS (57).

Furthermore, specific phenotypes of *L. meyenii*, particularly red *L. meyenii*, have been associated with anti-inflammatory and reproductive benefits. Research indicates that red *L. meyenii* can enhance ovarian function and improve overall reproductive health in women with PCOS (56). The active plant part of *L. meyenii* is its hypocotyl-root axis. This is particularly relevant as PCOS is often accompanied by chronic low-grade inflammation, which can exacerbate metabolic and hormonal imbalances (58).

Tribulus terrestris L. (Zygophyllaceae)

Tribulus terrestris, commonly known as puncture vine, has been traditionally used in various cultures for its purported benefits on reproductive health, including the management of female infertility and menstrual disorders (59). In an animal study with the observation periods divided into 7 and 14 days, administration of pure *T. terrestris* extract to immature female rats significantly increased the number and diameter of the corpus luteum, thickness of the theca-interna layer, and the number of secondary and Graafian follicles, indicating a stimulatory effect on folliculogenesis and corpus luteum formation through an LH-like activity (60). These findings suggest that *T. terrestris* may support the initiation of puberty and ovulatory cycles by mimicking or enhancing endogenous gonadotropin activity (60). Recent comprehensive reviews have highlighted that the secondary metabolites of *T. terrestris*, particularly steroidal saponins like protodioscin, are responsible for its positive effects on the female reproductive system, including improvements in ovarian histology, modulation of sex hormone levels, and enhancement of ovulation (61). Additionally, clinical and preclinical studies have demonstrated that *T. terrestris* supplementation can promote regular ovulation, reduce ovarian cysts, and restore hormonal balance in PCOS models, with evidence of improved menstrual regularity and reduced hyperandrogenic symptoms (59,61). The herb also exhibits antioxidant properties, which may help mitigate oxidative stress associated with PCOS and further support ovarian function (62). Overall, while more robust clinical trials are needed, current evidence supports the potential of an ethanolic fruit extract of *T. terrestris* as a complementary approach for improving ovarian health and managing PCOS-related symptoms in women (62).

The therapeutic effects of *T. terrestris* in PCOS are attributed to several mechanisms (59). One proposed mechanism is the normalization of hormonal balance and induction of ovulation through antiestrogenic action, which helps restore regular estrous cycles and ovulatory function in PCOS models (59). Studies in animal models have shown that *T. terrestris* extract can reverse letrozole-induced hormonal disturbances by increasing FSH levels and decreasing LH, estradiol, and testosterone levels, thereby promoting follicular development and reducing ovarian cysts (60,61). Additionally, *T. terrestris* has demonstrated significant effects on metabolic parameters associated with PCOS (62). It enhances insulin sensitivity by modulating metabolic factors, including p-Akt, GLUT8, and insulin receptor expression, in ovarian tissue. This leads to lower serum glucose and insulin levels, thereby improving insulin resistance. The herb also exerts antioxidant effects, reducing oxidative stress in ovarian tissue, which is crucial for normal folliculogenesis and ovulation (60,62).

Curcuma longa L. (Zingiberaceae) and *Phyllanthus emblica* L. (Phyllanthaceae)

The combination of *Curcuma longa* and *Phyllanthus emblica* has been investigated for its potential benefits in managing PCOS as a common endocrinopathy associated with insulin resistance, hyperandrogenism, and ovarian dysfunction (63,64). These plants, known for their antioxidant, anti-inflammatory, and insulin-sensitizing properties, have been traditionally used in Ayurvedic medicine to manage conditions such as diabetes, and their combination has shown promise in addressing the metabolic and hormonal disturbances associated with PCOS (65,66). In an open-label, randomized, active-controlled exploratory clinical study, Gupte et al. (2023) evaluated the effects of a combination of *C. longa* (rhizome) and *P. emblica* (fruit) prepared with either a traditional method (TF—Traditional Formulation) or a standardized extraction method (PNAE—Pharmanza Nisha Amalaki Extract) on glucose metabolism, inflammatory markers, and

reproductive hormones in women with PCOS. Participants were randomized into groups and received either traditional formulations or standardized extracts of *C. longa* and *P. emblica*, both with or without metformin, for 90 days. The study results indicated improvements in several key markers associated with PCOS (63).

The molecular mechanisms underlying the effects of *C. longa* and *P. emblica* in PCOS involve the modulation of key pathways related to insulin sensitivity, inflammation, and hormone regulation. Curcumin, the active compound in *C. longa*, activates PPAR-γ, a nuclear receptor that enhances insulin sensitivity and suppresses pro-inflammatory cytokines such as IL-6 and TNF-α, which are elevated in PCOS. Additionally, *P. emblica* has been shown to influence the AMPK pathway, improving glucose uptake and metabolic homeostasis, while reducing oxidative stress, a contributor to PCOS-related inflammation and metabolic dysfunction (63).

Taraxacum officinale F.H.Wigg. (Asteraceae)

Taraxacum officinale, commonly known as dandelion, is recognized for its diverse bioactive compounds, including polysaccharides, phenolic acids, and phytosterols, which contribute to its antihyperglycemic and anti-inflammatory properties (67). *T. officinale* root extracts showed no glucose-lowering effect in normoglycemic mice, but the aqueous extract exhibited clear antidiabetic activity in alloxan-induced mice, although weaker than metformin (68).

Beyond metabolic effects, *T. officinale* has been studied for its influence on ovarian cell biology. *In vitro* research using human ovarian granulosa cells found that *T. officinale* leaf extract promotes cell proliferation, upregulates the expression of hormone receptors, including *FSH, LH*, and *IGF-1* receptors, and enhances estradiol and progesterone secretion (69). These findings suggest that *T. officinale* may support ovarian steroidogenesis and endocrine function, which are often impaired in PCOS (69).

Although direct clinical evidence in women with PCOS is currently lacking, the demonstrated antidiabetic, antioxidant, and ovarian-supportive effects of *T. officinale* highlight its promise as a complementary agent for addressing insulin resistance, hormonal imbalance, and reproductive dysfunction associated with PCOS (69).

Nigella sativa L. (Ranunculaceae)

Nigella sativa (black seed, medicinal part: seed (70)) has been traditionally used in Middle Eastern and South Asian medicine for its various therapeutic properties. Its bioactive components, especially thymoquinone, possess potent antioxidant, anti-inflammatory, and insulin-sensitizing effects, making it a promising candidate for managing PCOS (71). *N. sativa* is also effective in improving gonadotropins and sex hormones, which are commonly disrupted in women with PCOS.

A randomized controlled trial involving 103 adolescent girls with PCOS demonstrated that daily supplementation with 1000 mg of *N. sativa* extract (plant part not specified) for 16 weeks resulted in significant clinical and hormonal improvements compared to a control group receiving medroxyprogesterone (72). The intervention group exhibited a significant modulation of serum reproductive hormones, including notable reductions in LH and testosterone levels, as well as a decrease in bilateral ovarian volume (72). Additionally, participants experienced an alleviation of clinical symptoms, characterized by reduced hirsutism severity and improvements in menstrual cycle irregularities such as oligomenorrhea and amenorrhea (72). These findings suggest that short-term *N. sativa* supplementation may effectively modulate hormonal imbalances and alleviate menstrual disturbances in adolescents with PCOS, likely due to the phytoestrogen compounds present in the extract (72).

Mahmoudian et al. conducted a randomized controlled trial assessing the effects of *N. sativa* supplementation on glycemic control in adolescent girls with PCOS. The study found that 16 weeks of *N. sativa* supplementation resulted in significant reductions in fasting plasma glucose and 2-hour postprandial glucose levels compared to the control group. These findings suggest that *N. sativa* may improve glycemic parameters in adolescents with PCOS (73).

Beyond hormonal and metabolic regulation, *N. sativa* has demonstrated benefits in improving reproductive cell quality. An experimental study using a PCOS-induced mouse model revealed that administration of *N. sativa* hydroalcoholic extract significantly enhanced oocyte maturation, fertilization, and blastocyst formation rates (74). These improvements were associated with elevated glutathione levels, increased glutathione peroxidase expression, and reduced reactive oxygen species in oocytes. Additionally, the extract upregulated genes involved in oocyte development and epigenetic regulation, including *Dnmt1, Hdac1, Mapk*, and *Cdk1*, while downregulating pro-inflammatory markers like *Cox2* (74). These findings support the potential of *N. sativa* to enhance reproductive competence in PCOS beyond systemic effects. Furthermore, recent evidence suggests that *N. sativa* may influence gene expression involved in oocyte maturation and epigenetic regulation, contributing to improved reproductive outcomes in PCOS (74).

Further insights come from an experimental letrozole-induced PCOS rat model, where thymoquinone, as a key bioactive component of *N. sativa*, was administered after PCOS induction (75). Rats received letrozole for 28 days, followed by intraperitoneal thymoquinone at 5 mg/kg or 10 mg/kg every 3 days for 30 days. The treatment restored disrupted folliculogenesis, reduced oxidative stress and apoptosis, and normalized hormone levels. These findings suggest that thymoquinone may improve ovarian function primarily through antioxidant and anti-apoptotic mechanisms, positioning N. sativa as a promising natural adjunct in PCOS management (75).

Vitex negundo L. (Lamiaceae)

Vitex negundo, commonly known as the five-leaved chaste tree, has been traditionally used to treat gynecological disorders, with recent preclinical research supporting its potential for managing PCOS. In a letrozole-induced PCOS rat model, both aqueous and hydroalcoholic extracts of *V. negundo* seeds (200–400 mg/kg) were administered over a total study period of 66 days, including PCOS induction and treatment phases. The extracts significantly restored regular estrous cyclicity, normalized the LH/FSH ratio by reducing elevated LH levels and increasing reduced FSH levels, and improved serum concentrations of estrogen and progesterone. Histological analysis showed decreased ovarian cysts and improved follicular development after treatment. In addition, treatment ameliorated hyperglycemia and dyslipidemia, suggesting beneficial metabolic effects, with aqueous extracts showing greater efficacy. Importantly, toxicity markers (SGPT, SGOT, creatinine) remained within the normal range even after prolonged administration, indicating a favorable safety profile (76).

A recent study by Kar et al. (2024) further supports the therapeutic potential of *V. negundo* seeds in PCOS management. Administration of the hydroethanolic extract of *V. negundo* seeds in a letrozole-induced PCOS rat model not only improved hormonal profiles but also reduced oxidative stress markers such as superoxide dismutase and catalase, and lowered inflammatory cytokines including TNF-α and IL-6. Treatment with *V. negundo* seeds resulted in a significant reduction in body weight compared to untreated PCOS rats, which exhibited marked weight gain after letrozole induction ($p < 0.001$). *V. negundo* seeds also modulated gonadotropin levels and corrected the LH/FSH ratio by regulating steroidogenic enzymes, ER-α signaling, and downregulating *NR3C4* expression, contributing to restored hormonal balance. Molecular docking analyses confirmed interactions between active phytochemicals—cinnamic acid, plumbagin, and nigundin B—and hormone/inflammatory signaling pathways. In addition to improvements in follicular morphology and reduced theca-layer thickness, histomorphological evaluation of the ovaries evidenced pronounced amelioration of PCOS pathology. These findings highlight *V. negundo* seed extract as a multifaceted, holistic therapeutic candidate for PCOS, owing to its antiandrogenic, antioxidant, and anti-inflammatory actions driven by key phytocompounds (77).

The pharmacological activity of *V. negundo* appears to be mediated through multiple mechanisms. Phytochemical screening revealed the presence of flavonoids and triterpenoids, known for their antioxidant and anti-inflammatory effects (76,77). The flavonoid-rich extracts are believed to exert antiandrogenic effects and modulate steroidogenic enzymes, potentially lowering intra-ovarian androgen levels (76). Additionally, improvements in insulin sensitivity and glucose tolerance were observed, suggesting an insulin-sensitizing effect which may be key in mitigating metabolic complications of PCOS (76,77). Collectively, these effects contribute to the normalization of endocrine function and ovarian morphology, making *V. negundo* a promising natural alternative in PCOS therapy.

Vitex agnus-castus L. (Lamiaceae)

Vitex agnus-castus, or chaste tree, has been traditionally used to regulate female reproductive hormones. Findings of both preclinical and clinical studies support its application in PCOS treatment. In a letrozole-induced PCOS rat model, administration of 365 mg/kg of *V. agnus-castus* ethanolic fruit extract for 30 days led to normalization of serum LH and testosterone, while increasing FSH, estradiol, and progesterone levels. Ovarian histology confirmed reduced cystic follicles, the reappearance of corpora lutea, and normalized theca and granulosa layers. Importantly, the extract significantly downregulated hypothalamic *KISS1* gene expression, which was previously elevated in the PCOS model, suggesting neuroendocrine modulation via the hypothalamic-pituitary-gonadal axis (78).

In addition to these findings in animals, a systematic review of randomized controlled trials showed that *V. agnus-castus* significantly improved premenstrual syndrome (PMS) symptoms, outperforming a placebo, vitamin B6, and magnesium in multiple trials (79).

V. agnus-castus contains dopaminergic diterpenes, such as casticin, that act as D2 receptor agonists in the anterior pituitary, thereby reducing prolactin secretion. This action supports luteal phase function and regulates the hypothalamic-pituitary-gonadal axis. Additional flavonoids

and iridoid glycosides possess anti-inflammatory and hormone-modulating properties (78,79).

Glycyrrhiza glabra L. (Fabaceae)

Glycyrrhiza glabra, commonly known as licorice (medicinal part: root/radix (80)), has demonstrated significant therapeutic potential in PCOS management due to its antiandrogenic, antioxidant, and hormonal regulatory properties. In a letrozole-induced PCOS rat model, oral administration of the methanolic extract of *G. glabra* (300 mg/kg/day for 21 days) significantly reduced LH and testosterone levels while increasing FSH, estradiol, and progesterone levels. Histopathological evaluation revealed a reduction in cystic follicles and a greater presence of corpora lutea and developing follicles, suggesting the restoration of ovulation. Additionally, *G. glabra* improved uterine and ovarian weights and normalized estrous cyclicity, confirming its broad reproductive benefits in experimental models (81).

The clinical efficacy of *G. glabra* has also been supported by human trials. In a randomized, double-blind, placebo-controlled study, *G. glabra* root extract administered alongside spironolactone for 8 weeks in women with PCOS enhanced the antiandrogenic effect of spironolactone, leading to a greater reduction in serum testosterone levels and improvements in hirsutism and acne. Importantly, this combination did not cause significant side effects, suggesting *G. glabra* may be a safe and effective adjunct therapy in the clinical management of hyperandrogenism (82).

Furthermore, *G. glabra* in combination with *Paeonia spp*. L. (Paeoniaceae) has been traditionally used in Eastern medicine for hormonal regulation. In a DHEA-induced PCOS mouse model, treatment with the *G. glabra–Paeonia* combination (50 mg/kg and 100 mg/kg) significantly reduced testosterone and fasting insulin levels and improved ovarian morphology by increasing the number of healthy primary and Graafian follicles. Animals treated with the higher dose also showed signs of ovulation, such as the presence of corpora lutea and normalized estrous cycles. These effects were associated with fewer cysts and better overall ovarian health. The beneficial outcomes are thought to be due to the combined action of bioactive compounds in both plants, which helps balance hormones and support healthy follicular development (83).

The bioactivity of *G. glabra* is primarily attributed to its active compounds, including glycyrrhizin, liquiritigenin, and flavonoids, which exhibit potent antioxidant and anti-inflammatory effects. These constituents support hormone balance by modulating the hypothalamic-pituitary-gonadal (HPG) axis and regulating ovarian steroidogenesis. *G. glabra* also reduces androgen levels by inhibiting key enzymes involved in testosterone synthesis, such as 17-β-hydroxysteroid dehydrogenase and 17,20-lyase. When used alongside pharmacologic agents such as spironolactone or combined with other herbs such as *Paeonia* L., it may further enhance therapeutic outcomes, particularly in managing both hormonal and metabolic disturbances associated with PCOS (81,82,83).

Zingiber officinale Roscoe (Zingiberaceae)

Zingiber officinale rhizome extract and its major active constituent, 6-gingerol, exhibit significant therapeutic potential in managing PCOS through their antioxidant and anti-inflammatory properties. In a rat model of induced PCOS, administration of *Z. officinale* extract at doses of 100 mg/kg and 200 mg/kg and 6-gingerol at 200 µg/kg and 400 µg/kg for 28 days produced marked improvements in reproductive hormone balance compared with untreated PCOS controls. Treatment significantly reduced LH levels across all experimental groups and decreased FSH concentration, with the most pronounced effect observed at 400 µg/kg of 6-gingerol. Both *Z. officinale* extract and 6-gingerol significantly decreased serum estradiol and testosterone levels ($p < 0.001$), while increasing progesterone concentrations ($p < 0.05$). These hormonal modulations were accompanied by improved ovarian follicular development, suggesting restoration of ovulatory function. Collectively, the findings support the potential of *Z. officinale* and 6-gingerol as natural therapeutic agents for ameliorating PCOS through endocrine regulation and ovarian recovery (84).

Clinical evidence also supports the utility of *Z. officinale*. In a randomized controlled trial, a multi-herbal formulation containing *Z. officinale* (rhizome) significantly reduced LH, the LH/FSH ratio, total and free testosterone, and HOMA-IR, compared to clomiphene citrate alone. Improvements were also seen in lipid parameters, with reductions in total cholesterol, triglycerides, LDL, and VLDL, and an increase in HDL (85). Furthermore, another study using 6-gingerol, derived from *Z. officinale* rhizome, at 50 mg/kg and 100 mg/kg in rats showed normalization of estrous cyclicity, restoration of ovarian and uterine weights, decreased cystic follicles, and increased corpus luteum formation, along with improved levels of FSH, estrogen, and progesterone (86).

The pleiotropic effects of ginger in PCOS are mediated through several mechanisms. Gingerols, shogaols, and zingerone, its principal phenolic compounds, exert potent antioxidant effects by increasing GSH and SOD, while

reducing MDA levels, thus protecting ovarian tissues from oxidative damage (84,86). *Z. officinale* also downregulates pro-inflammatory markers such as COX-2 and likely TNF-α and IL-6, reducing ovarian inflammation (86). These actions help improve insulin sensitivity. Metabolically, ginger has antidiabetic properties, enhancing insulin secretion (partly through serotonin receptor stimulation on pancreatic β-cells), lowering blood glucose levels, and improving lipid metabolism (85,86). Additionally, it regulates the hypothalamic-pituitary-ovarian axis, leading to balanced gonadotropin secretion (lower LH/FSH ratio), normalized steroidogenesis, and restoration of ovulation (84,85,86).

Trifolium pratense L. (Fabaceae)

Trifolium pratense, commonly known as red clover, is a leguminous plant rich in isoflavones—phytoestrogens structurally similar to endogenous estrogens—which have demonstrated therapeutic potential in the management of PCOS due to their antioxidant, anti-inflammatory, and hormone-modulating effects. In a letrozole-induced PCOS rat model, administration of *T. pratense* extract (70% ethanolic macerate; plant part not specified in the article) at doses of 500 mg/kg and 750 mg/kg for 30 days significantly improved key reproductive parameters compared to untreated PCOS rats. The treatment led to decreases in ovarian weight, ovarian volume, and number of cysts, while increasing the number of healthy oocytes. Biochemically, *T. pratense* extract decreased serum testosterone and increased estradiol levels, indicating both anti-androgenic and estrogenic activity. Ovarian histopathology revealed restoration of normal follicular architecture and a reduction in cystic follicles, with improved granulosa and theca cell layers (87).

T. pratense also exerts potent antioxidant effects in PCOS models, as evidenced by increased levels of GSH, SOD, and CAT, and reduced levels of MDA and NO, which are markers of oxidative stress (87). *T. pratense* has been widely investigated in animals and in clinical trials, most notably for menopausal symptoms, but direct evidence in PCOS is limited. Beyond a single letrozole-induced rat study showing histological, hormonal, and antioxidative improvements with *T. pratense* extract, robust clinical trials in women with PCOS are still lacking. Well-designed randomized trials are therefore needed to define the efficacy, dosing, and safety of *T. pratense* specifically in PCOS.

The evidence summarized in this narrative review suggests that a variety of medicinal plants, including *C. sinensis, M. spicata, M. arvensis, C. sativus, C. verum, T. foenum-graecum, A. graveolens, L. meyenii, T. terrestris, C. longa, P. emblica, N. sativa, V. negundo, V. agnus-castus, G. glabra, Z. officinale*, and *T. pratense*, may offer potential benefits for women with PCOS. Findings from preclinical and clinical studies indicate that these plants may influence several key pathophysiological mechanisms involved in PCOS, including insulin resistance, hyperandrogenism, chronic inflammation, and oxidative stress, thereby contributing to improvements in metabolic, hormonal, and reproductive parameters.

From a clinical perspective, these findings highlight the potential role of medicinal plants as complementary therapeutic approaches in PCOS management, particularly for women seeking alternative or adjunct options alongside conventional pharmacological treatments. Many plant-derived compounds appear to target multiple biological pathways simultaneously, which may be particularly relevant given the complex and multifactorial nature of PCOS.

However, despite these promising findings, the currently available evidence remains limited and heterogeneous. Many studies involve small sample sizes, short intervention durations, and variability in plant preparations, dosages, and study designs. Furthermore, for several plants, the available data are primarily derived from preclinical or experimental models, while well-designed randomized controlled trials in humans remain relatively scarce.

Future research should therefore focus on conducting rigorously designed clinical trials, standardizing herbal preparations and dosages, and further elucidating the molecular mechanisms underlying the observed therapeutic effects. Such efforts are essential to better define the safety, efficacy, and clinical applicability of medicinal plants and to support their potential integration into evidence-based strategies for PCOS management.

Abbreviations

AKT1: AKT serine/threonine kinase 1
AMPK: AMP-activated protein kinase
CAT: Catalase
CC: Clomiphene citrate
COX-2 : Cyclooxygenase-2
CRP: C-reactive protein
EGCG: Epigallocatechin gallate
ER-α: Estrogen receptor alpha
FSH: Follicle-stimulating hormone
FSHR: Follicle-stimulating hormone receptor
GLUT8: Glucose transporter type 8
GPx: Glutathione peroxidase
GSH: Glutathione
GST: Glutathione S-transferase
HDL: High-density lipoprotein
HOMA-IR: Homeostatic Model Assessment of Insulin Resistance
HPG: Hypothalamic-pituitary-gonadal
IGF-1: Insulin-like growth factor 1 receptor
IL-1β: Interleukin-1 beta
IL-6: Interleukin-6
KISS1: Kisspeptin-1
LDL: Low-density lipoprotein
LH: Luteinizing hormone
LH/FSH: Luteinizing hormone to follicle-stimulating hormone ratio
LHR: Luteinizing hormone receptor
MAPK: Mitogen-activated protein kinase
MAPK1: Mitogen-activated protein kinase 1
MAPK3: Mitogen-activated protein kinase 3
MDA: Malondialdehyde
NO: Nitric oxide
NR3C4: Nuclear receptor subfamily 3 group C member 4 (androgen receptor gene)
p-Akt: phosphorylated Akt
PCOS: Polycystic ovary syndrome
PI3K: Phosphoinositide 3-kinase
PPAR-γ: Peroxisome proliferator-activated receptor gamma
RCT: Randomized controlled trial
ROS: Reactive oxygen species
SGOT: Serum glutamic-oxaloacetic transaminase (AST)
SGPT: Serum glutamic-pyruvic transaminase (ALT)
SOD: Superoxide dismutase
SREBP1: Sterol regulatory element-binding protein 1
STAT3: Signal transducer and activator of transcription 3
TNF-α: Tumor necrosis factor alpha
VLDL: Very-low-density lipoprotein

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Author Contributions

Conceptualization: G.Ž. and J.J.J.; Methodology: J.J.J., N.J., and S.R.; Formal analysis: S.R., N.J., J.J.J., and V.J.; Investigation: G.Ž., A.Ć., and R.Č.Ć.; Data curation: G.Ž., A.Ć., and R.Č.Ć.; Writing – Original Draft Preparation: G.Ž., A.Ć., and R.Č.Ć.; Writing – Review and Editing: V.J., S.R., N.J., and J.J.J.; Supervision: J.J.J. All authors have read and approved the published version of the manuscript.

Statement of Ethics

Not applicable. This study is an original review based exclusively on previously published literature and did not involve any direct interventions with human participants or animals.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availability

All data analyzed during this study were retrieved from previously published, publicly available, peer-reviewed articles and databases and are included in the cited literature.

Statement of Generative AI Technologies Use

No generative AI was used.

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RISK FACTORS FOR SPINAL ANESTHESIA-INDUCED HYPOTENSION DURING ELECTIVE CESAREAN SECTION

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Spinal anesthesia is widely considered the "gold standard" for elective cesarean sections due to its rapid onset and lower risk of complications compared to general anesthesia. However, hypotension is a common adverse effect, influenced by maternal and procedural factors. Despite various preventive measures, predicting hypotension based on patient characteristics remains challenging. This study investigated the relationship between hypotension and variables such as age, BMI, height, preoperative fasting, and hypertension during pregnancy. A retrospective analysis was conducted on 123 patients who underwent elective cesarean section under spinal anesthesia between April and August 2023 at the Clinic of Gynecology and Obstetrics, Novi Sad. Demographic data, anesthetic dose, timing of surgery, and blood pressure trends were collected. Patients were categorized according to delivery time, height, occurrence of hypotension, and history of hypertension. Statistical analyses included Student's t-test, Mann–Whitney U, Shapiro–Wilk, chi-square tests, ANOVA, and correlation analysis. Hypotension occurred in 45.5% of patients. Older age was significantly associated with a higher incidence of hypotension ($p = 0.031$), whereas hypertension during pregnancy correlated with a lower incidence ($p = 0.032$). Height, BMI, and surgery timing had no significant impact on hypotension risk. Taller patients received a larger volume of anesthetic ($p = 0.005$), with a moderate correlation between height and dose ($r_s = 0.427$, $p < 0.001$); however, anesthetic dose was not predictive of hypotension. Most hypotensive events occurred within 10 minutes following spinal anesthesia. In conclusion, hypotension was associated with older age, while hypertension during pregnancy appeared to have a protective effect. No significant associations were found with height, BMI, or fasting duration. These findings highlight the need for individualized monitoring and larger-scale studies to optimize spinal anesthesia management in cesarean delivery.

Keywords: spinal anesthesia, hypotension, cesarean delivery

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INTRODUCTION

Neuraxial analgesia (spinal, epidural, or combined spinal epidural anesthesia) is the "gold standard" for labor pain relief (1). The choice of anesthetic technique for cesarean delivery is based on the safety and health benefits to the mother and fetus, the experience level of the practitioner, and the availability of drugs and equipment (2). Spinal anesthesia has been favored as the best choice for elective, uncomplicated cesarean deliveries (1,2,3). Compared to epidural anesthesia, spinal anesthesia allows for earlier onset of surgery and requires approximately ten times less anesthetic to achieve a comparable level of anesthesia for a cesarean section (3). It offers better health-related outcomes than general anesthesia, minimizing complications such as failed intubation, failed ventilation, and aspiration of gastric contents; moreover, maternal mortality is significantly reduced when general anesthesia is avoided (2,4). Potential adverse effects common to both spinal and epidural anesthetic techniques include: maternal hypotension, failure to provide adequate anesthesia, post-dural puncture headache (PDPH), which may be associated with meningitis, intracranial hemorrhage, subdural hematoma, itching, and transient neurologic symptoms over the injection site (3,5).

Among the reported side effects, hypotension is the most common, with incidence varying widely across studies, ranging from 7.4% to 74.1% (6). Spinal block-induced sympatholysis causes arterial and venous vasodilatation, leading to a decreased systemic vascular resistance (6,7). The incidence of hypotension may be influenced by a range of environmental and individual factors, including hypovolemia, preoperative hypertension, block level related to vertebral column height, age over 40 years, obesity, and the use of combined general and spinal anesthesia (8,9). Some studies have suggested that anesthetic dosing may be adjusted based on patient characteristics, although this approach remains under ongoing investigation (10). While some anesthetists prefer a fixed dosage regimen, others tailor the anesthetic dose according to patient-specific characteristics such as height, weight, and body mass index (10,11). However, despite their frequent use, these parameters have shown limited reliability in accurately predicting the spread of spinal anesthesia (11,12).

A systematic review by Klöhr et al. revealed significant variability in how spinal anesthesia-induced hypotension is defined. Among 63 studies, they identified 15 different definitions, with the most common being a drop to 80% of baseline systolic arterial pressure (SAP), or a combination of SAP ≤ 100 mmHg and ≤ 80% of baseline (6). Baseline blood pressure is commonly defined as the initial reading obtained upon arrival in the operating room (13). A consensus published in 2018 recommends maintaining SAP at or above 90% of baseline to reduce maternal side effects (14).

Spinal anesthesia-induced hypotension is commonly associated with maternal nausea and vomiting, which may lead to aspiration of gastric contents. In rare cases, it can result in cardiovascular collapse, loss of consciousness, cardiac arrest, and even maternal death (15,16). If prolonged, maternal hypotension can impair uteroplacental blood flow, potentially leading to neonatal acidosis and, in severe cases, neurological injury or intrauterine fetal death (15). However, the extent to which reduced uteroplacental perfusion affects fetal outcomes remains a subject of ongoing debate. Nonetheless, there is a broad consensus on the importance of closely monitoring hemodynamic changes to ensure the well-being of both mother and fetus, as well as the value of implementing preventive measures against hypotension (6,16,17).

In order to prevent the nearly inevitable hypotension and other known side effects associated with spinal anesthesia, a range of strategies has been developed (17, 18). Leg wrapping for venodilation and left lateral tilt can help alleviate aortocaval compression caused by the gravid uterus on the abdominal aorta and inferior vena cava, which has been associated with reduced hemodynamic changes (18,19). Antiemetic prophylaxis is typically administered after clamping the umbilical cord to reduce nausea and vomiting (20). Although intravenous fluid loading has been tested and found to be only marginally effective, IV co-loading (administering fluids in parallel with the spinal block) appears to be the most effective strategy (18). The type of intravenous fluid (crystalloid or colloid) used is not a primary factor causing hypotension, allowing staff to select fluids based on the patient's individual needs while focusing on other contributing factors (21). The use of vasopressors is crucial in managing spinal hypotension, with phenylephrine currently the vasopressor of choice to compensate for arteriolar vasodilation (18,22). The use of a low dose of spinal bupivacaine has been associated with a reduction in maternal side effects. However, careful dose selection is crucial, as insufficient anesthesia can lead to the need for general anesthesia, increasing the risk of maternal and neonatal complications (23).

The aim of this study was to examine the association between risk factors and the occurrence of hypotension in pregnant women who had an elective cesarean section under spinal anesthesia. The following hypotheses were proposed: that pregnant women with shorter body height experience hypotension more frequently during elective cesarean section under spinal anesthesia, that the duration of preoperative fasting affects the occurrence of hypotension during elective cesarean section under spinal anesthesia, and that participants diagnosed with arterial hypertension during pregnancy experience hypotension less frequently during elective cesarean section under spinal anesthesia.

METHODS

Study design

This retrospective study analyzed data from the medical records of pregnant women who underwent elective cesarean section under spinal anesthesia at the Clinic of Gynecology and Obstetrics in Novi Sad between April and August 2023. It included 123 pregnant women whose cesarean deliveries were completed entirely under spinal anesthesia. Cases where surgery was initiated under spinal anesthesia but converted to general anesthesia were excluded. Data were obtained from photocopies of anesthesia records. Demographic data collected included patient age, height, weight, and Body Mass Index (BMI). For each patient, the level of the spine at which the local anesthetic was administered and the total dose given were recorded. Based on the surgical schedule and operation time, patients were grouped by delivery time intervals: 8:00 AM–10:00 AM, 10:00 AM–12:00 PM, and after 12:00 PM. Those operated on later had a longer preoperative fasting period, which was a relevant variable in this study. Anesthesia records were also used to collect data on arterial blood pressure at the time of entering the operating room, as well as the lowest recorded systolic, diastolic, and mean arterial pressure during surgery. For patients who experienced a drop in blood pressure below 90 mmHg, the time elapsed between administration of the local anesthetic and the onset of hypotension was noted. It was also recorded whether the patient had been diagnosed with hypertension during pregnancy. In hypotensive cases, the amounts of phenylephrine and adrenaline administered by the anesthesiologist were documented. Data were compiled in a Microsoft Excel (Office 2019) database. This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Clinical Center of Vojvodina (approval number 00-113, issued on 23 June, 2023).

Statistical analysis

All data were analyzed using JASP 0.17.2.1. and IBM® SPSS® Statistics 26. Within the sample of 123 patients, groups were formed based on height: patients taller than 165 cm were classified as the tall group (Group V), while those shorter than 165 cm were classified as Group M. Based on the lowest recorded systolic pressure during cesarean section, patients were divided into hypotensive (Group H), with systolic pressure below 90 mmHg, and normotensive (Group N), with systolic pressure remaining above 90 mmHg throughout the procedure.

Descriptive statistics were used to summarize all demographic data. For continuous variables with one categorical independent variable (with only two levels), Student's *t*-test was used when the Shapiro-Wilk test indicated a normal distribution. If the Shapiro-Wilk test showed deviation from normality, the non-parametric Mann–Whitney U test was applied. The chi-square test (χ^2) was used to assess differences in frequencies of categorical variables. Analysis of Variance (ANOVA) was used when there were three levels of an independent variable, or when there was more than one independent variable. Pearson's linear correlation coefficient (*r*) was applied for normally distributed continuous variables. When data deviated from a normal distribution, Spearman's rank correlation (r_s) was used instead. A p-value < 0.05 was considered statistically significant.

RESULTS

The study included 123 female patients. Group V (patients whose height was greater than 165 cm) consisted of 78 participants, while Group M (patients whose height was less than 165 cm) included 45 participants. In the group of pregnant women who had a blood pressure lower than 90 mmHg during surgery (Group H), there were 56 participants, while in 67 participants, the systolic blood pressure during surgery did not fall below 90 mmHg (Group N).

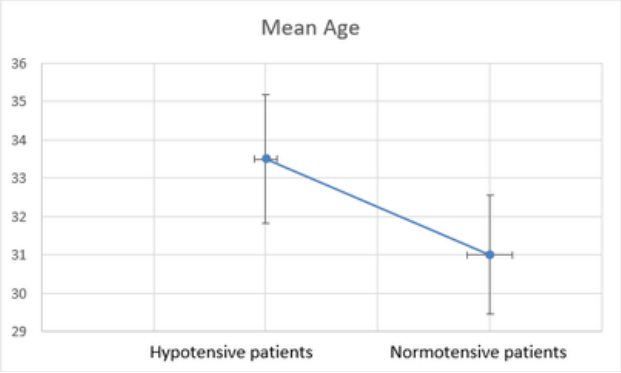

Figure 1. *Distribution of female patients according to age*

Relationship between patient age and the occurrence of hypotension during cesarean section

The average age of patients scheduled for an elective cesarean section was 32 years (SD = 6), with the youngest patient being 20 and the oldest 45 years old. In the hypotensive (H) group, the average age was 33.5 years (SD = 5.5), while in the normotensive (N) group, the average age was 31 years (SD = 6). Using Student's t-test, a statistically significant difference between the groups was found (t = 2.183, p = 0.031). On average, patients who experienced hypotension during surgery were older, as shown in Figure 1.

Association between body weight and the occurrence of hypotension during cesarean section

The average body weight of the participants was 85 kg (SD = 15), with a maximum of 126 kg and a minimum of 57 kg. However, the average weight within the group of hypotensive and normotensive patients differed. Among hypotensive patients, the average weight was 87.2 kg (SD = 15.3), while in normotensive patients, the average weight was lower, at 83.3 kg (SD = 15). The Shapiro-Wilk test, used to assess deviation from normality, showed a statistically significant difference ($p < 0.001$) between the weight distribution in Group N and the normal distribution. No statistically significant difference was observed for Group H ($p = 0.220$).

To assess differences in body weight between the N (normotensive) and H (hypotensive) groups, the Mann-Whitney U test was applied. The results indicated no statistically significant difference ($U = 2207.500$, $p = 0.093$). Accordingly, body weight was not identified as a predictive factor for the occurrence of hypotension.

Association of height with the occurrence of hypotension during cesarean section

Among 123 participants, the average height was 167 cm, with the shortest woman at 150 cm and the tallest woman at 183 cm. In the hypotensive group, the average height was 167.8 cm (SD = 6.2), while in the normotensive group, it was 166.4 cm (SD = 6.7). The Shapiro-Wilk test showed a statistically significant deviation from normal distribution in the hypotensive group ($p = 0.023$), but not in the normotensive group ($p = 0.836$). The Mann-Whitney *U* test revealed no statistically significant difference between the groups ($U = 2052.500$, $p = 0.371$). Therefore, height was not identified as a predictive factor for the occurrence of hypotension.

Association of Body Mass Index (BMI) with the occurrence of hypotension during cesarean section

The average BMI was 30.5 (SD = 5.4), ranging from a minimum of 20.9 to a maximum of 44.81. In the hypotensive group, the average BMI was 30.995 (SD = 5.392), and in the normotensive group, it was 30.130 (SD = 5.354). The Mann-Whitney U test showed no statistically significant difference ($p = 0.277$). Hence, BMI is not a predictive factor for the development of hypotension.

Effect of the amount of local anesthetic on hypotension and its relation to height

The local anesthetic used was 0.5% hyperbaric bupivacaine. As shown in Table 1, the average volume of anesthetic administered to shorter hypotensive patients (with systolic pressure < 90 mmHg) was 2.526 ml, while for taller hypotensive patients it was 2.603 ml.

Using the ANOVA test, which was employed to examine differences in the total amount of administered anesthetic in relation to the factors of height and presence of hypotension, it was determined that there was no statistically significant interaction between these two factors ($p = 0.812$), indicating that there was no interaction effect of height and hypotension on the total volume of anesthetic administered. It was also found that there was no statistically significant difference in the amount of anesthetic

Table 1. *Average volume of anesthetic administered by patient height and hypotension status*

Height group	Presence of hypotension	N	Average volume of anesthetic (ml)	Standard deviation (SD)
Short participants	Hypotensive	19	2.526	0.073
	Normotensive	26	2.500	0.160
Tall participants	Hypotensive	37	2.603	0.155
	Normotensive	41	2.590	0.174

administered with respect to the presence of hypotension alone (p = 0.506). However, when the effect of height was examined, a statistically significant difference was found (p = 0.005). This finding suggested that taller patients received a higher amount of anesthetic, regardless of whether hypotension was recorded during cesarean section under spinal anesthesia.

Correlation between height and volume of anesthetic administered

The analysis tested whether there was a linear, logarithmic, or logistic relationship between height and anesthetic volume. The question was whether the administered amount of anesthetic could be better predicted based on height by accounting for the curvature of the function. For the logarithmic model, the correlation (R) was 0.463, and R^2 = 0.214, meaning that about 22% of the variation in anesthetic volume could be explained by height. Adding the logarithmic model to the linear model yielded a small, non-significant improvement (R^2 change = 0.004, p = 0.393). The logistic model showed similar non-significant results (R^2 change = 0.003, p = 0.420).

Using Spearman's correlation, which was chosen over Pearson's due to a non-normal distribution, a moderate and statistically significant correlation was found (r_s = 0.427, p < 0.001).

Surgery time and association between hypotension and prolonged preoperative fasting

Patients were divided into 3 groups based on the time of surgery: 8 AM–10 AM (47 patients), 10 AM–12 PM (34 patients), and after 12 PM (42 patients). The chi-square test (χ^2 = 2.098, p = 0.350) showed no significant difference in the distribution of patients across these time slots.

Further analysis (χ^2 = 0.481, p = 0.786) showed no association between later surgery (after 12 PM) and a higher frequency of hypotension (Table 2).

Time from anesthetic administration to onset of hypotension

Among the 123 participants, 56 experienced hypotension. The time from anesthetic administration to the onset of hypotension was recorded (Table 3).

In the majority of patients (60.7%), a drop in systolic blood pressure was observed at the 10-minute measurement. The earliest drop was observed 5 minutes following administration of the local anesthetic, while in only one patient, the drop in blood pressure occurred 40 minutes after administration.

Table 2. *Frequency of hypotension by time of surgery*

Time of surgery		Presence of hypotension		Total
		Hypotensive patients	Normotensive patients	
8-10 AM	Number of patients	23	24	47
	% of patients out of all during this time	48.936	51.064	100
	% of patients out of all within the group	41.071	35.821	37.333
10-12 PM	Number of patients	14	20	34
	% of patients out of all during this time	41.176	58.824	100
	% of patients out of all within the group	25	29.851	27.642
After 12 PM	Number of patients	19	23	42
	% of patients out of all during this time	45.238	54.762	100
	% of patients out of all within the group	33.929	34.328	34.146
Total	Number of patients	56	67	123
	% relative to hypotension	45.528	54.472	100

Comparison of blood pressure at the beginning of surgery with the lowest recorded blood pressures in relation to the total amount of administered anesthetic

During a cesarean section under spinal anesthesia, the anesthesiologist recorded both systolic and diastolic blood pressure in the anesthesia protocol. Blood pressure was first measured upon entry into the operating room (baseline blood pressure). Next, it was recorded at the time of local anesthetic administration and subsequently every 2–5 minutes. The average difference between baseline systolic blood pressure and the lowest recorded systolic pressure was 46 mmHg (SD = 14). The average difference between baseline diastolic pressure and the lowest recorded diastolic pressure was 24 mmHg (SD = 11.5).

When these average differences in blood pressure were compared with the total amount of administered anesthetic

Table 3. *Time to onset of hypotension*

Time	Frequency	%
5 minutes	15	26.79
10 minutes	34	60.71
15 minutes	3	5.36
20 minutes	2	3.57
25 minutes	1	1.79
40 minutes	1	1.79

using Pearson's test, no statistically significant association was found, neither for systolic pressure (p = 0.621), nor for diastolic pressure (p = 0.734).

Administration of medication during cesarean section

In patients who experienced hypotension during the cesarean section, the anesthesia protocol copies included the documented amounts of phenylephrine and adrenaline administered during the procedure. Among these patients, the average amount of phenylephrine administered was 288.704 µg (SD = 176.721), while the average amount of adrenaline was 23.824 µg (SD = 21.036).

Using Pearson correlation, no statistically significant association was found between patient height (groups V and M) and the total amount of phenylephrine (p = 0.538) or adrenaline (p = 0.857) administered. Both groups received comparable amounts of medication during the surgery.

Frequency of hypotension during surgery in relation to maternal height

Out of a total of 123 participants, hypotension occurred an average of 1.9 times during the surgery (SD = 1.6) in 56 participants. Of these, 33 women experienced hypotension only once, while only one woman experienced hypotension 10 times. Blood pressure lower than 90 mmHg was recorded 2 times on average (SD = 2.172) in the group of shorter hypotensive women (n = 19), while in the group of taller hypotensive women (n = 37), hypotension occurred 1.7 times on average (SD = 1.234). The Mann-Whitney U test did not show a statistically significant difference (U = 358.000, p = 0.906). There was no correlation between maternal height and the occurrence of hypotension.

Correlation between hypertension during pregnancy and the occurrence of hypotension during spinal anesthesia in cesarean section

Table 4. *Presence of hypertension during pregnancy in relation to blood pressure recorded during surgery*

Diagnosis of hypertension in pregnancy		Arterial tension during surgery		Total
		Hypotensive participants	Normotensive participants	
Yes	Number of participants	4	14	18
	Expected number	8	10	18
	% for row	22.2	77.8	100
	% for column	7.1	20.9	14.6
No	Number of participants	52	53	105
	Expected number	48	57	105
	% for row	49.5	50.5	100
	% for column	92.9	79.1	85
Total	Number of participants	56	67	123
	Expected number	56	67	123
	% for row	45.5	54.5	100
	% for column	100	100	100

As shown in Table 4, out of 123 pregnant women, 18 (15%) were diagnosed with hypertension during pregnancy. Using the Chi-square test, a statistically significant difference was found between the groups (χ^2 = 4.618, p = 0.032). Among women without hypertension during pregnancy, almost half were hypotensive (49%), and the other half were normotensive (50%). Among women with hypertension during pregnancy, there were more normotensive women (78%) than hypotensive women (22%).

DISCUSSION

Hypotension during spinal anesthesia is a frequently discussed complication in cesarean sections. The incidence of hypotension varies across studies, with reports indicating that, in the absence of pharmacological prophylaxis, hypotension occurs in 70-80% of cases (17). In our study, hypotension was recorded in 45.53% of participants, which prompted us to consider other risk factors that may have contributed to its occurrence.

Maternal age as a risk factor for hypotension during cesarean section

Several studies have indicated that maternal age is a reliable predictor of hypotension during cesarean sections under spinal anesthesia (24–26). Our findings aligned

with these reports, showing that women who developed hypotension during the procedure were, on average, older. This is consistent with the study by Brenck et al., which established that the significant age cut-off for a higher incidence of hypotension was ≥ 35 years (24). Future research should further examine the characteristics of patients in the ≥ 35-year group to determine which factors may predispose them to a higher risk of hypotension.

Weight and Body Mass Index (BMI) as risk factors for hypotension during cesarean section

In examining the correlation between body weight, Body Mass Index (BMI), and the occurrence of hypotension, studies have shown that patients with higher BMIs are at greater risk for developing hypotension during cesarean sections under spinal anesthesia. Research specifically designed to investigate this correlation has found that the BMI cut-off value ranges from 25 to 29 kg/m^2 (24, 27). In their research, Ohpasanon and colleagues used BMI to predict the spread of local anesthetic after administration (26). Their results indicated that both the size of the uterus and a BMI ≥ 35 contributed to increased intra-abdominal pressure, which led to compression of the subarachnoid space. This compression caused the local anesthetic to spread over a larger area, resulting in more frequent occurrence of hypotension. However, in our study, no correlation between body weight and BMI with the occurrence of hypotension during the cesarean section was observed. A possible reason for this could be the relatively low average BMI of all participants (30.5), as well as the minimal variation between the hypotensive and normotensive groups.

Maternal height and block level

The quality of anesthesia and the incidence of hypotension are closely linked to the level of the spinal block, which is influenced by the amount of local anesthetic administered into the subarachnoid space. When lower doses of local anesthetics are used, the level of block may also depend on the maternal height (12). Our findings indicate that maternal height significantly influenced the block level achieved. Shorter patients predominantly had a block at the L3-L4 level, while taller patients had blocks at both L3-L4 and L2-L3 levels. Although this was not the focus of our study, other studies have noted the significance of maternal height in relation to the spinal column length and the local spread and potency of spinal anesthesia (11,28).

In a 1990 study, Norris investigated the correlation between local anesthetic dose and the distribution of sensory anesthesia, concluding that no dose adjustment was necessary for heights between 146.9 and 174.0 cm (29). However, more recent studies suggest that dosage should be adjusted based on maternal height (10, 12). Our study did not find a statistically significant interaction between the amount of anesthetic administered and maternal height regarding the incidence of hypotension. When comparing the average differences between baseline blood pressure and the lowest recorded blood pressure values in relation to the total amount of anesthetic administered, no statistically significant differences were found for either systolic or diastolic blood pressure. However, the study showed that taller patients received higher amounts of anesthetic, regardless of whether hypotension occurred during spinal anesthesia for the cesarean section. In the group of shorter hypotensive patients (n = 19), an average of 2 instances of blood pressure readings below 90 mmHg was recorded. In the group of taller hypotensive patients (n = 37), hypotension was observed an average of 1.7 times. Therefore, it was concluded that there was no correlation between maternal height and the occurrence of hypotension.

Using a logarithmic function to predict the required amount of local anesthetic, we found that it was significantly simpler to determine the appropriate dosage when the height of the patients was known. Regarding the effect of the anesthetic on hypotension, we also investigated the most common time interval between the administration of the local anesthetic and the onset of hypotension. Hypotension most commonly occurred 10 minutes after the local anesthetic administration. The shortest recorded time was 5 minutes, while in 1 patient, hypotension occurred 40 minutes after the administration. The reason for the delayed onset of hypotension is not necessarily related to the use of local anesthetics. To prevent such causes of hypotension, appropriate monitoring and close cooperation between the anesthesiologist and surgeon during the cesarean section are essential (30).

As part of the study, we also compared the amounts of medication used during the cesarean section in relation to maternal height. In both the tall and short groups of participants, we did not find a statistically significant difference in the total amount of phenylephrine and adrenaline administered. Both groups received an equal amount of medication for hypotension management. In the

literature, phenylephrine is described as a potent, fast-acting vasopressor with a short duration of action, which is highly effective and easily titrated. The combination of high doses of phenylephrine and crystalloid infusion has been shown to be an effective technique for controlling hypotension (30).

Preoperative fasting duration as a risk factor for the development of hypotension

One of the most common methods for preventing hypotension induced by spinal anesthesia is fluid preloading. This technique involves the prophylactic intravenous administration of crystalloid solutions at a volume of 10–20 mL/kg approximately 15–20 minutes prior to local anesthetic injection (31). However, the efficacy of this approach is increasingly called into question because preloading with crystalloids leads to rapid fluid redistribution into the extravascular space, resulting in only a transient increase in intravascular volume. This acute volume expansion can trigger atrial natriuretic peptide secretion, which results in peripheral vasodilation and accelerates the excretion of the previously administered fluids. It has also been established that hypotension mainly occurs shortly after the application of spinal anesthesia, and even a rapid bolus fluid infusion (a technique known as "co-loading") cannot prevent this. There is a research consensus that even large volumes of intravenous fluids cannot prevent hypotension during spinal anesthesia (31).

In our study, we evaluated the development of hypotension during cesarean sections relative to preoperative fasting duration, which was calculated based on the scheduled operation time. The cesarean section surgeries were equally distributed into 3 time intervals: 8:00 AM–10:00 AM, 10:00 AM–12:00 PM, and after 12:00 PM. We observed that patients operated on during the latest interval (after 12:00 PM) were not more likely to experience intraoperative hypotension. Similarly, Husaini and Russell (32) compared a group receiving 1 L of Ringer's solution 10 minutes before spinal anesthesia to a parallel control group receiving no preoperative fluids. In both groups, prophylactic ephedrine infusion was administered immediately after the injection of the local anesthetic. There was no significant difference in the need for ephedrine later, nor did the incidence of hypotension differ between the groups (32). As an improvement to this study, it would be beneficial to compare the data on the quantity of fluids administered preoperatively with the time of surgery and the occurrence of hypotension.

Correlation of hypertension during pregnancy with the development of hypotension during cesarean section

In our study, we also recorded data on whether participants had been diagnosed with hypertension during pregnancy. When comparing hypertension during pregnancy with the occurrence of hypotension during the cesarean section, the results showed that the number of patients who did not have hypertension during pregnancy was almost equal to the number of those who later became hypotensive (49%) and those who remained normotensive (50%). Among patients who had hypertension during pregnancy, there were more normotensive (78%) than hypotensive (22%) women. In a study conducted by Aya et al., patients with severe preeclampsia had a lower incidence of hypotension during cesarean sections under spinal anesthesia (33). Blood pressure was measured and recorded before the application of spinal anesthesia, and every 2 minutes over a period of half an hour afterwards. Despite receiving a smaller volume of fluid and a higher dose of local anesthetic, patients with severe preeclampsia were less likely to experience hypotension. The risk of hypotension was almost six times lower in patients with severe preeclampsia compared to pregnant women with normal blood pressure (33). Animal studies under normal physiological conditions have demonstrated that uteroplacental blood flow exceeds fetal oxygen demand, providing a safety margin against fluctuations in perfusion. Similarly, a retrospective review by Maayan-Metzger et al. of 919 term pregnancies undergoing elective cesarean delivery under spinal anesthesia found that even maternal hypotension exceeding 30–50% from baseline was not associated with adverse perinatal outcomes, supporting the concept of a protective buffer in placental oxygen delivery (16, 25). Other studies suggest that the duration of hypotension may be more critical than its severity, indicating that maternal hypotension lasting longer than 4 minutes could be associated with neurobehavioral changes (6). The data we used for comparison were solely related to the presence of a hypertension diagnosis during pregnancy and the development of hypotension during the cesarean section. A potential topic for future research could be defining preoperative blood pressure values that influence the reduced occurrence of hypotension, as well as variations in the amount of local anesthetic that can be administered to patients who are hypertensive before the onset of anesthesia, compared with those who are not.

The results of our research show that older patients were, on average, more frequently hypotensive during cesarean section under spinal anesthesia. Patients who had hypertension during pregnancy were, on average, less likely to experience hypotension during cesarean section compared with those in whom hypertension was not diagnosed during pregnancy. The participants in the lower-height group were not statistically significantly more likely to be hypotensive than those in the taller group. In our study, the duration of preoperative fasting was not a risk factor for the development of hypotension during cesarean section under spinal anesthesia. Further studies are needed with a larger number of participants to obtain a more representative sample and to allow greater variation among groups.

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Author Contributions

Conceptualization: S.Dj. and A.U.; Methodology, investigation, and data curation: S.Dj.; Formal analysis: S.Dj., A.U., and N.G.; Writing – original draft: S.Dj.; Writing review & editing: A.U. and N.G. 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 the Clinical Center of Vojvodina, approval number 00-113, issued on 23 June 2023. The study was conducted in accordance with the Declaration of Helsinki. As this was a retrospective study based on existing, anonymized anesthesia records, the requirement for individual informed consent was waived by the Ethics Committee.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availability

All data analyzed during this study are included in this published article.

Statement of Generative AI Technologies Use

No generative AI was used.

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AFMN BIOMEDICINE

ORIGINAL ARTICLE

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VACCINE HESITANCY IN PARENTS OF CHILDREN WITH CONGENITAL UROGENITAL ANOMALIES WITH HYPOSPADIAS AND UNDESCENDED TESTIS

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Parental vaccine hesitancy (VH) toward childhood immunization is a critical public health challenge affecting individual pediatric health and broader societal immunity. This study aimed to evaluate VH among parents of male children by comparing those whose children had congenital urogenital anomalies (CUA), specifically hypospadias and undescended testicles, with those of children without malformations. The assessment also incorporated family demographics, health literacy levels, and adherence to preventive health practices. This descriptive-analytical study included 409 participants, divided into two groups: parents of children presenting for elective circumcision without malformations (CNM) and parents of children diagnosed with CUA. Data were collected using a socio-demographic form, the Turkey Health Literacy Scale (TSOY-32), and the Vaccine Hesitancy Scale (VHS). High vaccine hesitancy was defined as scores in the upper quartile of the VHS (VHS-uQ). The overall vaccination rate among the children was 96.6% (n = 395). In the CUA group, the absence of routine vitamin D supplementation was associated with 25-fold higher odds of VHS-uQ ($p < 0.001$). Mothers with a high school education or higher demonstrated a 4.69-fold higher odds of VHS-uQ ($p < 0.001$). Conversely, COVID-19 vaccination of both parents was associated with lower VHS-uQ rates in both groups ($p < 0.001$). Interestingly, higher TSOY-32 scores were associated with a 4.57-fold higher odds of VHS-uQ ($p = 0.034$). This study identified maternal health literacy and adherence to routine preventive care as key determinants of VH among parents of children with CUA. indings suggest that fostering trust through continuous healthcare engagement and evidence-based counseling is a vital pathway to improving vaccine acceptance in this specific population.

Keywords: vaccine hesitancy, childhood vaccination, parental vaccine hesitancy, hypospadias, undescended testicles, congenital urogenital anomalies

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INTRODUCTION

Hypospadias and undescended testes represent the most prevalent congenital urogenital anomalies (CUA) in male children (1,2). The diagnosis of a congenital anomaly, combined with the necessity for surgical intervention and the potential for complications, often induces significant anxiety and fear in parents. Such emotional stressors can profoundly influence the healthcare-related decisions parents make for their children (3,4). Parents typically acquire health information from mass media, their social environment, and healthcare professionals to manage their children's daily care and long-term well-being (5,6). Within the spectrum of pediatric healthcare, vaccination remains a fundamental and highly effective primary public health strategy for disease prevention (7).

Substantial progress has been achieved through routine childhood immunization, which has targeted infections that were the primary causes of pediatric morbidity and mortality in the last century. It is estimated that global vaccination programs, which have led to the eradication of smallpox and elimination of polio in numerous regions, save more than 3 million children annually (8). However, vaccine hesitancy (VH), identified by the World Health Organization (WHO) in 2019 as one of the top ten global health threats, has increased gradually. This trend has intensified following the COVID-19 pandemic, incited by social media misinformation regarding both COVID-19 and routine childhood vaccines (9,10). VH among parents striving to make optimal health decisions for their children poses a significant challenge, impacting not only individual pediatric health but also collective societal immunity. Evaluating determinants of VH, such as sociocultural influences, economic conditions, environmental factors, and media exposure, is essential for providing timely, evidence-based guidance to parents (9,11,12).

In this study, we aimed to evaluate and compare VH levels among parents of male children diagnosed with CUA (hypospadias and undescended testes) and those of children presenting for elective circumcision without malformations (CNM).

METHODS

Study design and settings

This multicenter, descriptive-analytical study was conducted between February 2024 and May 2024 across four consortium centers in Turkey (Aksaray, Rize, Trabzon, and Kilis). These centers represent three distinct regions, according to the Nomenclature of Territorial Units for Statistics (NUTS-1) and the Turkey Demographic and Health Survey (TDHS) (13).

Study population and sampling

A total of 492 parents of male children aged 0–7 years, who presented to pediatric surgery outpatient clinics for hypospadias, undescended testes, or elective circumcision, were initially recruited using stratified sampling. Participants were categorized into two groups: those with CUA and those seeking CNM.

Exclusion criteria were as follows: 35 participants with comorbidities, 9 who provided uniform (identical) answers to all scale items, 33 patients aged 98 months or older, and 6 who provided contradictory responses. Consequently, the final analysis was performed on 409 participants.

Data collection and instruments

Data were collected through face-to-face interviews conducted by pediatric surgeons at the respective centers. The research instrument consisted of a 31-item structured questionnaire (Supplementary File 1), the Turkey Health Literacy Scale (TSOY-32) (14), and the Vaccine Hesitancy Scale (VHS) (15).

The structured questionnaire comprised five sections:

1. Sociodemographic information: parental education levels, ages, and child-specific data (birth order, weight, and gestational age).
2. Healthcare practices: adherence to routine infant-child-adolescent monitoring protocols of the Turkish Ministry of Health (16), including iron/vitamin D supplementation, antenatal care visits, and maternal vaccination status.
3. Vaccination history and attitudes: perspectives on routine and private vaccines (e.g., rotavirus, meningococci), COVID-19 vaccination status, and the impact of social media on vaccine perception (17-19).
4. Information sources: preferred sources for health information (e.g., pediatricians, family physicians, social media, bloggers, and official websites).

TSOY-32 scale

Maternal health literacy (HL) was assessed using the TSOY-32 scale. This 32-item, 5-point Likert-type scale evaluates four dimensions—accessing, understanding, evaluating, and applying health information—across two domains: "Treatment and Service Utilization" and "Disease Prevention/Health Promotion" (20,21). For the score

calculation of the scale consisting of 32 items on a 5-point Likert scale (1=Very easy, 2=Easy, 3=Difficult, 4=Very difficult, 5=No idea') for each item, it was coded as suggested in the literature, and a value of 0 was assigned to the 'no idea' code. Scores ranging from 0 (lowest HL) to 50 (highest HL) were categorized into three levels: 0–25, >25–33, and >33–50 points.

Vaccine hesitancy scale

The Turkish version of the 9-item VHS was administered to assess parental hesitancy (15). Items are scored on a 5-point Likert scale, with total scores ranging from 9 to 45; higher scores indicate greater hesitancy. For analysis, participants were dichotomized into two groups based on the upper quartile: lower quartile (VHS-lQ; <25 points) and upper quartile (VHS-uQ; ≥25 points).

Ethics

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Hacettepe University non-interventional Ethics Committee (Approval No. GO 2024/05-42) on January 23, 2024.

Statistical analyses

In the study, the data were collected in Excel format, and analyses were performed using SPSS 22.0 (IBM, USA).

Participants were divided into two groups: those with congenital urogenital anomalies (CUA) and the CNM group.

VHS (Vaccine Hesitancy Scale) scores were evaluated in quartiles, divided into the upper quartile (VHS-uQ) (Q4) and lower quartiles (Q1-3) (VHS-lQ).

The normal distribution of the data was evaluated by the Shapiro-Wilk test. Categorical variables were expressed as n (%) and continuous variables showing normal distribution were expressed as mean ± standard deviation. The Chi-square test was used to compare the percentage distribution of categorical data between groups.

Variables with an association to VH indicated by a p-value of <0.10 were selected for further analysis. Multiple logistic regression analysis was then used to explore associations between child-family parameters (such as sociodemographic characteristics, vaccination practices, healthcare practices, and health information sources) and the VHS-uQ in both the CUA and CNM groups. Additionally, data from the overall group were analyzed to identify associations between VH and child-family parameters, including TSOY-32 scale scores and the different study groups. Adjusted odds ratios with 95% confidence intervals were calculated, and a p-value of <0.05 was considered statistically significant.

RESULTS

Sociodemographic characteristics

In this study, 36.7% (n = 150) of the children were first-borns. Regarding age distribution, 48.9% were younger than 24 months, 29.3% were aged 24–47 months, and 21.8% were 48 months or older. Routine vitamin D supplementation was administered to 90.7% of the participants, while 74.4% adhered to the standard iron supplementation regimen starting at 4 months of age (Table 1).

Maternal age was distributed as follows: 10.3% were under 25 years, 65.3% were between 25 and 34 years, and 24.4% were 35 years or older. In contrast, 55% of the fathers were under 25 years old. The sociodemographic profiles of the CUA and CNM groups were largely comparable, with no significant differences in maternal age (p = 0.585), paternal age (p = 0.544), gestational duration (p = 0.085), child age distribution (p = 0.264), or birth weight (p = 0.232). However, maternal education levels differed significantly: 54.2% of mothers in the CUA group had eight years or less of education compared to 41.9% in the CNM group (p = 0.015) (Table 1).

Vaccination rates and attitudes

The overall childhood vaccination rate was 96.6% (n = 395). While the CUA group showed a slightly higher routine vaccination rate (98%) compared to the CNM group (94.4%), this difference was at the threshold of significance (p = 0.050). Parental COVID-19 vaccination rates were similar between groups (CUA fathers: 61.0% vs. CNM fathers: 63.7%, p = 0.582; overall mothers: 66.5%).

Post-pandemic attitudes revealed that while 37.7% of parents viewed routine childhood vaccines as more essential after COVID-19, a substantial minority (24.9% overall) reported increased hesitancy. Negative impacts from social media news regarding vaccines were reported by 16.4% of parents, with a higher but not statistically significant prevalence in the CUA group (18.5% vs. 13.1%, p = 0.215). Uptake for special vaccines (rotavirus/meningococcal) remained low at 12.5% across both groups (p = 0.550).

Table 1. *Evaluation of socio-demographic, health practice, and vaccination information of parents*

Features	Overall n (%)	CUA* (n = 249)	Control (n = 160)	pÆ
Child characteristics				
First child				
1st	150 (36.7)	86 (34.5)	64 (40.0)	0.263
2nd and more	259 (63.3)	163 (65.5)	96 (60.0)	
Child age (month)				
<24	200 (48.9)	114 (45.8)	86 (53.8)	0.264
24-47	120 (29.3)	76 (30.5)	44 (27.5)	
≥48	89 (21.8)	59 (23.7)	30 (18.8)	
Gestational duration				
Mature	339 (82.9)	213 (85.5)	146 (91.3)	0.085
Premature	70 (17.1)	36 (14.5)	14 (8.8)	
Birth weight				
SGAз	50 (12.2)	18 (14.4)	16 (9.3)	0.232
No	359 (87.8)	122 (85.6)	170 (90.7)	
Hospitalization				
Yes	112 (27.4)	74 (29.7)	38 (23.8)	0.186
No	297 (72.6)	175 (70.3)	122 (76.3)	
Childhood vaccination§				
Yes	395 (96.6)	244 (98.0)	151 (94.4)	**0.05**
No	14 (3.4)	5 (2.0)	9 (5.6)	
At least 1 dose of special vaccine&				
Yes	51 (12.5)	33 (13.3)	18 (11.3)	0.55
No	358 (87.5)	216 (86.7)	142 (88.8)	
Parent characteristics				
Maternal age				
<25	42 (10.3)	23 (9.2)	19 (11.9)	0.585
25-34	267 (65.3)	162 (65.1)	105 (65.6)	
≥35	100 (24.4)	64 (25.7)	36 (22.5)	
Paternal age				
<35	225 (55.0)	134 (53.8)	91 (56.9)	0.544
≥35	184 (45.0)	115 (46.2)	69 (43.1)	
Maternal education				
8 years and lessß	202 (49.4)	135 (54.2)	67 (41.9)	**0.015**
High school and above	207 (50.6)	134 (53.8)	93 (58.1)	
Paternal education				
8 years and lessß	186 (45.6)	115 (46.2)	71 (44.4)	0.72
High school and above	223 (54.5)	79 (63.2)	89 (55.6)	
Health care practices				
Antenatal care visit				
1-3 times	42 (10.5)	29 (11.8)	13 (8.3)	0.264
4 and more	359 (89.5)	216 (88.2)	143 (91.7)	
Vaccination during pregnancy				
Yes	308 (75.3)	182 (73.1)	126 (78.8)	0.195
No	101 (24.7)	67 (26.9)	34 (21.3)	
Standard vitamin D supplementation regimen during infancy				
Yes	371 (90.7)	228 (91.2)	144 (90.0)	0.692
No	38 (9.3)	22 (8.8)	16 (10.0)	
Standard iron supplementation regimen during infancy				
Yes	300 (73.3)	172 (69.1)	128 (80.0)	**0.015**
No	109 (26.7)	77 (30.9)	32 (20.0)	

Standard vitamin D supplementation regimen during pregnancy				
Yes	365 (89.2)	226 (90.8)	139 (86.9)	0.216
No	44 (10.8)	23 (9.2)	21 (13.1)	
Standard iron supplementation regimen during pregnancy				
Yes	327 (80.0)	201 (80.7)	126 (78.8)	0.627
No	82 (20.0)	48 (19.3)	34 (21.3)	
Vaccine history and ideas of parent				
Childhood vaccination§ idea				
Protects the child	313 (76.5)	187 (75.1)	126 (78.8)	0.447
I'm hesitant Ↄ	77 (18.8)	50 (20.1)	27 (16.9)	
Not safeϴ	12 (2.9)	9 (3.6)	3 (1.9)	
Refuse	7 (1.7)	3 (1.2)	4 (2.5)	
Childhood vaccination§ idea after COVID-19				
Yes, necessary	154 (37.7)	93 (37.3)	61 (38.1)	0.811
Yes, hesitant	102 (24.9)	60 (24.1)	42 (26.3)	
No	153 (37.4)	96 (38.6)	57 (35.6)	
COVID-19 vaccination				
Mother	66 (16.1)	45 (18.1)	21 (13.1)	0.454
Father	48 (11.7)	26 (10.4)	22 (13.8)	
Parent	206 (50.4)	126 (50.6)	80 (50.0)	
No	89 (21.8)	52 (20.9)	37 (23.1)	
Maternal COVID-19 vaccination				
Yes	272 (66.5)	171 (68.7)	101 (63.1)	0.246
No	137 (33.5)	78 (31.3)	59 (36.9)	
Paternal COVID-19 vaccination				
Yes	254 (62.1)	152 (61.0)	102 (63.7)	0.582
No	155 (37.9)	97 (39.0)	58 (36.3)	
Influenced by social media news about vaccines				
Yes, hesitant	114 (27.9)	72 (28.9)	42 (26.3)	0.215
Yes, negative	67 (16.4)	46 (18.5)	21 (13.1)	
No, not affect	228 (55.7)	131 (52.6)	97 (60.6)	
Health information sources				
Family elders as a SHI†				
Reliable	274 (67.0)	164 (65.9)	110 (68.8)	0.545
Others	135 (33.0)	85 (34.1)	50 (31.3)	
Pediatrician as a SHI†				
Reliable	400 (97.8)	241 (96.8)	159 (99.4)	0.082
Others	9 (2.2)	8 (3.2)	1 (0.6)	
Family physician as a SHI†				
Reliable	382 (93.4)	232 (93.2)	150 (93.8)	0.819
Others	27 (6.6)	17 (6.8)	10 (6.3)	
Family nurse/midwife as a SHI†				
Reliable	352 (86.1)	215 (86.3)	137 (85.6)	0.837
Others	57 (13.9)	34 (13.7)	23 (14.4)	
Both members of the family medicine unit as a SHI†				
Reliable	344 (84.1)	208 (83.5)	136 (85.0)	0.5
One of them is reliable	46 (11.2)	31 (12.4)	15 (9.4)	
Others	19 (4.6)	10 (4.0)	9 (5.6)	
Social media as a SHI†				
Reliable	18 (4.4)	11 (4.4)	7 (4.4)	0.984
Others	391 (95.6)	238 (95.6)	153 (95.6)	

Government and university website as a SHI†				
Reliable	87 (21.3)	47 (18.9)	40 (25.0)	0.14
Others	322 (78.7)	202 (81.1)	120 (75.0)	
Social media blogger as a SHI†				
Reliable	34 (8.3)	22 (8.8)	12 (7.5)	0.633
Others	375 (91.7)	227 (91.2)	148 (92.5)	
Book as a SHI†				
Reliable	197 (48.2)	118 (47.4)	79 (49.4)	0.695
Others	212 (51.8)	131 (52.6)	81 (50.6)	
Television as a SHI†				
Reliable	46 (11.2)	31 (12.4)	15 (9.4)	0.337
Others	363 (88.8)	218 (87.6)	145 (90.6)	
Scales				
VHS Score π				
Lower Qα	306 (74.8)	182 (73.1)	124 (77.5)	
Upper Qα	103 (25.2)	67 (26.9)	36 (22.5)	0.316
TSOY**				
0-25	84 (43.3)	34 (39.1)	50 (46.7)	
>25-33	58 (29.9)	22 (25.3)	36 (33.6)	**0.042**
>33	52 (26.8)	31 (35.6)	21 (19.6)	

*=Congenital urogenital anomaly, **Æ=** Chi-square test was used to compare the CUA and control group. ß= Primary and secondary education (8 years), **§=**Childhood routine vaccination programme, **†=** source of health information, ɜSGA= Small gestational age, **§=**Childhood routine vaccination programme, **π=** Vaccine hesitancy score, α= Lower Q group: <25 points; Upper Q group: ≥25 points **&=**Rota or meningococcal vaccine, Ɔ=I'm hesitant, but there's a vaccine tracking system. Θ=Not safe, but there's a vaccine tracking system, **†=** Source of health information, **= Turkish Health Literacy Scale

Factors associated with the vaccine hesitancy scale (VHS)

Vaccine hesitancy (VHS-uQ) was significantly lower in children under 24 months (15.5%) compared to older age groups ($p < 0.001$). Furthermore, while only 14.4% of parents who expressed confidence in the national immunization program were in the VHS-uQ group, this rate rose to 59.7% among those who were hesitant and 91.7% among those who perceived vaccines as unsafe ($p < 0.001$) (Table 2).

In the CUA group, maternal education level was a significant predictor: 36.8% of mothers with at least a high school education were in the VHS-uQ group compared to 18.5% of those with lower education levels ($p = 0.001$). Additionally, higher VHS scores were significantly associated with non-adherence to routine preventive care, including lack of routine vitamin D (72.7%) or iron supplementation (69.1%) during infancy ($p < 0.001$ and $p = 0.004$, respectively), and absence of maternal prenatal supplementation ($p < 0.001$). Distrust in the family medicine team was also linked to higher hesitancy ($p = 0.001$) (Table 2).

In the CNM group, 45.2% of those who developed vaccine hesitancy post-pandemic fell into the VHS-uQ category ($p < 0.001$). Parents who were not vaccinated against COVID-19 showed significantly higher hesitancy rates (48.6%) compared to their vaccinated counterparts (11.3%, $p < 0.001$) (Table 2).

Logistic regression analysis

Multivariable analysis of the entire cohort showed no significant association between VH and urogenital anomaly status or basic healthcare practices (all $p > 0.05$). However, significantly higher odds of VH were observed among participants with higher TSOY-32 scores (OR = 4.57, 95% CI: 1.12–18.65) and parents of children aged ≥48 months (OR = 4.13, 95% CI: 1.32–12.97). Conversely, parental COVID-19 vaccination was a protective factor against VH (Table 3).

In the CUA group, the odds of high vaccine hesitancy were 6.31 times higher among mothers with at least a high school education ($p < 0.001$). Lack of routine vitamin D supplementation for the infant was associated with a 25-fold increase in the odds of VH (95% CI: 4.04–156.5). Vaccination of both parents against COVID-19 was strongly associated with lower VH scores (OR: 0.07, 95% CI: 0.02–0.24).

In the CNM group, paternal education (high school or above) was associated with 7.52 times higher odds of VH ($p < 0.001$). Parents who reported increased hesitancy specifically after the COVID-19 pandemic had 11.43 times higher odds of being in the VHS-uQ group (95% CI: 2.52–51.84, $p = 0.002$) (Table 4).

Table 2. *Evaluation of parents' vaccination hesitancy using the Vaccine Hesitancy Scale*

	All			CUA			Control		
Features	**Overall** n = 409	**VHS π Upper Qα**		**Overall Cua** n = 249	**VHS π Upper Qα**		**Overall Control** n = 160	**VHS π Upper Qα**	
	n (%)	**%****	**p**	**n (%)**	**%****	**p**	**n (%)**	**%****	**P**
Child characteristics									
First child			0.958			0.52			0.315
1st	150 (36.7)	25.3		86 (34.5)	24.4		64 (40.0)	26.6	
2nd and more	259 (63.3)	25.1		163 (65.5)	28.2		96 (60.0)	19.8	
Child age (month)			**<0.001**			**0.003**			**0.02**
<24	200 (48.9)	15.5		114 (45.8)	16.7		86 (53.8)	14	
24-47	120 (29.3)	32.5		76 (30.5)	32.9		44 (27.5)	31.8	
≥48	89 (21.8)	37.1		59 (23.7)	26.9		30 (18.7)	33.3	
Gestational duration			0.678			0.553			0.139
Mature	339 (82.9)	24.8		206 (82.7)	27.7		133 (83.1)	20.3	
Premature	70 (17.1)	27.1		43 (17.3)	23.3		27 (16.9)	33.3	
Birth weight			0.367			0.493			0.441
SGA*	50 (12.2)	20		36 (14.5)			14 (8.8)	14.3	
No	359 (87.8)	25.9		213 (85.5)			146 (91.2)	23.3	
Hospitalization			0.475			0.49			0.201
Yes	112 (27.4)	27.7		74 (29.7)	32.4		38 (23.8)	18.4	
No	297 (72.6)	24.2		175 (70.3)	24.6		122 (76.2)	23.8	
Childhood vaccination§			0.339			0.725			0.4
Yes	395 (96.6)	25.6		244 (98.0)	27		151 (94.4)	23.2	
No	14 (3.4)	14.3		5 (2.0)	20		9 (5.6)	11.1	
At least 1 dose of special vaccine&			0.771			0.428			0.569
Yes	51 (12.5)	23.5		33 (13.3)	21.2		18 (11.2)	27.8	
No	358 (87.5)	25.4		216 (86.7)	27.8		142 (88.8)	21.8	
Parent characteristics									
Maternal age			0.276			0.573			0.182
<25	42 (10.3)	35.7		23 (9.2)	39.1		19 (11.9)	31.6	
25-34	267 (65.3)	25.1		162 (65.1)	27.2		105 (65.6)	21.9	
≥35	100 (24.4)	21		64 (25.7)	21.9		36 (22.5)	19.4	
Paternal age			0.987			**0.013**			0.147
<35	225 (55.0)	28		134 (53.8)	26.9		91 (56.9)	29.7	
≥35	184 (45.0)	21.7		115 (46.2)	27		69 (43.1)	13	
Maternal education			**0.002**			**0.001**			0.238
8 years and lessß	202 (49.4)	18.3		135 (54.2)	18.5		67 (41.9)	17.9	
High school and above	219 (50.6)	31.9		114 (45.8)	36.8		93 (58.1)	25.8	
Paternal education			**0.024**			0.088			0.13
8 years and lessß	186 (45.6)	19.9		115 (46.2)	21.7		71 (44.4)	16.9	
High school and above	223 (54.5)	29.6		134 (53.8)	31.3		89 (55.6)	27	
Health care practices									
Antenatal care visit			**<0.001**			0.492			**<0.001**
1-3 times	42 (10.5)	57.1		29 (11.8)	69		13 (8.3)	30.8	
4 and more	359 (89.5)	21.4		216 (88.2)	20.8		143 (91.7)	22.4	
Vaccination during pregnancy			**0.012**			**0.001**			0.764
Yes	308 (75.3)	22.1		182 (73.1)	21.4		126 (78.8)	23	
No	101 (24.7)	34.7		67 (26.9)	41.8		34 (21.2)	20.6	

Standard vitamin D supplementation regimen during infancy			**<0.001**			**<0.001**			0.377
Yes	371 (90.7)	22.1		227 (91.2)	22.5		144 (90.0)	21.5	
No	38 (9.3)	55.3		22 (8.8)	72.7		16 (10.0)	31.3	
Standard iron supplementation regimen during infancy			**0.003**			**0.004**			0.394
Yes	300 (73.3)	21.3		172 (69.1)	21.5		128 (80)	21.1	
No	109 (26.7)	35.8		77 (33.1)	39		32 (20)	28.1	
Standard vitamin D supplementation regimen during pregnancy			**<0.001**			**0.001**			**0.017**
Yes	365 (89.2)	22.2		226 (90.8)	23.9		139 (86.9)	19.4	
No	44 (10.8)	50		23 (92.4)	56.5		21 (13.1)	42.9	
Standard iron supplementation regimen during pregnancy			**<0.001**			**<0.001**			**0.013**
Yes	327 (80.0)	19		201 (80.7)	19.4		126 (78.8)	18.3	
No	82 (20.0)	50		48 (19.3)	58.3		34 (21.2)	38.2	
Vaccine history and ideas of parents									
Childhood vaccination§ Idea			**<0.001**			**<0.001**			**<0.001**
Protects the child	313 (76.5)	14.4		187 (75.1)	13.4		126 (78.8)	15.9	
I'm hesitant Ɔ	77 (18.8)	59.7		50 (20.1)	68		27 (16.9)	44.4	
Not safeϴ	12 (2.9)	91.7		9 (3.6)	88.9		3 (1.8)	100	
Refuse	7 (1.7)	14.3		3 (1.2)	0		4 (2.5)	25	
Childhood Vaccination§ Idea after COVID-19			**<0.001**			**<0.001**			**<0.001**
Yes, necessary	154 (37.7)	12.3		93 (37.4)	11.8		61 (38.1)	13.1	
Yes, hesitant	102 (24.9)	52.9		60 (24.1)	58.3		42 (26.3)	45.2	
No	153 (37.4)	19.6		96 (38.6)	21.9		57 (35.6)	15.8	
Parental COVID-19 vaccination status			**<0.001**			**<0.001**			**<0.001**
Only mother vaccinated	66 (16.1)	19.7		45 (18.1)	22.2		21 (13.1)	14.3	
Only father vaccinated	48 (11.7)	29.2		26 (10.4)	30.8		22 (13.8)	27.3	
Both parents vaccinated	206 (50.4)	14.1		126 (50.6)	15.9		80 (50.0)	11.3	
Neither parent vaccinated	89 (21.8)	52.8		52 (20.9)	55.8		37 (23.1)	48.6	
Maternal COVID-19 vaccination status			**<0.001**			**<0.001**			**<0.001**
Yes	272 (66.5)	15.4		171 (68.7)	17.5		101 (63.1)	11.9	
No	137 (33.5)	44.5		78 (31.3)	47.4		59 (36.9)	40.7	
Paternal COVID19 vaccination status			**<0.001**			**<0.001**			**0.002**
Yes	254 (62.1)	22.5		152 (61.1)	18.4		102 (63.8)	14.7	
No	155 (37.9)	16.9		97 (38.9)	40.2		58 (36.2)	36.2	
Influenced by social media news about vaccines			0.102			0.054			**0.009**
Yes, hesitant	114 (27.9)	32.5		72 (28.9)	34.7		42 (26.2)	28.6	
Yes, negative	67 (16.4)	32.8		46 (18.5)	30.4		21 (13.1)	38.1	
No, not affect	228 (55.7)	19.3		131 (52.6)	21.4		97 (60.6)	16.5	
Health information sources									
Family elders as a SHI†			0.332			0.213			0.919
Reliable	274 (67.0)	23.7		164 (65.9)	24.4		110 (68.8)	22.7	
Others	135 (33.0)	28.1		85 (34.1)	31.8		50 (31.2)	22	
Pediatrician as a SHI†			0.325			0.35			0.589
Reliable	400 (97.8)	25.5		241 (96.8)	27.4		159 (99.4)	22.6	
Others	9 (2.2)	11.1		8 (3.2)	12.5		1 (0.6)	0	
Family physician as a SHI†			**<0.001**			**<0.001**			**0.031**
Reliable	382 (93.4)	22.8		232 (93.2)	24.1		150 (93.7)	20.7	
Others	27 (6.6)	59.3		17 (6.8)	64.7		10 (6.3)	50	

Family nurse/midwife as a SHIŧ			**0.029**			0.109			0.127
Reliable	352 (86.1)	23.3		215 (86.3)	25.1		137 (85.6)	20.4	
Others	57 (13.9)	36.8		34 (13.7)	38.2		23 (14.4)	34.8	
Both members of the family medicine unit as a SHI			**<0.001**			**0.001**			0.133
Reliable	344 (84.1)	22.7		208 (83.5)	24.5		136 (85.0)	22.5	
1 of them is reliable	46 (11.2)	28.3		31 (12.5)	25.8		15 (9.4)	33.3	
Others	19 (4.7)	63.2		10 (4.0)	80		9 (5.6)	44.4	
Social media as a SHIŧ			**0.05**			0.173			0.145
Reliable	18 (4.4)	5.6		11 (4.4)	9.1		7 (4.4)	0	
Others	391 (95.6)	26.1		238 (95.6)	27.7		153 (95.6)	100	
Government and university website as a SHIŧ			0.054			0.548			**0.029**
Reliable	87 (21.3)	17.2		47 (18.9)	23.4		40 (25.0)	10	
Others	322 (78.7)	27.3		202 (81.1)	27.7		120 (75.0)	26.7	
Social media blogger as a SHIŧ			0.29			0.643			0.222
Reliable	34 (8.3)	17.6		22 (8.8)	22.7		12 (7.5)	8.3	
Others	375 (91.7)	25.9		227 (91.2)	27.3		148 (92.5)	23.6	
Book as a SHIŧ			0.752			0.283			**0.048**
Reliable	197 (48.2)	25.9		118 (47.3)	23.7		79 (49.4)	29.1	
Others	212 (51.8)	24.5		131 (52.7)	29.8		81 (50.6)	16	
Television as a SHIŧ			0.384			0.473			0.685
Reliable	46 (11.2)	30.4		31 (12.4)	32.3		15 (9.4)	26.7	
Others	363 (88.8)	24.5		218 (87.6)	26.1		145 (90.6)	22.1	
TSOY-32 V			0.079						
0-25	84 (43.3)	26.2							
>25-33	58 (29.9)	27.6							
>33	52 (26.8)	11.5							
Disease groups			0.351						
CUA*	249 (60.9)	26.9							
Control	160 (39.1)	22.5							

*=Congenital urogenital anomaly, **= According to the row variable ß= Primary and secondary education (8 years), **§=**Childhood routine vaccination programme **ŧ=** source of health information, ɜSGA= Small gestational age, **§=**Childhood routine vaccination programme, **π=** Vaccine Hesitancy Score, α= Upper Q group: ≥25 points in the VHS **&=**Rota or meningococcal vaccine, Ɔ=I'm hesitant, but there's a vaccine tracking system. Θ=Not safe, but there's a vaccine tracking system, **ŧ=** source of health information, **V** = Turkey Health Literacy Scale.

DISCUSSION

To the best of our knowledge, this study is the first to evaluate VH specifically among parents of children with CUA.

Our findings reveal that parents in the CUA group who did not routinely administer vitamin D to their infants had 25 times higher odds of VH, suggesting that vaccine refusal may be part of a broader pattern of non-adherence to preventive healthcare. Interestingly, a higher socio-economic and educational profile did not translate to increased vaccine acceptance; mothers with a high school education or higher demonstrated 6.31 times higher odds of VH ($p < 0.001$), and higher TSOY-32 scores were associated with a 4.57-fold increase in hesitancy.

The age of a child also emerged as a significant factor, with parents of children under 24 months showing lower hesitancy rates (15.5%) compared to older age groups ($p = 0.005$), likely due to more frequent healthcare engagement during early infancy. Furthermore, the COVID-19 pandemic significantly reshaped parental attitudes; those who developed hesitancy post-pandemic showed 3.99-fold higher VHS-uQ rates in the CUA group and 11.43-fold higher rates in the CNM group. Conversely, having both parents vaccinated against COVID-19 was strongly associated with lower VH scores in both cohorts ($p < 0.001$), emphasizing that trust in public health interventions remains a cornerstone of routine childhood immunization compliance.

Table 3. *Logistic regression analysis of factors affecting vaccine hesitancy in all participants*

	All			
Features (subgroups (1) vs subgroups)	**Sig.**	**Exp (B)**	**95% C.I.**	
			Lower	Upper
Disease (CUA vs control)	0.25	1.74	0.68	4.46
Father's education (High school and above vs 8 years and lessß)	**0.001**	6.96	2.19	22.14
Child age	**0.041**			
Child age (24-47 vs <24)	0.087	2.88	0.86	9.67
Child age (≥48 vs <24)	**0.015**	4.13	1.32	12.97
Father's age (≥35 vs <35)	**0.04**	0.35	0.13	0.95
Antenatal Care Visit (>4 times vs 1-3 times)				
Standard vitamin D supplementation regimen during pregnancy (No vs Yes)	0.95	1.06	0.17	6.84
Standard iron supplementation regimen during pregnancy (No vs Yes)	0.22	2.56	0.58	11.33
Standard vitamin D supplementation regimen during infancy (No vs Yes)	0.556	1.89	0.23	15.62
Standard iron supplementation regimen during infancy (No vs Yes)	0.401	0.52	0.11	2.42
Childhood Vaccination§ Idea after COVID-19	**0.005**			
Childhood Vaccination§ Idea after COVID-19 (hesitant vs unaffected)	**0.007**	5.34	1.58	18.11
Childhood Vaccination§ Idea after COVID-19 (necessary vs unaffected)	0.762	0.83	0.25	2.79
Parental COVID-19 vaccine status	**0.002**			
COVID-19 vaccine (mother vs none)	**0.007**	0.11	0.22	0.55
COVID-19 vaccine (father vs none)	**0.038**	0.18	0.37	0.91
COVID-19 vaccine (both parent vs none)	**<0.001**	0.08	0.02	0.29
Influenced by social media news about vaccines	0.7			
Influenced by social media news about vaccines (hesitant vs others)	0.498	0.67	0.21	2.16
Influenced by social media news about vaccines (negative vs others)	0.442	0.62	0.18	2.12
Family Nurse/midwife as a SHI† (reliable vs others)	0.658	1.36	0.35	5.37
Government and university website as a SHI† (reliable vs others)	0.382	0.56	0.15	2.06
Book as a SHI† (reliable vs others)				
Vaccination during pregnancy (No vs Yes)	0.123	2.53	0.78	8.21
TSOY	0.056			
(≥33 vs 0-25)	**0.034**	4.57	1.12	18.65
(25-33 vs 0-25)	**0.022**	5.52	1.27	23.93
Constant	0.002	0.03		

Health literacy (HL) is a critical determinant in parental vaccine decision-making, as it requires parents to effectively interpret and evaluate complex risk-benefit information regarding their children's health. VH remains a global public health challenge that transcends individual pediatric health, impacting collective societal immunity (22). The literature reveals varying rates of VH worldwide; for instance, a large-scale study involving 4,445 parents reported a 6.1% indecision rate regarding routine vaccinations (23), whereas a study in Nigeria found that 31.6% of participants expressed hesitancy (24). The relationship between HL and vaccination status remains a subject of debate in the international literature. While research in the USA found no significant correlation between HL and vaccination adherence (25), a similar study in India demonstrated that maternal HL was directly associated with DTP_3 immunization status (26). A notable finding in our study was that VH was 4.57 times higher among participants with higher TSOY-32 scores (≥33), and this hesitancy was significantly more pronounced among those sceptical of childhood vaccination programs ($p < 0.001$). Despite these identified levels of hesitancy, the overall vaccination rate in our cohort remained high at 96.6%. This discrepancy suggests that a robust vaccination tracking system is an effective tool for maintaining high coverage and supporting parental decision-making, even in the presence of underlying hesitancy.

Table 4. *Logistic regression analysis of factors affecting vaccine hesitancy in the congenital urogenital anomaly and control group*

	CUA*				Control			
Features (subgroups (1) vs subgroups)	Sig.	Exp (B)	95% C.I.		Sig.	Exp (B)	95% C.I.	
			Lower	Upper			Lower	Upper
Maternal education (High school and above vs 8 years and lessß)	**<0.001**	6.31	2.32	17.19				
Paternal education (High school and above vs 8 years and lessß)	0.508	1.41	0.51	3.91	**0.005**	7.52	1.85	30.61
Child age	**0.017**				0.103			
Child age (24-47 vs <24)	0.396	1.58	0.55	4.56	**0.044**	3.45	1.03	11.53
Child age (≥48 vs <24)	**0.005**	4.8	1.62	14.19	0.142	2.86	0.7	11.59
Paternal age (≥35 vs <35)					**0.006**	0.21	0.07	0.64
Antenatal care visit (>4 times vs 1-3 times)	0.465	0.6	0.15	2.37				
Standard vitamin D supplementation regimen during pregnancy (No vs Yes)	0.339	0.48	0.1	2.19	0.921	0.91	0.15	5.47
Standard iron supplementation regimen during pregnancy (No vs Yes)	0.409	1.69	0.49	5.89	0.084	4.1	0.83	20.29
Standard vitamin D supplementation regimen during infancy (No vs Yes)	0.001	25.15	4.04	156.5				
Standard iron supplementation regimen during infancy (No vs Yes)	0.938	0.96	0.3	3.05				
Childhood Vaccination§ Idea after COVID-19	**<0.001**				**0.001**			
Childhood Vaccination§ Idea after COVID-19 (hesitant vs unaffected)	**0.009**	3.99	1.42	11.21	**0.002**	11.43	2.52	51.84
Childhood Vaccination§ Idea after COVID-19 (necessary vs unaffected)	**0.005**	0.18	0.06	0.6	0.67	0.76	0.21	2.69
Parental COVID-19 vaccine status	**<0.001**				**0.005**			
COVID-19 vaccine (mother vs none)	**0.002**	0.11	0.03	0.45	**0.025**	0.14	0.02	0.78
COVID-19 vaccine (father vs none)	**0.011**	0.14	0.03	0.64	**0.035**	0.19	0.04	0.89
COVID-19 vaccine (both parent vs none)	**<0.001**	0.07	0.02	0.24	**<0.001**	0.08	0.02	0.32
Influenced by social media news about vaccines	0.894				0.499			
Influenced by social media news about vaccines (hesitant vs others)	0.639	1.28	0.46	3.52	0.446	0.61	0.17	2.2
Influenced by social media news about vaccines (negative vs others)	0.809	1.16	0.35	3.81	0.249	0.38	0.07	1.98
Family Nurse/midwife as a SHI† (reliable vs others)	0.596	1.41	0.4	4.94				
Government and university website as a SHI† (reliable vs others)					0.27	0.46	0.12	1.82
Book as a SHI† (reliable vs others)					0.907	1.06	0.38	2.97
Vaccination during pregnancy (No vs Yes)	0.384	1.55	0.58	4.15				
Constant	0.195	0.25			0.044	0.17		

*=Congenital Urogenital Anomaly, ß= Primary and Secondary Education (8 years), **§**=Childhood routine vaccination programme, **†**= source of health information

A woman's journey into motherhood, characterized by a continuous period of learning and adaptation, commences during pregnancy. Throughout this phase, mothers strive to access the most accurate information to make informed and appropriate healthcare decisions for both themselves and their unborn children (27). Enhancing a mother's ability to comprehend health information is critical, as it directly influences her child's long-term well-being and life trajectory. Investing in maternal health literacy and providing access to timely, evidence-based information are essential strategies that yield benefits not only for the individual child but for society as a whole (6,28,29).

Current literature highlights a complex relationship between parental education levels and vaccine confidence. For instance, a study involving 2,214 parents in Rwanda demonstrated that individuals with lower education levels were 3.3 times more likely to express confidence in childhood vaccinations (30). Similarly, research conducted in Saudi Arabia found that VH was 1.71 times higher among those with an education level exceeding high school (31). Our findings align with and reinforce this trend, as we observed that mothers in the CUA group with a high school education or higher had 6.31 times higher odds of VH. This suggests that in more complex clinical contexts like CUA,

higher educational attainment may be associated with increased questioning or heightened sensitivity to vaccine-related information.

The sources of information accessed by parents regarding child health play a pivotal role in shaping their healthcare attitudes and decisions (7,32). In our study, healthcare professionals emerged as the most trusted sources, with parents reporting high levels of reliance on pediatricians (98.0%), family physicians (93.4%), and family medicine midwives (86.7%). This high level of trust in clinical experts is a significant asset for public health, as these professionals serve as the primary line of defence against vaccine misinformation. However, the rapid proliferation of technology, the internet, and social media platforms has created a dual-edged sword: while access to information is easier than ever, distinguishing between evidence-based facts and inaccurate claims has become increasingly challenging for parents, often leading to vaccine hesitancy (32-34). For instance, during the wave of misinformation regarding the MMR vaccine, research indicated that only a quarter of parents consulted their family physicians to verify the information they encountered (35). Similarly, studies on HPV vaccination have shown that relying on information from family and friends can increase the prevalence of insufficient knowledge by 4.34 times (36). Interestingly, our study found that exposure to health information from sources such as social media, family elders, books, television, and public or university websites had no statistically significant effect on vaccine hesitancy in either the CUA or CNM groups, suggesting that for this specific population, clinical trust may outweigh external influences.

The COVID-19 pandemic, along with the intense global debates surrounding COVID-19 vaccines, has reignited concerns regarding routine childhood immunization. Factors such as perceived side effects, vaccine ingredients, the nature of vaccine pathogens, institutional distrust, religious or cultural beliefs, and a lack of comprehensive information have historically fueled VH and continue to bring these discussions to the forefront (9,37,38). Conversely, addressing specific concerns of families regarding vaccine safety with evidence-based, scientific answers is recognized as a crucial strategy for restoring and increasing vaccine confidence (39,40). The impact of the pandemic on parental risk perception is further illustrated in studies such as that by Salazar et al., which reported that parents with higher levels of VH tended to be less concerned about the clinical risks of COVID-19 (41). Our findings contribute to this narrative by demonstrating that while VH rates were significantly lower in families where both parents were vaccinated against COVID-19, exposure to negative vaccine news led to a 3.99-fold increase in the odds of high hesitancy (VHS-uQ) within the CUA group. Based on these results, we suggest that positive outcomes from personal engagement in health practices and the acquisition of reliable health knowledge serve as protective factors, ultimately reducing VH and fostering parental trust in childhood immunization programs.

Strengths and limitations

A primary strength of this study is its multicenter design, encompassing diverse sociocultural regions, which allowed for a more comprehensive evaluation of VH among parents of children with hypospadias and undescended testes. To our knowledge, this is the first study to specifically address VH within the context of CUA. By exploring the unique psychological and clinical landscape of these families, our research provides essential guidance and a novel perspective for physicians and surgeons operating in this specialized field, highlighting the intersection of surgical diagnosis and public health adherence.

Despite these strengths, certain limitations must be acknowledged. First, while multicentric within a national context, the study lacks international scope, which limits the generalizability of the findings to different global socioeconomic and cultural environments. Additionally, the cross-sectional nature of the research precludes a longitudinal assessment of parental attitudes. As the study was conducted post-pandemic, we were unable to utilize a long-term cohort design starting prior to the COVID-19 era, which would have allowed for a more precise temporal analysis of the pandemic's direct impact on shifting vaccination perceptions.

This study identifies significant predictors of VH among mothers of children with CUA. Multivariate analysis reveals that elevated VH is associated with higher maternal education levels and non-adherence to routine infant vitamin D supplementation protocols. Conversely, protective effects were observed in families where both parents were vaccinated against COVID-19 and among those who perceived an increased necessity for childhood vaccinations following the pandemic. These findings suggest that in the specific context of CUA, VH is often intertwined with broader patterns of healthcare engagement and subjective risk assessment.

Clinically, reduced VH correlates strongly with consistent participation in preventive care, the establishment of a robust therapeutic alliance with healthcare providers, and the receipt of evidence-based health education during

clinical encounters. To improve vaccination acceptance in this population, targeted interventions should prioritize three key areas: enhancing provider-parent communication through trusted clinical relationships, delivering science-based immunization information during routine pediatric visits, and developing tailored educational strategies that specifically address the nuanced vaccine concerns of parents with higher health literacy.

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Author Contributions

Conceptualization: S.S.Y and M.S.D.; Methodology: S.S.Y, M.T., and M.S.D; Software: S.S.Y and M.S.D.; Validation: M.S.D and S.S.Y.; Formal analysis: S.S.Y; Investigation: M.S.D.; Resources: M.T., I.O., A.S.C., D.B., and S.S.; Data curation: M.S.D.; Writing – Original Draft Preparation: M.T., M.S.D, and S.S.Y.; Writing – Review and Editing: M.S.D and S.S.Y.; Visualization: M.S.D. and S.S.Y.; Supervision: S.S.Y.; Project administration: M.T., M.S.D, and S.S.Y. All 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 and was approved by the Hacettepe University non-interventional Ethics Committee (Approval No. GO 2024/05-42) on January 23, 2024.

Statement of Competing Interest

The authors declare no conflict of interest.

Statement of Data Availability

All data analyzed during this study are included in this published article and available from the corresponding author in the form of anonymized data upon reasonable request.

Statement of Generative AI Use

Not applicable.

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ORIGINAL ARTICLE

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TRANSITION CHALLENGE: THE ROUGH ROAD TO ADULTHOOD IN JUVENILE IDIOPATHIC ARTHRITIS—EXPERIENCE FROM TWO CENTERS

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The transition process to adulthood is a challenging but crucial period for the future well-being of patients with chronic diseases. The objective of this study was to evaluate whether specific clinical variables and disease activity status are associated with improved transition readiness among patients with juvenile idiopathic arthritis (JIA) and their parents. JIA patients aged 12–18 years were enrolled in this cross-sectional study. Patient characteristics and laboratory data were collected, and disease activity was assessed using the Juvenile Arthritis Disease Activity Score (JADAS-10 and JADAS-27). All patients and their parents completed the Serbian version of the Transition Readiness Assessment Questionnaire (TRAQ) simultaneously. A total of 91 JIA patients (including 27 males and 64 females; median age 15.32 years, range 11.58–18 years), along with their respective parents, were enrolled in the study. Our results demonstrated that increased patient age and the use of biologic therapy, particularly etanercept, were significantly associated with higher TRAQ scores and improved transition readiness in both JIA patients ($p < 0.001$; $p = 0.038$) and their parents ($p < 0.001$; $p = 0.035$). Patient and parent TRAQ scores showed a strong positive correlation ($\rho = 0.676$, $p < 0.001$). No significant associations were found between other clinical variables (gender, JIA disease subtype, disease duration, disease activity status, extraarticular comorbidities, and autoimmune diseases in the patient's family) and transition readiness. Older patient age and the use of biologic therapy, particularly etanercept, are positively associated with transition readiness in both JIA patients and their parents.

Keywords: transition readiness, juvenile idiopathic arthritis, disease activity, biologics

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INTRODUCTION

The most common chronic rheumatic condition in children is juvenile idiopathic arthritis (JIA) (1). It is a lifelong disease requiring continuous, regular appointments with both pediatric and adult rheumatologists until a definitive transition to the adult healthcare system occurs. Unfortunately, over 50% of JIA patients have active disease when entering adulthood. Despite this, many stop taking medications and attending regular appointments with adult rheumatologists, resulting in worse disease outcomes (2–11). To prevent permanent disability, there is an urgent need to improve transitional healthcare and ensure the successful transfer of young JIA patients to the adult healthcare system.

This transition is a complex, long-lasting, and challenging process (12). It occurs during a particularly vulnerable period of life when young people are growing up, trying to build their personal identity and become fully independent from their parents. Over the last two decades, significant advances have been made in recognizing the needs of JIA patients and their families. Detailed analyses have identified the problems and barriers to a successful transition. Nowadays, we are fully aware of the importance of providing continuous medical and psychosocial support to young people and their families, guiding them toward becoming independent and responsible adults. It is important to start education early, provide psycho-emotional and mental health support through well-defined multidisciplinary teams, and improve communication between pediatric and adult healthcare providers with individualized, well-organized transitional programs (13–17).

In recent years, assessing the transition readiness of young people with chronic diseases has become possible thanks to the development of transition readiness tools, such as the Transition Readiness Assessment Questionnaire (TRAQ) (18–20). In the current study, we aimed to extend our previous research, in which we showed that biologic use and higher academic achievement are associated with better transition readiness (21). Specifically, we aimed to evaluate the transition readiness of patients with JIA and their parents and to determine whether certain clinical variables, different biologic therapies, and disease activity status could be predictive of this challenging process.

METHODS

This cross-sectional study was approved by the Ethics Committees of the Institute of Rheumatology, Belgrade (No. 29/8, Date: 24/03/2021) and the University Clinical Centre Niš (No. 38788/8, Date: 21/12/2021), and was conducted in accordance with the principles of the Declaration of Helsinki. Between April 2021 and February 2022, we enrolled 91 patients with JIA (27 males and 64 females; median age: 15.32 years; range: 11.58–18 years) from two outpatient pediatric rheumatology clinics, along with their parents. All participants provided written informed consent after receiving a detailed explanation of the study objectives and procedures. Patient characteristics (gender, age, JIA subtype, disease duration, disease activity, extra-articular comorbidities, ongoing therapies, and family history of autoimmune diseases) were obtained from medical records. All patients and their parents completed the Serbian version of the Transition Readiness Assessment Questionnaire (TRAQ) simultaneously. During the same visit, blood samples were collected for monitoring treatment efficacy and safety. Disease activity was assessed using the Juvenile Arthritis Disease Activity Score (JADAS-10 and JADAS-27) (22).

Statistical Analysis

Data were analyzed using IBM SPSS Statistics, version 20.0 (IBM Corp., Armonk, NY, USA). Continuous and categorical variables were evaluated. Continuous variables were presented as mean ± standard deviation (SD) or median (range), depending on the data distribution. Categorical variables were expressed as absolute numbers and percentages. Comparisons between categorical variables were performed using the Chi-square (χ^2) test. Student's t-test, the Mann–Whitney U test, and ANOVA were used to compare continuous variables, as appropriate. Associations between numerical variables were assessed using Spearman's rank correlation coefficient (ρ). Statistical significance was set at $p < 0.05$.

RESULTS

A total of 91 patients with JIA were enrolled, with a female predominance (64, 70.3%). Among them, 25 (27.5%) had concomitant diseases, and 17 (18.7%) had uveitis, which was the most common extra-articular disease manifestation. A family history of autoimmune diseases

was recorded in 24 (26.4%) patients. All patients received disease-modifying antirheumatic drugs (DMARDs), while 51 (56.0%) underwent treatment escalation with biologic therapies. During the follow-up period, biologic agents were switched in some patients due to therapeutic ineffectiveness; overall, 56 patients received TNF inhibitors (etanercept, n = 28; adalimumab, n = 28), and 14 received the IL-6 inhibitor tocilizumab. The analyzed data showed a strong correlation between patient and parent total TRAQ scores ($\rho = 0.676$, $p < 0.001$) (Figure 1).

Patient age and the use of biologic therapy were shown to have a statistically significant impact on TRAQ scores, resulting in higher scores and greater readiness for transition among patients with JIA ($p < 0.001$ and $p = 0.038$, respectively) and their parents ($p < 0.001$ and $p = 0.035$, respectively). We also found that total TRAQ score values were statistically significantly higher among patients with JIA treated with etanercept and their parents ($p = 0.012$; $p = 0.018$) (Table 1).

There was no statistically significant association between clinical disease variables (JIA subtype, gender, disease duration, extra-articular comorbidities, family history of autoimmune diseases, and disease activity status assessed using JADAS-10 and JADAS-27) and total TRAQ scores or readiness for transition in either group.

DISCUSSION

In recent decades, significant efforts have been made to improve the transition process. Nowadays, we know that transition should occur gradually, beginning several years in advance, and be facilitated by well-organized multidisciplinary teams whose role is to provide psychosocial and mental health education and support for young adults and their families. This approach helps them accept the new adult rheumatologist and increase their level of self-responsibility for their health. Transition represents a challenging bridge between childhood and adulthood that children with chronic conditions, including JIA, must cross together with their families. Throughout this journey, pediatric and adult rheumatologists must support young people with chronic rheumatic conditions to help them achieve a successful transition and become responsible adults with regard to their health.

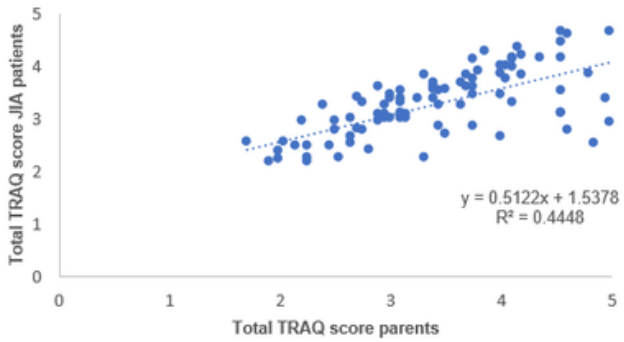

Figure 1. *Strong positive correlation between total Transition Readiness Assessment Questionnaire (TRAQ) scores among JIA patients and their parents*

The main focus of our study was to identify clinical variables associated with JIA that positively predict transition readiness in patients with JIA and their parents. To our knowledge, this is the first study to evaluate transition readiness among parents of patients with JIA. Our results revealed that total TRAQ scores did not differ significantly between patients with JIA and their parents and that the scores were strongly correlated, which may be explained by the tendency of parents to be overprotective of children with chronic diseases (23). This finding is consistent with those reported by a group of Turkish authors (24). Our results also suggest that age improves self-management skills and transition readiness among patients with JIA, indicating that older patients have a higher level of responsibility. Similar findings have been reported by other authors (23, 25–27). In contrast, Jensen et al. (28) and Sonmez et al. (24) found that increasing patient age was not associated with better transition readiness.

Table 1. *Effect of different biologic therapies on Transition Readiness Assessment Questionnaire (TRAQ) scores in patients with juvenile idiopathic arthritis (JIA) and their parents*

Biologic treatment usage	TRAQ JIA patients	TRAQ parents
Yes	3.4 ± 0.62	3.48 ± 0.83
No	3.13 ± 0.61	3.3 ± 0.81
p	0.038	0.035
Adalimumab		
Yes	3.16 ± 0.55	3.13 ± 0.77
No	3.34 ± 0.66	3.52 ± 0.82
p	0.228	0.335
Etanercept		
Yes	3.53 ± 0.57	3.71 ± 0.75
No	3.17 ± 0.62	3.27 ± 0.82
p	0.012	0.018
Tocilizumab		
Yes	3.48 ± 0.86	3.31 ± 0.63
No	3.51 ± 0.82	3.62 ± 0.66
p	0.867	0.678

Data are expressed as mean ± standard deviation; $p < 0.05$ was considered statistically significant.

We also found that the use of biologics (21), particularly etanercept, had a statistically significant positive effect on transition readiness in both patients with JIA and their parents. This may be explained by the fact that biologic therapy can lead to better disease control and more rapid achievement of remission. Similar to Batu et al. (29), we found no significant association between disease activity scores (JADAS-10 and JADAS-27) and transition readiness. In contrast, a group of Turkish authors reported that active JIA was a predictor of lower transition readiness, while a group of Thai authors found that disease inactivity predicted lower transition readiness (30). Our data demonstrated that patient gender was not significantly associated with TRAQ scores or transition readiness, which is consistent with findings reported by other authors (23–25, 28). However, some studies have shown that female patients with JIA have better transition readiness than males (27, 31–33). Other clinical variables (JIA subtype, disease duration, extra-articular comorbidities, and family history of autoimmune diseases) also showed no association with TRAQ scores or transition readiness. In contrast, Bingham et al. (26) reported that longer disease duration, extra-articular comorbidities, and a family history of autoimmune diseases were associated with higher TRAQ scores and greater transition readiness.

Transition is a highly challenging process that requires cooperation not only among healthcare professionals but also between patients with JIA and their families. This sensitive process takes time and requires patience and understanding of the evolving needs of young people with chronic diseases, supported by appropriate medical and psychosocial care. Transition should be individualized for each patient and family and adjusted to the healthcare system of each country.

In summary, we conclude that older age and the use of biologic therapy, particularly etanercept, have a positive impact on transition readiness among both patients with JIA and their parents. Future studies should include larger patient cohorts and well-organized transitional clinics to facilitate and improve this challenging and complex process.

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Author Contributions

Conceptualization: D.L.; Methodology: D.L., G.S., and H.S.; Investigation and data curation: D.N., D.L., and S.Dj.; Formal analysis: H.S. and S.Dj.; Literature search: M.Z.; Writing – original draft: D.L.; Writing – review & editing: D.N. and S.Dj. 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 Institute of Rheumatology, Belgrade (approval No. 29/8, issued on March 24, 2021) and the Ethics Committee of the University Clinical Centre Niš (approval No. 38788/8, issued on December 21, 2021). The study was conducted in accordance with the ethical principles of the Declaration of Helsinki, and written informed consent was obtained from all participants.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availability

All data analyzed in this study are presented in the Results section. Additional data are available from the corresponding author upon reasonable request.

Statement of Generative AI Technologies Use

No generative AI was used.

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THE EFFECT OF SGLT-2 INHIBITORS ON HEMOGLOBIN AND HEMATOCRIT LEVELS IN PATIENTS WITH TYPE 2 DIABETES: A RETROSPECTIVE REAL-WORLD ANALYSIS

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SGLT-2 inhibitors have been associated with increases in hemoglobin and hematocrit levels; however, the independence of this effect from confounding factors remains unclear in real-world settings. This study aimed to determine whether hemoglobin and hematocrit levels differed between patients with type 2 diabetes mellitus using SGLT-2 inhibitors and the control group and to investigate whether this effect was independent of factors such as age, sex, ferritin level, glycemic control, and renal function. Data were retrospectively collected from 205 adult patients at the Internal Medicine Clinic of Düziçi State Hospital. Patients were divided into two groups: those using an SGLT-2 inhibitor for at least three months (n = 103) and the control group (n = 102). Hemoglobin, hematocrit, ferritin, HbA1c, and eGFR levels were compared. Subgroup analyses and propensity score matching (PSM) were performed. Median hemoglobin (14.90 (12.6–18.2) vs. 13.00 (12.0–16.06) g/dL, $p < 0.001$) and hematocrit (45.20 (37.6–54.1) vs. 40.00 (32.2–49.6)%, $p < 0.001$) were significantly higher in the SGLT-2 group. This increase remained significant across all subgroups (sex, age, ferritin, HbA1c) and persisted after PSM ($p < 0.001$). Higher hematological parameters were observed with a treatment duration of ≥12 months. SGLT-2 inhibitors are associated with significantly higher hemoglobin and hematocrit levels in patients with type 2 diabetes, independent of iron stores and glycemic control. The underlying mechanism likely involves increased erythropoiesis beyond hemoconcentration. Periodic hematological monitoring is recommended.

Keywords: SGLT-2 inhibitors, hemoglobin, hematocrit, erythrocytosis, type 2 diabetes

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INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disease that is not limited to glycemic imbalance but also affects the hematological and renal systems, presenting with micro- and macrovascular complications (1). The coexistence of anemia and reduced renal function in patients with diabetes significantly increases the risk of cardiovascular mortality (2).

Sodium-glucose co-transporter 2 (SGLT-2) inhibitors (e.g., empagliflozin and dapagliflozin) are oral antidiabetic agents that exert their effects by blocking glucose reabsorption in the proximal renal tubule. Large-scale clinical trials conducted in recent years (EMPA-REG OUTCOME, DAPA-HF) have demonstrated that these drugs not only provide glycemic control (3,4), but also reduce cardiovascular events and renal progression (5,6).

An increase in Hb and Hct levels has been consistently observed with SGLT-2 inhibitor use in randomized controlled trials and meta-analyses (7–9). Although initially considered a consequence of hemoconcentration due to osmotic diuresis, recent data suggest that increased erythropoietin (EPO) production and erythropoiesis stimulation may also play a role (10,11). The proposed mechanism involves SGLT-2 inhibition reducing metabolic stress in the renal cortex, thereby activating the hypoxia-inducible factor-2α (HIF-2α) pathway in peritubular fibroblasts, which in turn stimulates EPO synthesis and augments iron utilisation (7,11). This pathway is distinct from and additive to hemoconcentration. The hypothesis that this hematological effect may represent a potential mechanism mediating the cardiovascular and renal benefits of SGLT-2 inhibitors is currently an area of active investigation.

While randomized trials have established the association between SGLT-2 inhibitor use and increased Hb/Hct, real-world data confirming this effect across diverse subgroups and after adjustment for multiple confounders are still needed. The extent to which this association persists independently of iron stores, renal function, and glycemic control in routine clinical practice warrants further real-world evaluation.

This study aimed to provide real-world confirmatory evidence for the association between SGLT-2 inhibitor use and changes in Hb and Hct levels in patients with type 2 diabetes, and to examine whether this association is independent of potential confounding factors such as age, sex, ferritin, and glycemic control. Unlike previous studies, this analysis specifically evaluated the effect within a low-ferritin subgroup, examined the impact of treatment duration on hematological response, and performed propensity score matching to support the independence of these findings from key demographic and glycemic variables.

METHODS

Study design and population

This retrospective cross-sectional study was conducted at the Internal Medicine Clinic of Düziçi State Hospital and reported in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines. Approval was obtained from the Scientific Research Ethics Committee of Adana City Training and Research Hospital (Decision No: 508, Date: August 5, 2025). The study was conducted in accordance with the principles of the Declaration of Helsinki.

A total of 205 adults with type 2 diabetes were included. Patients were divided into two groups:

·SGLT-2 inhibitor group (n = 103): patients who had been regularly using empagliflozin or dapagliflozin for at least three months;

·Control group (n = 102): patients with type 2 diabetes mellitus not using SGLT-2 inhibitors.

Exclusion criteria: type 1 diabetes, known hematological malignancies, active bleeding, a history of major surgery or blood transfusion within the last three months, chronic liver disease, and incomplete laboratory data.

Sample size justification

The sample size of 205 patients was determined based on a priori power analysis. Using previously published effect sizes for Hb differences between SGLT-2 inhibitor users and controls (mean difference approximately 1.5–2.0 g/dL, SD approximately 1.5 g/dL) (9), with α = 0.05 and 80% power, a minimum of 90 patients per group was required. The final sample of n = 103 (SGLT-2) and n = 102 (control) slightly exceeded this minimum, providing adequate power to detect clinically meaningful differences.

Variables and definitions

Demographic data (age, sex) and laboratory parameters (Hb, Hct, ferritin, HbA1c, creatinine) were retrieved from the hospital information management system. The eGFR was calculated using the CKD-EPI formula. The type and duration of SGLT-2 inhibitor use were recorded for the SGLT-2 inhibitor group. Ferritin values were measured contemporaneously with Hb and Hct as part of the same outpatient blood panel.

The ferritin threshold of <30 ng/mL was adopted from established clinical guidelines for iron deficiency in patients with diabetes and chronic disease, as this value has been associated with impaired erythropoietic response independent of absolute anemia (12). This threshold was used to identify a subgroup in whom iron deficiency would be a plausible alternative explanation for lower Hb levels, thus permitting a more stringent test of the SGLT-2 inhibitor effect.

Statistical analysis

Data analysis was performed using IBM SPSS Statistics 25.0. Normality of continuous variables was examined using the Kolmogorov-Smirnov test. Non-normally distributed data are presented as median (min–max); the Mann-Whitney U test was used for intergroup comparisons. Normally distributed data are presented as mean ± SD. Categorical variables were analyzed using Pearson's chi-square test.

To address potential confounding, two complementary analytical approaches were used. First, Propensity Score Matching (PSM) was applied using age, sex, and HbA1c as matching variables (1:1 nearest-neighbour matching; balanced sample n = 47 vs. n = 47). Second, multivariable linear regression analyses were conducted with Hb and Hct as dependent variables, adjusting for age, sex, HbA1c, eGFR, ferritin, and SGLT-2 inhibitor use. Effect sizes are reported as median differences with 95% confidence intervals where applicable. Given the exploratory nature of subgroup analyses (sex, age, ferritin, HbA1c, treatment duration), Bonferroni correction was applied; the adjusted significance threshold for subgroup comparisons was set at $p < 0.010$. Statistical significance for primary analyses was set at $p < 0.05$.

RESULTS

The demographic data of the 205 patients are presented in Table 1. No significant difference was found between groups in terms of mean age (57.75 ± 6.79 vs. 55.85 ± 7.77; p = 0.063). The proportion of male patients was significantly higher in the SGLT-2 inhibitor group (57.3%) than in the control group (39.2%) (p = 0.010).

Both Hb and Hct values were significantly higher in patients using SGLT-2 inhibitors compared to the control group (Table 2). Median Hb was 14.90 g/dL in the SGLT-2 inhibitor group and 13.00 g/dL in the control group (median difference: +1.90 g/dL, 95% CI: 1.50–2.30 g/dL; p < 0.001). Median Hct was 45.20% in the SGLT-2 inhibitor group versus 40.00% in the control group (median difference: +5.20%, 95% CI: 4.20–6.20%; p < 0.001).

Table 1. *Comparison of sociodemographic variables between groups*

Variables	SGLT-2 Inhibitor N = 103	Control N = 102	p
Age			
Mean ± SD	57.75 ± 6.79	55.85 ± 7.77	0.063[a]
Median (min–max)	57.9 (42–70)	56.5 (37–69)	
<58 years	46 (44.7%)	54 (52.9%)	0.236[b]
≥58 years	57 (55.3%)	48 (47.1%)	
Sex			
Male	59 (57.3%)	40 (39.2%)	0.010[b]
Female	44 (42.7%)	62 (60.8%)	

[a]Independent samples t-test; [b]Pearson Chi-Square test. P < 0.05 is considered statistically significant.

Table 2. *Comparison of hemoglobin and hematocrit values between groups*

Variables	SGLT-2 N = 103 Median (min–max)	Control N = 102 Median (min–max)	Median difference (95% CI)	p
Hemoglobin (Hb, g/dL)	14.90 (12.6–18.2)	13.00 (12.0–16.06)	+1.90 (1.50–2.30)	<0.001
Hematocrit (Hct, %)	45.20 (37.6–54.1)	40.00 (32.2–49.6)	+5.20 (4.20–6.20)	<0.001

Mann-Whitney U test. P < 0.05 is considered statistically significant. Hb: hemoglobin; Hct: hematocrit; CI: confidence interval.

Subgroup analyses demonstrated that SGLT-2 use was associated with higher Hb and Hct levels in both male ($p < 0.001$) and female ($p < 0.001$) patients. When the study population was divided by median age (<58 and ≥58 years), Hb and Hct values were significantly higher in both age subgroups among SGLT-2 inhibitor users ($p < 0.001$). Of note, even in patients with low ferritin levels (<30 ng/mL), Hb and Hct values remained significantly higher in the SGLT-2 inhibitor group ($p < 0.001$), suggesting that the observed association is not solely attributable to iron stores. In both good (HbA1c <7%) and poor (HbA1c ≥7%) glycemic control subgroups, SGLT-2 inhibitor use was associated with increased hematological parameters (Table 3). All subgroup findings remained significant after Bonferroni correction.

In patients who used SGLT-2 inhibitors for more than 12 months (n = 48), Hb (14.9 g/dL) and Hct (45.3%) values were significantly higher than those in patients who used them for less than 12 months (n = 55; Hb: 13.4 g/dL, Hct: 40.6%) ($p < 0.001$) (Table 4). This duration-dependent effect suggests

Table 3. *Comparison of hemoglobin and hematocrit levels between the SGLT-2 inhibitor and control groups according to subgroups*

Subgroups	Variable	SGLT-2 Inhibitor median	Control median	p
Sex				
Male (SGLT-2: n = 59; Control: n = 40)	Hb (g/dL)	15	14	<0.001
	Hct (%)	46	42	<0.001
Female (SGLT-2: n = 44; Control: n = 62)	Hb (g/dL)	14	12	<0.001
	Hct (%)	42	38	<0.001
Age				
<58 years (SGLT-2: n = 46; Control: n = 54)	Hb (g/dL)	15	13.3	<0.001
	Hct (%)	45	40	<0.001
≥58 years (SGLT-2: n = 57; Control: n = 48)	Hb (g/dL)	14	12	<0.001
	Hct (%)	45	39	<0.001
Ferritin level				
<30 ng/mL (SGLT-2: n = 14; Control: n = 18)	Hb (g/dL)	14	12.7	<0.001
	Hct (%)	44	39	<0.001
≥30 ng/mL (SGLT-2: n = 89; Control: n = 84)	Hb (g/dL)	15	13	<0.001
	Hct (%)	45	40	<0.001
Glycemic control (HbA1c)				
<7% (SGLT-2: n = 17; Control: n = 18)	Hb (g/dL)	15	13.7	0.008
	Hct (%)	45	41	0.007
≥7% (SGLT-2: n = 86; Control: n = 84)	Hb (g/dL)	14	13	<0.001
	Hct (%)	45	39	<0.001

Mann-Whitney U test. Bonferroni-corrected significance threshold: $p < 0.010$. All subgroup differences remain significant after correction. Hb: hemoglobin; Hct: hematocrit; HbA1c: glycated hemoglobin.

Table 4. *Comparison of hemoglobin and hematocrit values according to SGLT-2 inhibitor duration of use (SGLT-2 group only, n = 103)*

Variables	<12 months N = 55 Median	≥12 months N = 48 Median	p
Hemoglobin (Hb, g/dL)	13.4	14.9	<0.001
Hematocrit (Hct, %)	40.6	45.3	<0.001

Mann-Whitney U test. $P < 0.05$ is considered statistically significant. Analysis performed within the SGLT-2 inhibitor group only (n = 103). Hb: hemoglobin; Hct: hematocrit.

Table 5. *Propensity score matching results*

Variable	SGLT-2 Inhibitor N = 47	Control N = 47	p
Hb (g/dL) – Median (IQR)	14.80 (13.95–15.65)	13.30 (12.40–14.45)	<0.001
Hct (%) – Mean ± SD	44.92 ± 3.78	40.91 ± 3.70	<0.001

Mann-Whitney U test for Hb; independent samples t-test for Hct. $p < 0.05$ is considered statistically significant. Matching variables: age, sex, HbA1c. Hb: hemoglobin; Hct: hematocrit; IQR: interquartile range; SD: standard deviation.

a sustained and potentially progressive hematological response. There was no significant difference between empagliflozin and dapagliflozin subgroups regarding Hb and Hct levels.

After propensity score matching based on age, sex, and HbA1c (n = 47 SGLT-2 inhibitor vs. n = 47 control), the SGLT-2 inhibitor group continued to have significantly higher Hb (14.80 vs. 13.30 g/dL) and Hct (44.92 ± 3.78% vs. 40.91 ± 3.70%) values than the control group ($p < 0.001$) (Table 5). Multivariable linear regression, adjusting for age, sex, HbA1c, eGFR, and ferritin, confirmed that SGLT-2 inhibitor use was independently associated with higher Hb ($\beta = 1.74$, 95% CI: 1.30–2.18, $p < 0.001$) and higher Hct ($\beta = 4.88$, 95% CI: 3.60–6.16, $p < 0.001$) (Table 6).

In the eGFR subgroup analysis, the association between SGLT-2 inhibitor use and increased Hb/Hct was observed regardless of whether eGFR was above or below 60 mL/min/1.73m^2. Although direct statistical comparisons across eGFR strata were limited by subgroup sample sizes, the consistency of effect across renal function categories provides preliminary real-world support for the notion that the hematological association is not confined to patients with preserved renal function.

Table 6. *Multivariable linear regression analysis for hemoglobin and hematocrit*

Dependent Variable	β (SE)	95% CI	p
Hemoglobin (g/dL)	1.74 (0.22)	1.30–2.18	<0.001
Hematocrit (%)	4.88 (0.65)	3.60–6.16	<0.001

Unstandardized regression coefficients (β) for SGLT-2 inhibitor use (reference: control group). Model adjusted for age, sex, HbA1c, eGFR, and ferritin. SE: standard error; CI: confidence interval; Hb: hemoglobin; Hct: hematocrit; eGFR: estimated glomerular filtration rate.

DISCUSSION

This real-world study demonstrates that Hb and Hct levels are significantly higher in patients with type 2 diabetes who use SGLT-2 inhibitors than in non-users, and that this association persists after adjustment for age, sex, ferritin level, and glycemic control. These findings are consistent with and extend the existing literature from randomized controlled trials and meta-analyses, offering confirmatory evidence in routine clinical settings.

The present study provides real-world confirmatory evidence of an already-established association, while adding novel observations about subgroup consistency and treatment duration effects. The association between SGLT-2 inhibitor use and increased Hb/Hct has been well documented in randomized trials such as EMPA-REG OUTCOME and DAPA-HF, and confirmed in meta-analyses (9). The present study does not claim mechanistic novelty but contributes to external validity by confirming these findings in an unselected real-world cohort from a single community-level institution in Turkey, where patients with comorbidities typically excluded from trials are represented. Additionally, the ferritin-stratified subgroup analysis and treatment duration comparison represent analytical dimensions not uniformly addressed in the prior literature.

The Hb increase (+1.90 g/dL) and Hct increase (+5.20%) observed in our study are consistent with those reported in previous meta-analyses. The meta-analysis by Tian et al. (9) reported an average Hb increase of approximately +0.62 g/dL; the larger absolute difference in our study may reflect the cross-sectional design (comparing prevalent users to non-users) rather than within-patient change, and the relatively longer mean duration of SGLT-2 inhibitor use in our cohort.

The mechanism by which SGLT-2 inhibitors increase Hct was initially attributed to plasma volume reduction (hemoconcentration) due to osmotic diuresis (7). However, several lines of evidence suggest a concurrent erythropoietic mechanism. Mazer et al. (11) demonstrated that empagliflozin significantly increases EPO levels and optimizes iron utilisation in patients with type 2 diabetes and coronary artery disease. The proposed pathway involves SGLT-2 inhibition reducing glucose and sodium reabsorption in the proximal tubule, thereby decreasing the oxygen demand of the Na-K-ATPase–dependent transport machinery. This reduction in tubular metabolic stress improves cortical oxygenation and activates the HIF-2α pathway in peritubular fibroblasts, leading to augmented EPO synthesis (7,11). Additionally, SGLT-2 inhibitors may improve iron bioavailability by modulating hepcidin regulation and optimizing erythropoietic iron utilization (11). The observation in our study that Hb and Hct remained significantly higher in SGLT-2 inhibitor users even among patients with low ferritin levels (<30 ng/mL), and that the effect was more pronounced with longer treatment duration (≥12 months), is consistent with this erythropoietic hypothesis rather than simple hemoconcentration. However, the absence of direct EPO measurements in our study limits mechanistic inference, and these observations should be interpreted as associative rather than causal.

Regarding the class effect, both empagliflozin and dapagliflozin were associated with comparable hematological changes in our cohort, with no significant difference between the two agents. This is consistent with prior pharmacological data suggesting a shared mechanism. However, since canagliflozin was not evaluated in this study, conclusions about a class effect should be drawn cautiously and would require further validation including other SGLT-2 inhibitors.

Clinically, the observed Hb and Hct increases have dual implications. Improved oxygen-carrying capacity may contribute to the cardioprotective and renoprotective benefits of SGLT-2 inhibitors (9). Conversely, polycythaemia-related risks—including increased blood viscosity and potential thrombotic complications—warrant attention (12,13). In our cohort, median Hct in the SGLT-2 group was 45.2%, within safe limits; however, values up to 54.1% were observed. Periodic complete blood count monitoring is therefore recommended, particularly in male patients with high baseline Hb values.

Limitations of this study include its retrospective design, single-centre nature, absence of direct EPO measurements, and the inability to account for all potential confounders (e.g., erythropoiesis-stimulating agent use, dietary factors, physical activity). The PSM model was limited to age, sex, and HbA1c due to sample

size constraints. The cross-sectional design precludes causal inference; all findings should be interpreted as associations. Selection bias inherent in retrospective single-center studies limits generalizability. The multivariable regression model strengthens the independence claim but cannot fully exclude residual confounding.

SGLT-2 inhibitor use is significantly associated with increased Hb and Hct levels in patients with type 2 diabetes in a real-world clinical setting, independently of iron stores and glycemic control. This association was consistent across sex, age, and glycemic control subgroups and was more pronounced with longer treatment duration. No significant difference was observed between empagliflozin and dapagliflozin. These findings provide confirmatory real-world support for the hematological effects of SGLT-2 inhibitors and are consistent with an erythropoietic mechanism, although causal inference cannot be established from this design. Clinicians should consider potential hematological changes when prescribing these agents and perform appropriate follow-up.

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Author Contributions

Conceptualization: A.T. and M.S.B.; Methodology: A.T. and M.S.B.; Formal analysis: A.T.; Investigation: A.T. and M.S.B.; Writing – Original draft: A.T. and M.S.B.; Writing – review & editing: A.T. All authors have read and agreed to the published version of the manuscript.

Statement of Ethics

This study protocol was reviewed and approved by the Scientific Research Ethics Committee of Adana City Training and Research Hospital, approval number 508, issued on august 5, 2025. This study was conducted in accordance with the principles of the Declaration of Helsinki. Due to the retrospective nature of the study and the use of anonymized data from medical records, the ethics committee approved this study without requiring individual informed consent.

Statement of Competing Interest

The authors declare no relevant 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

Generative artificial intelligence (Claude, Anthropic) was used to assist with translation and English language editing during manuscript preparation. The authors take full responsibility for the accuracy and integrity of all content.

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FUSOBACTERIUM SPECIES IN ORAL AND MAXILLOFACIAL INFECTIONS: MICROBIAL ECOLOGY, COMMUNITY DYNAMICS, AND ANTIMICROBIAL SUSCEPTIBILITY

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Oral and maxillofacial infections (OMIs) are polymicrobial conditions in which obligate anaerobes, particularly *Fusobacterium* spp., play an important pathogenic role. Despite their relevance, the species distribution, ecological context, and antimicrobial susceptibility of *Fusobacterium* (F. -hereinafter) in OMIs remain incompletely understood. This study aimed to characterize their occurrence, co-isolation patterns, and resistance profiles to improve diagnostic accuracy and guide empirical therapy. Clinical specimens collected between October 2018 and August 2023 from 41 patients were cultured under aerobic and anaerobic conditions. *Fusobacterium* isolates were identified using MALDI-TOF MS, and susceptibility was reinterpreted according to EUCAST version 16 (2026). In total, 42 isolates were recovered, predominantly *F. nucleatum* (66.7%) and *F. necrophorum* (26.2%), with *F. periodonticum*, *F. gonidiaformans*, and *F. canifelinum* each representing 2.4%. Species distribution varied by specimen type: among 28 stomatognathic samples, *F. nucleatum* was found in 85.7%, *F. necrophorum* in 10.7%, and *F. periodonticum* in 3.6%. Peritonsillar abscesses were dominated by *F. necrophorum* (80%). All maxillary sinus samples exclusively contained *F. nucleatum*. Polymicrobial infections occurred in 85.4% of cases, frequently involving *Streptococcus* spp. (including the *S. anginosus* group) and members of the *Prevotellaceae* family, whereas peritonsillar abscesses more often exhibited monomicrobial growth. Isolates showed high susceptibility to β-lactam/β-lactamase inhibitor combinations and carbapenems, while metronidazole and clindamycin displayed broader MIC distributions. These findings highlight the importance of assessing the co-occurrence of *Fusobacterium* species with other members of the polymicrobial community within OMIs, as understanding these interspecies associations is crucial for selecting effective empirical therapy.

Keywords: anaerobic bacteria, antimicrobial susceptibility, biofilm, *Fusobacterium* spp., oral and maxillofacial infection, polymicrobial infections

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INTRODUCTION

Oral and maxillofacial infections (OMIs) remain a frequent and clinically significant problem in dentistry and otolaryngology. Most OMIs originate from odontogenic foci; however, infections related to trauma, surgical procedures, diseases of the paranasal sinuses, and the upper respiratory tract are also commonly encountered. When inadequately treated or when source control is delayed, OMIs may lead to deep neck infections, mediastinitis, sepsis, cavernous sinus thrombosis, intracranial or orbital abscesses, and osteomyelitis (1). Because empiric antimicrobial therapy is often initiated before definitive microbiological results are available, a robust and accurate definition of the typical polymicrobial spectrum, including anaerobic contributors, is decisive for early therapeutic decision-making.

According to current knowledge, anaerobic bacteria are prominent contributors to OMIs, with *Prevotella* and *Porphyromonas* among the genera most frequently recovered from odontogenic and deep-space infections (2). Species of the genus *Fusobacterium* are Gram-negative, obligate anaerobes that colonize the oral cavity and, to a lesser extent, the gastrointestinal tract. Their increasing recognition in clinical practice likely reflects both improvements in anaerobic diagnostic workflows and greater awareness of their pathogenic potential in polymicrobial infections (3). *F. nucleatum* is particularly prominent within the oral niche. It functions as a bridging organism within dental biofilms, mediating co-aggregation between early and late colonizers via multiple adhesins (RadD, Fap2, FadA, CmpA, FomA) and related virulence-associated factors (Figure 1). These ecological functions support the development and stability of complex microbial communities and have been linked to inflammatory and tissue-destructive processes in periodontal and deep-space infections (4, 5). Although *Fusobacterium* spp. are most commonly associated with periodontal and odontogenic infections, they have also been implicated in a spectrum of extraoral diseases, e.g., pleuropulmonary liver abscesses, intracranial infections, and intra-abdominal pathology (6-9). Besides *F. nucleatum*, other clinically relevant species are *F. necrophorum, F. gonidiaformans, and F. periodonticum* (10-13). Notably, *F. necrophorum* is classically associated with Lemierre syndrome and is also recovered from severe head and neck infections in both pediatric and adult populations (10). Despite its considerable clinical significance, the local epidemiology, species distribution and antimicrobial susceptibility patterns of the *Fusobacterium* species involved in OMIs remain poorly understood. This is particularly evident in routine clinical settings, where the fastidious nature of anaerobic organisms continues to hinder reliable diagnostics. The need for optimal sampling further exacerbates this issue, including timely transport under anaerobic conditions, accurate species-level identification, and appropriate antimicrobial susceptibility testing. In addition, the interpretation of antibiotic susceptibility data over time is influenced by the evolution of antibiotic susceptibility testing (AST) methodologies and breakpoint criteria for anaerobes, which are systematically updated by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical

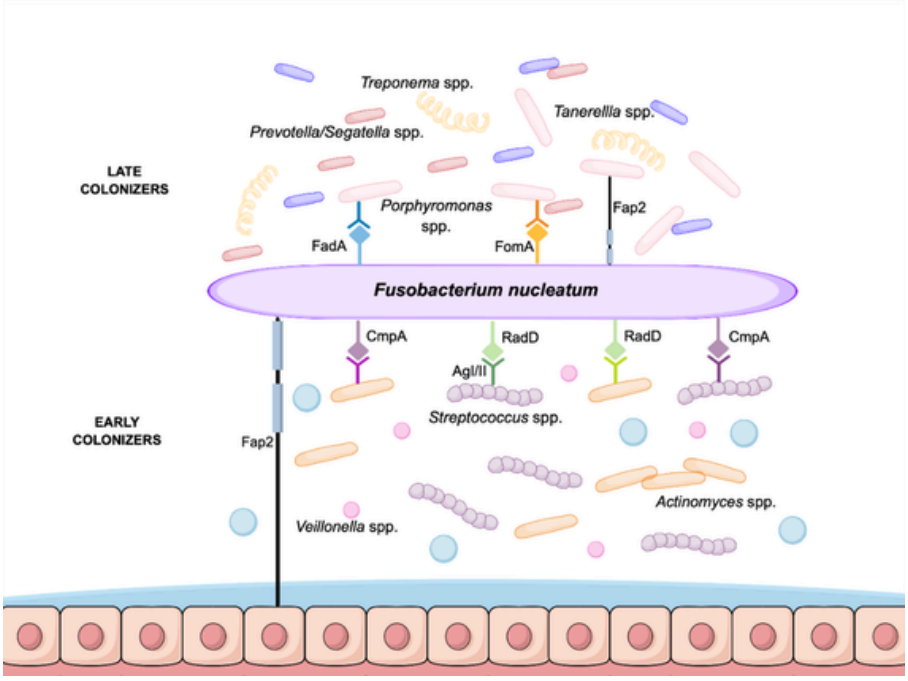

Figure 1. *F. nucleatum as a bridging organism linking early and late oral biofilm colonizers* *F. nucleatum* mediates co-aggregation between early colonizers (e.g., *Streptococcus, Actinomyces, Veillonella)* and late colonizers (e.g., *Porphyromonas, Prevotella/Segatella, Treponema, Tannerella)* through multiple adhesins, including FadA, FomA, Fap2, RadD, CmpA.

and Laboratory Standards Institute (CLSI) to improve standardization and clinical relevance. Although these revisions strengthen the reliability of contemporary interpretations, they also limit direct comparability with historical datasets, as apparent temporal changes may reflect shifts in interpretive criteria rather than true epidemiological trends (14, 15).

This study aimed to investigate the prevalence and species distribution of *Fusobacterium* spp. in oral and maxillofacial infections and sinusitis, characterize their co-occurrence with other microorganisms, and to evaluate the antimicrobial susceptibility of *Fusobacterium* isolates with the aim of improving diagnostic and therapeutic decision-making in polymicrobial OMIs.

METHODS

A retrospective analysis of microbiological results was conducted using data obtained from clinical specimens collected from patients diagnosed with infections of the stomatognathic system and other maxillofacial tissues. Between October 2018 and August 2023, *Fusobacterium* spp. was identified in samples obtained from 41 hospitalized patients with OMIs. Patients were treated at the Department of Craniofacial and Maxillofacial Surgery (N = 33) and the Department of Pediatric Otolaryngology (N = 8), both located within a tertiary care hospital in Poland. Clinical specimens were collected by aspiration or surgical drainage, deep tissue biopsy from the affected sites, or deep wound swabbing. All samples were obtained in accordance with standard clinical procedures and transported under anaerobic conditions. Specimens were categorized according to anatomical site and patient age group (adult/pediatric) as follows: stomatognathic system infections (pus, biopsy tissue, deep wound swabs; 27/1), maxillary sinus infections (aspirates; 4/0), and peritonsillar abscesses (swabs; 2/7). Only microbiological results in which *Fusobacterium* spp. were identified, either as a sole pathogen or as part of a polymicrobial culture, were included in the analysis. All specimens were cultured under aerobic and anaerobic conditions using routine microbiological methods. Anaerobic cultures were performed on Schaedler agar supplemented with vitamin K1 and 5% sheep blood and incubated at 37°C for 48-72 h in an anaerobic chamber with a gas mixture of 85% N_2, 10% CO_2, and 5% H_2. Aerobic cultures were grown on Columbia blood agar with 5% sheep blood and on MacConkey agar, incubated at 37°C for at least 24 h. Fungal cultures were performed on Sabouraud agar, incubated aerobically at 30°C for 48 h. All culture media were supplied by bioMérieux, France. Species identification was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Bruker, Germany). Antibiotic susceptibility testing was performed using the Epsilometer test (bioMérieux, France) and interpreted according to EUCAST recommendations applicable at the time of testing (versions 8.0–13.1). Periodic revisions of EUCAST guidelines, especially between 2018 and 2021, limit the direct comparability of antimicrobial susceptibility results across time. The minimum inhibitory concentration (MIC) values derived from routine diagnostic testing were available only for some *Fusobacterium* spp. isolates. Routine MIC testing was not consistently performed during the earlier study period, partly because interpretive criteria were not yet established. To ensure comparability, all available MIC results were reinterpreted according to the current EUCAST criteria (version 16.0, 2026).

Data distribution was assessed using the Shapiro-Wilk test. Categorical variables, including comparisons between age groups, were analyzed using Fisher's exact test. Statistical significance was defined as $P < 0.05$. Statistical analyses and data visualization were performed using R software version 4.5.3 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Specimen characteristics

During the study period, 41 clinical specimens were analyzed. Most samples originated from the stomatognathic system (28/41; 68.3%), including pus aspirates (13; 31.7%), deep wound swabs (12; 29.3%), and biopsy tissue (3; 7.3%). Additional specimens comprised swabs from peritonsillar abscesses (9/41; 22.0%) and maxillary sinus biopsies (4/41; 9.8%).

Patient demographics

The adult cohort included 17 females (51.5%) and 16 males (48.5%). The pediatric cohort showed a similar sex distribution (50/50%). The median age of adult patients was 40.5 years (IQR: 36.8–66.8), and the median age of pediatric patients was 17 years (IQR: 9.5-17.25).

Fusobacterial infections were most frequently observed in the 30–39 years (24%) and 70–79 years (20%) age groups; however, age-group distribution did not differ significantly ($P = 0.7902$).

Patient characteristics varied by infection site, with marked differences in age distribution across stomatognathic

infections, sinusitis, and peritonsillar abscess. Stomatognathic infections (N = 28): the mean age was 49.0 years, and the median age was 48.0 years (IQR 33.0–70.0); peritonsillar abscess (N = 9): the mean age was 17.56 years, and the median age was 17.0 years (IQR 9.0–25.5), sinusitis (N = 4): the mean age was 48.25 years, and the median age was 42.0 years (IQR 35.0–61.5).

Microbiological findings and species distribution

Overall, 25 microbial species were detected across the biological samples analyzed, including pathogens and commensal taxa, known to contribute to opportunistic infections.

In total, 42 *Fusobacterium* isolates were recovered from 41 specimens taken from 41 patients. The most frequently isolated species were *F. nucleatum* (66.7%) and *F. necrophorum* (26.2%), followed by *F. periodonticum, F. gonidiaformans*, and *F. canifelinum* (2.4%).

Species distribution varied by specimen type. Among stomatognathic samples (28), *F. nucleatum* was identified in 24 (85.7%), *F. necrophorum* in 3 (10.7%), and *F. periodonticum* in 1 (3.6%) sample. Isolates from peritonsillar abscesses included *F. necrophorum* (8/80%), *F. gonidiaformans* (1/10%), and *F. canifelinum* (1/10%). Two *Fusobacterium* species were isolated from a sample taken from one patient: *F. necrophorum* and *F. gonidiaformans*.

All maxillary sinus samples yielded *F. nucleatum* (4/100%).

Co-infection patterns

Polymicrobial growth was observed in 85.4% of specimens ($P < 0.001$). The majority of co-detected organisms were anaerobic bacteria. In stomatognathic infections, the most common co-isolated taxa were *Streptococcus* spp., particularly *Streptococcus anginosus* group (SAG: *S. anginosus, S. constellatus*) detected in 17 specimens (60.7%) from 16 patients. Other streptococci included *S. mitis/oralis* (N = 3), *S. parasanguinis* (N = 2), *S. gordonii* (N = 1), and *S. salivarius* (N = 1). Members of the *Prevotellaceae* family (*Prevotella* and *Segatella*) were detected in 15 specimens (53.6%). It is important to note that some species classified as *Prevotella* by MALDI-TOF MS have been renamed *Segatella*, including *Seg. buccae, Seg. maculosa, Seg. oris*, and *Seg. baroniae*. The most frequently cultured species was *P. nigrescens* (N = 6), followed by *Seg. buccae* (N = 3), *Seg. oris* (N = 3), *P. intermedia* (N = 2), *P. denticola* (N = 2), *Seg. baroniae* (N = 2), *Seg. maculosa* (N = 1), *P. jejuni* (N = 1), and one unidentified *Prevotella* sp. Other anaerobes detected in ≥3 cases included *Parvimonas micra, Actinomyces* spp. (*A. denticolens, A. graevenitzii*), *Dialister pneumosintes*, and *Gemella* spp. (*G. morbillorum, G. haemolysans*).

From clinical specimens of peritonsillar abscesses, *F. necrophorum* was isolated in both adult and pediatric patients, while monomicrobial *F. necrophorum* growth was detected in 55.6% of cases. In polymicrobial peritonsillar infections, isolates from clinical specimens of one adult patient included *F. necrophorum, F. gonidiaformans, S. anginosus,* and *Bulleidia extructa*. Among pediatric patients, isolates from peritonsillar abscesses included *F. necrophorum* in combination with *Klebsiella pneumoniae, Veillonella dispar*, or a mixed microbiota comprising *S. intermedius, Eikenella corrodens*, and *Aggregatibacter aphrophilus*.

From maxillary sinus specimens, *F. nucleatum* was isolated in all cases, with each specimen showing polymicrobial growth. Associated taxa included *S. anginosus* (50%), *Parvimonas micra* (50%), *Prevotella*/*Segatella* spp. (50%), and *Peptoniphilus* spp. (25%).

The overall distribution of taxa by specimen type is shown in Figure 2. Figure 3 illustrates the frequency of co-occurrence of individual microorganisms with *Fusobacterium* species.

Antimicrobial susceptibility testing

The AST was interpreted according to the latest EUCAST version (v16.0; 2026) based on MIC values. Overall, *Fusobacterium* isolates showed 100% susceptibility to β-lactam/β-lactamase inhibitor combinations and carbapenems. In contrast, susceptibility to metronidazole and clindamycin was lower, with broader MIC distributions. The MICs of the antimicrobial agents and their distributions by species are presented in Table 1.

DISCUSSION

The findings presented in this study, together with available literature, underscore the importance of laboratory-based identification of bacteria and antimicrobial susceptibility testing in oral and maxillofacial infections. In our cohort, *Fusobacterium* spp. were co-detected with other microorganisms in 85% of cases, most commonly alongside *Prevotella* spp. (including taxa reclassified to *Segatella*) and notably with members of the SAG. Further investigation of these co-infections should be a research priority because they directly affect the understanding of disease mechanisms and treatment management. The repeated co-infections observed in this study appear to reflect ecological cooperation within polymicrobial communities rather than incidental colonization.

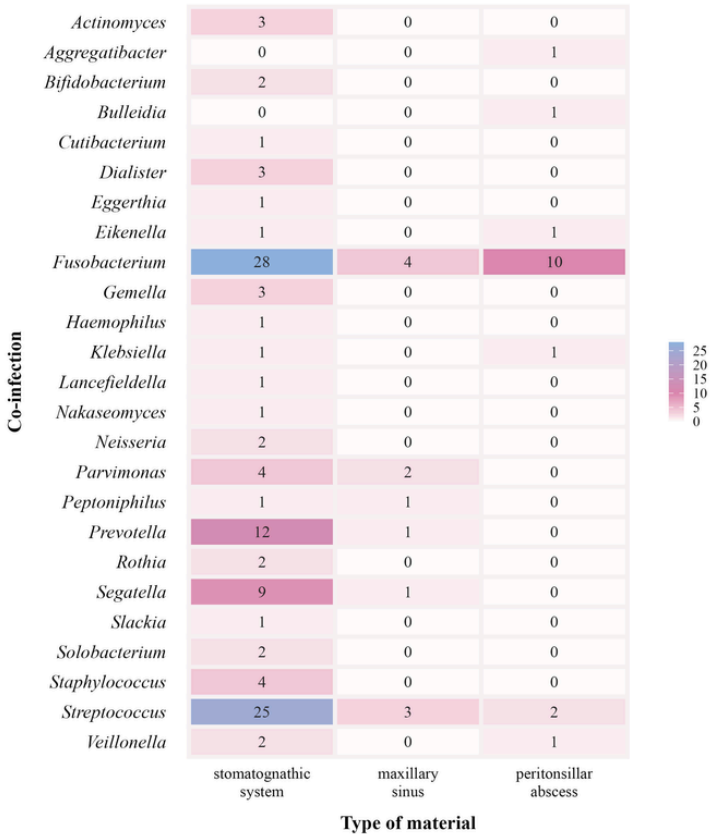

Figure 2. *Heatmap of co-infection species across different sample types. Species names are based on NCBI Taxonomy (16)*

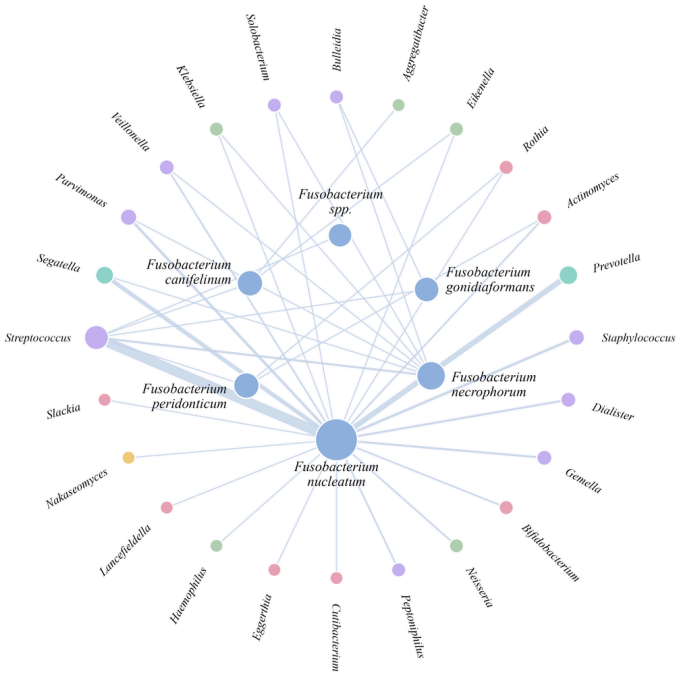

Figure 3. *Co-occurrence of Fusobacterium species with other taxa. Colors indicate bacterial phyla, and line thickness represents the frequency of co-isolation from clinical specimens*

Among fusobacteria, *F. nucleatum* warrants particular attention as a prevalent oral pathobiont repeatedly implicated in odontogenic and deep head-and-neck infections, including periodontal and peri-implant disease, dental abscesses, peritonsillar abscesses, and sinusitis (17). *F. nucleatum* has also been studied as an organism associated with extra-oral disease (including malignancy-associated dysbiosis); however, its relevance in the present context lies in its ability to persist in complex biofilms and to shape microbial community architecture at the mucosal surfaces (18, 19).

Mechanistically, *F. nucleatum* is a prototypical "bridging" organism: it co-aggregates with phylogenetically diverse taxa, promotes biofilm maturation, and modifies local physicochemical conditions (e.g., redox potential and pH) in ways that favor strict anaerobes (20). Single-species biofilms of *F. nucleatum* are typically less robust than multispecies communities, underscoring their reliance on metabolic complementarity. Quorum-sensing signals, such as autoinducer-2, further facilitate interspecies coaggregation with periodontal "red complex" organisms and can amplify inflammatory signaling (21, 22). Several surface adhesins (e.g., FadA, Fap2, RadD, FomA) contribute to attachment and-critically-co-aggregation with diverse oral taxa, thereby stabilizing multispecies biofilms (5, 20, 23). *Fusobacterium*-derived lipopolysaccharide and metabolic products can activate Toll-like receptor-linked pathways and induce the production of pro-inflammatory mediators (5, 24).

In vitro studies have shown that proteolytic activity in *F. nucleatum*, including autotransporter serine proteases such as fusolisin, can degrade host substrates and modulate host immune responses (25). Short-chain fatty acids are major metabolic products of supra- and subgingival bacteria, including *F. nucleatum*. Elevated butyrate levels have been detected in gingival crevicular fluid from patients with periodontitis, suggesting a potential role in the pathogenesis of oral infections (26). High concentrations of butyric acid have been shown to stimulate the production of reactive oxygen species in osteoblasts. This, in turn, stimulates the secretion

Table 1. *Antimicrobial susceptibility of Fusobacterium spp. MIC values determined by the Epsilometer test interpreted according to EUCAST recommendations (version 16; 2026)*

Antibiotic	*F. nucleatum*				*F. necrophorum*				*F. gonidiaformans*				*F. canifelinum*			
	N	N with MIC	S (%)	MIC (µg/mL)	N	N with MIC	S (%)	MIC (µg/mL)	N	N with MIC	S (%)	MIC (µg/mL)	N	N with MIC	S (%)	MIC (µg/mL)
AMC	30	10	100	0.016-0.19	9	9	100	0.016-0.25	1	1	100	0.016	1	1	100	0.016
PG	18	2	100	0.023-0.25	4	4	100	0.016-0.125	-	-	-	-	-	-	-	-
IMP	16	4	100	0.006-0.47	-	-	-	-	-	-	-	-	-	-	-	-
MEM	4	4	100	0.008-0.012	4	2	100	0.04-0.023	-	-	-	-	-	-	-	-
TZP	1	1	100	0.016	2	0	-	-	-	-	-	-	-	-	-	-
CM	26	10	80	0.016-256	9	5	80	0.016-0.5	1	1	100	0.032	1	1	100	0.064
MTR	23	7	100	0.016-1	10	7	86	0.016-0.75	1	1	100	0.047	-	-	-	-

Legend: AMC; amoxicillin/clavulanic acid, PG; benzylpenicillin, IMP; imipenem, MEM; meropenem, TZP; piperacillin/tazobactam, CM; clindamycin, MTR; metronidazole, N; number of tested isolates. N with MIC; the number of isolates for which the MIC was determined during routine testing; S; susceptible, MIC; minimum inhibitory concentration.

of 8-isoprostaglandin and matrix metalloproteinase-2, leading to bone destruction and affecting bone repair (27).

Prevotella spp. (including *Segatella*-reclassified species) are common oral commensals that frequently act as opportunistic pathogens in chronic periodontitis, pulpal infections, and odontogenic abscesses. Their pathogenic potential in polymicrobial infection may be enhanced by physical and metabolic integration into fusobacteria-centered biofilms. Both genera can modulate innate immune responses through Pathogen-Associated Molecular Patterns diversity and secreted factors, contributing to an inflammatory milieu that supports suppuration, tissue destruction, and clinical persistence (28-31).

Similarly, the co-detection of *Fusobacterium* spp. with SAG in our cases is likewise clinically meaningful. Although SAG members are often considered part of the commensal microbiota, they are well known for their propensity to form abscesses and cause invasive disease, including deep neck infections (32, 33). Experimental models suggest that co-inoculation of SAG with *F. nucleatum* yields greater virulence than monomicrobial infection, supporting synergistic pathogenicity (29, 34-36). The co-isolation of *Fusobacterium* spp. and SAG underscores the clinical relevance of their polymicrobial involvement, indicating that both taxa may participate in the infectious process rather than representing incidental co-occurrence.

Therapeutic management of OMIs should integrate prompt source control (drainage and debridement when indicated) with antimicrobials that cover the expected polymicrobial spectrum, including strict anaerobes. Commonly used options include β-lactams, β-lactam/β-lactamase inhibitor combinations, carbapenems in severe disease, and metronidazole as an anaerobic agent—typically as part of a regimen that also covers streptococci (30, 37). In our cohort, susceptibility profiles were generally favourable for β-lactam/β-lactamase inhibitor combinations and carbapenems, whereas reduced susceptibility was observed for clindamycin.

Advances in genomic methods have improved the detection of antimicrobial resistance, particularly in fastidious anaerobes, while providing a broader view of the resistome in polymicrobial infections. Resistance determinants have been reported in odontogenic abscess microbiomes and in collections of *Fusobacterium* and *Prevotella* isolates, including genes associated with resistance to tetracyclines (e.g., tet variants), β-lactams (β-lactamase production; *blaTEM, cfxA, cfxA2*), macrolides, lincosamides, and streptogramin B (MLS_B phenotype; e.g., *erm* genes), and—more rarely—nitroimidazoles, such as metronidazole (*nim*) (2, 30, 38, 39). High sequence identity between resistance genes found in anaerobes and in oral streptococci supports the plausibility of horizontal gene transfer within multispecies biofilms, providing a mechanistic explanation for the emergence and dissemination of resistance in polymicrobial niches (39).

Our findings suggest that when *Fusobacterium* spp. is detected in a sample, a polymicrobial infection should be considered rather than an isolated pathogen, since it is frequently recovered together with *Prevotella*/*Segatella* and SAG. Clinically meaningful characterization of these consortia depends on appropriate sampling (preferably aspirates or tissue rather than superficial swabs), rapid

transport under anaerobe-preserving conditions, species-level identification, and antibacterial susceptibility testing. The main advantage of this study is that it initiates the characterization of *Fusobacterium* species in multi-bacterial infections of the oral cavity, maxillofacial region, and sinuses, including clinically relevant co-infection patterns, which may form the basis for empirical and targeted treatment. Limitations include an insufficient sample size and the fact that it originated from a single hospital. Furthermore, the evolution of AST standards has made direct comparison of drug susceptibility with historical data sets difficult.

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None.

Author Contributions

Conceptualization: A.M.; Methodology: K.M-M. and M.K.; Formal analysis: K.M-M. and A.M.; Investigation: K.M-M. and M.K; Data curation: K.M-M., E.P. and M.K. Writing – Original Draft Preparation: K.M.-M.; Writing – Review and Editing: K.M-M., M.K., E.P. and A.M.; Supervision: A.M.

Statement of Ethics

This retrospective study was conducted using anonymized data extracted from medical records. According to local regulations, formal approval of the bioethics committee was not required. All procedures were performed in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availability

The datasets analyzed during the current study are not publicly available due to institutional restrictions, but they are available from the corresponding author on reasonable request and subject to applicable regulations.

Statement of Generative AI Use

No generative AI was used.

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THE IMPACT OF ANTENATAL CORTICOSTEROID ADMINISTRATION ON EARLY OUTCOME PARAMETERS AND LABORATORY FINDINGS IN NEONATES

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This study evaluates the impact of antenatal corticosteroid (ACS) administration on early neonatal outcomes and laboratory parameters in preterm infants born at 28–34 gestational weeks. It assesses the effects of ACS on early morbidity, respiratory support, NICU admission, and metabolic changes reflected in laboratory parameters. A retrospective cohort study analyzed medical records of preterm neonates born between January 2018 and July 2023. Neonatal outcomes were compared between ACS-exposed and non-exposed groups. Statistical analysis was performed using Chi-square and Fisher's exact tests ($p < 0.05$). A total of 150 infants were included; 74 (49.3%) received ACS. No significant differences were found in birth weight, gestational age, Apgar scores, NICU admission, resuscitation needs, respiratory support, or early morbidity. ACS exposure was associated with a lower, though non-significant, rate of critical illness (8.1% vs. 18.4%). Serum magnesium levels were significantly higher in the non-ACS group ($p = 0.003$), likely reflecting maternal magnesium sulfate administration. ACS benefits appear more pronounced at lower gestational ages. In infants born after 32 weeks, ACS appears to have a limited impact on respiratory and early clinical outcomes, while overall prognosis is primarily determined by gestational maturity and baseline neonatal characteristics. These findings highlight the need for further research to refine ACS administration strategies, particularly for late preterm neonates.

Keywords: antenatal corticosteroids, preterm neonates, respiratory distress, magnesium, neonatal outcomes

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INTRODUCTION

Around 15 million babies are born prematurely across the globe each year, with over a million of these infants not surviving (1). Premature birth remains a major health concern, with respiratory distress syndrome (RDS) being a leading cause of neonatal morbidity and mortality. Antenatal corticosteroids (ACS) significantly reduce the risk of RDS, intraventricular hemorrhage (IVH), necrotizing enterocolitis (NEC), early-onset sepsis, and neonatal death, with the greatest benefits observed when administered between 26 and 35 weeks of gestation (2). ACS has become a cornerstone in the management of pregnancies at risk of preterm delivery, following evidence from the first randomized controlled trial published in 1972 (3). Their role as a standard intervention was further reinforced in 1994, when the National Institutes of Health in the United States issued a consensus statement endorsing their widespread use for improving neonatal outcomes (4). The World Health Organization and multiple obstetric organizations recommend the use of ACS for pregnancies at risk of premature birth before 34 weeks. Some countries have taken this further, advising that ACS may also be beneficial for women at risk of delivering up to 36 weeks of gestation (5). Antenatal corticosteroids are a crucial therapy for improving neonatal outcomes in pregnancies at risk of preterm birth, with a single course recommended between 24 0/7 and 33 6/7 weeks of gestation, including cases with ruptured membranes and multiple gestations. In certain situations, corticosteroid administration may be considered as early as 23 0/7 weeks or up to 36 6/7 weeks for late preterm births, particularly if no prior course has been given (6). Despite extensive research supporting the benefits of ACS, its impact on early neonatal outcomes in preterm infants remains an area of continuous investigation. The objective of this study was to evaluate the effects of ACS administration on key early neonatal outcomes and laboratory parameters in preterm neonates (28–34 weeks), including the need for resuscitation at birth, aspects of neonatal intensive care unit (NICU) admission, oxygen therapy requirements, need for mechanical ventilation, surfactant administration, and the incidence of pathological conditions such as IVH, NEC, sepsis, and hemodynamic instability, as well as metabolic and physiological effects reflected in laboratory findings. Understanding these associations can help optimize ACS administration strategies and improve neonatal care in preterm populations.

METHODS

This retrospective cohort study analyzed medical records of preterm neonates born between 28+0 and 34+6 weeks of gestation, along with data from their mothers. All neonates within this gestational age range who were admitted to the NICU during the study period were eligible for inclusion. In cases of multiple gestation, each neonate was analyzed individually. Among 27 twin pregnancies (54 neonates), one twin neonate with early neonatal death was excluded from analysis, resulting in a final sample of 150 neonates. This monocentric retrospective cohort study was conducted at a single tertiary Neonatal Intensive Care Unit (NICU), using data collected from the hospital's electronic medical records and neonatal histories between January 1, 2018, and July 1, 2023, while the study period was defined from January 1, 2020, to December 31, 2022. The study compared neonatal outcomes between infants whose mothers received antenatal corticosteroids and those who did not. The standard antenatal corticosteroid regimen followed institutional protocols. It included either two intramuscular doses of 12 mg betamethasone administered 24 hours apart, or four intramuscular doses of 6 mg dexamethasone administered every 12 hours, depending on drug availability and clinical discretion. All women who received corticosteroids completed the regimen within 24 hours prior to delivery. All analyses were conducted at the level of individual neonates, including those from multiple gestations, as the dataset represents the full population of preterm births during the study period, with outcomes assessed per neonate.

Maternal characteristics

Maternal characteristics were assessed to identify potential confounding factors between the ACS and non-ACS groups. The recorded variables included parity, categorized as primiparous (one previous delivery) or multiparous (two or more deliveries); type of pregnancy, classified as singleton or multiple gestation; and mode of conception, whether natural or via in vitro fertilization (IVF). Additionally, mode of delivery (vaginal birth or cesarean section), medication use during pregnancy (including antihypertensives, antibiotics, and diabetes medications), and maternal comorbidities (such as hypertension, gestational diabetes, anemia, and other pathological conditions) were documented.

Neonatal characteristics and early outcomes

To evaluate the impact of ACS on neonatal health, the study assessed the following early neonatal outcomes: need for

resuscitation at birth, Apgar score at 1 and 5 minutes, and general condition at NICU admission, categorized as stable, mild respiratory distress, or critically ill (defined as requiring immediate mechanical ventilation and/or inotropic support due to respiratory and/or hemodynamic instability). The study also examined the requirement for mechanical ventilation, including the type of ventilation (invasive vs. non-invasive), and the need for surfactant therapy. Additionally, the presence of respiratory disorders, such as RDS, transient tachypnea of the newborn (TTN), and apnea of prematurity, as well as other respiratory complications, was analyzed. Furthermore, the study evaluated pathological conditions, including IVH, NEC, neonatal sepsis, and hemodynamic instability.

Statistical analysis

IBM SPSS Statistics for Windows, version 25 (Armonk, NY: IBM Corp., USA) was used for statistical analysis. Normality of distribution was assessed using the Shapiro–Wilk test, and homogeneity of variance was evaluated using Levene's test. Almost all variables violated the assumption of normality (Shapiro–Wilk $p < 0.05$) in at least one group. In addition, some variables also showed evidence of unequal variances (Levene's test $p < 0.05$). Therefore, the nonparametric Wilcoxon test was deemed the most appropriate method for all comparisons. Results are presented as numbers and percentages, arithmetic mean and standard deviation, and median and interquartile range. The χ^2 test (or Fisher's exact test when expected frequencies were insufficient), independent samples t-test, and Mann-Whitney U test were used to assess the significance of differences. Primary comparisons between exposure groups were initially assessed using univariate statistical tests (Mann–Whitney U test, χ^2 test, Fisher's exact test, as appropriate). In addition, multivariable logistic regression models were performed for six key neonatal outcomes (need for mechanical ventilation, intraventricular hemorrhage, sepsis, mortality, NICU admission, and critical illness at admission). These models were adjusted for specific maternal and pregnancy-related factors. The revised analyses also incorporated essential neonatal variables: gestational age, birth weight, sex, and mode of delivery. The threshold for statistical significance was set at $p = 0.05$. Those p-values that could not be expressed to three decimal places are reported as $p < 0.001$. All variables were complete unless otherwise noted. For medications, pathological conditions, and neonatal blood cultures, a small number of observations were missing due to unavailable documentation or a lack of clinical indication. No imputation was applied; analyses were based on available data. A priori power analysis was conducted to evaluate the adequacy of the sample size. For a two-sample t-test, assuming a medium effect size ($d = 0.5$), significance level of 0.05, and power of 0.80, a minimum of 64 participants per group was required. For chi-square tests with an expected medium effect size ($w = 0.3$), a total sample size of 88 (df = 1), 107 (df = 2), or 121 (df = 3) was needed to achieve 80% power at $\alpha = 0.05$. The final sample of 150 neonates met these criteria, supporting the statistical robustness of the analyses performed. NICU admission status was selected as the primary multivariate outcome because it reflects the neonate's overall clinical condition upon admission, including respiratory, cardiovascular, and hemodynamic parameters. This outcome was considered a more comprehensive indicator of early clinical severity than an isolated RDS diagnosis. A multinomial logistic regression model was used to analyze NICU admission condition (Vital, Moderate (Mild respiratory distress), Critical) as a three-category outcome, with "Vital" as the reference. The model was fitted using the multinom() function from the nnet package in R and included the following predictors: ACS exposure, maternal administration of $MgSO_4$ before birth, corticosteroids within 24 hours of delivery, maternal medications during pregnancy, pathological maternal conditions, and neonatal magnesium levels within the first 24 hours. Since the multinom() function does not support cluster-robust standard errors or generalized estimating equations (GEE), we conducted a sensitivity analysis using a reduced dataset that included only one randomly selected neonate per twin pair. This allowed us to ensure independence of observations. Results from both the full and reduced models are reported in the same comparison table. Categorical thresholds, including gestational age (cut-off at 32 weeks), birth weight bands (<1000 g, 1000–1999 g, ≥2000 g), and NICU length of stay (≤7, 8–14, ≥15 days), were pre-specified based on clinical relevance and were consistently applied in all statistical analyses. Gestational age was categorized using a predefined threshold at 32 weeks, in accordance with guidelines from the American Academy of Pediatrics and the World Health Organization, which define infants born before 32 completed weeks as very preterm (7,8). Birth weight categories (<1000 g, 1000–1999 g, and ≥2000 g) were also defined in advance. Although they differ slightly from the WHO classification, these bands reflect the distribution of our study population (64.7% between 1000 and 1999 g) and correspond to locally relevant clinical groupings used in NICU care (9). Based on clinical relevance and the observed distribution of NICU

length of stay in our cohort, this variable was categorized into three predefined groups: ≤7 days, 8–14 days, and ≥15 days. This approach allowed for more meaningful comparisons, given the skewed distribution of LOS and the need to maintain adequate subgroup sizes (n = 46, n = 72, and n = 32, respectively). In addition to the multinomial regression for NICU admission, we performed separate multivariable logistic regression analyses for other primary neonatal endpoints: need for mechanical ventilation, intraventricular hemorrhage (IVH), sepsis, mortality, and composite "critical illness" at admission. All models were adjusted a priori for gestational age (weeks), birth weight (grams), sex, delivery mode (vaginal vs. cesarean/induced), and maternal pathological conditions and medications during pregnancy. For outcomes with a low number of events, such as mortality, Firth's penalized logistic regression was used to reduce small-sample bias and improve model convergence. The results are presented as adjusted odds ratios (aOR) with 95% confidence intervals (CI) and p-values. Model fit was evaluated using the likelihood ratio chi-square test and Nagelkerke R^2, and classification accuracy, sensitivity, and specificity were calculated. For continuous outcomes with a statistically significant difference in the main analysis (serum magnesium levels), we repeated the Mann–Whitney U test on a subsample excluding one randomly selected infant from each twin pair to account for within-pair clustering. The results were compared to those from the full dataset to assess robustness.

RESULTS

A total of 124 mothers and 150 neonates were included in the analysis. The difference in sample size reflects the inclusion of 27 twin pregnancies, with each neonate analyzed as a separate observation. Among the 27 pregnant women with multiple pregnancies, antenatal corticosteroids were administered to 15 (25.4%), while 12 (18.5%) did not receive them. Neonatal outcomes were not stratified by type of pregnancy (singleton vs. multiple), but preliminary comparisons did not reveal significant differences between these groups. A total of 59 (47.6%) women received antenatal corticosteroids. The majority received betamethasone (n = 33; 55.9%), dexamethasone (n = 25; 42.4%), and only one received methylprednisolone. Most women received corticosteroids 24 hours before delivery (n = 52; 88.1%). Significantly more mothers who received antenatal corticosteroids during pregnancy also received other medications (Table 1). In other maternal parameters, specifically parity, type of pregnancy, mode of conception, type of delivery, and pathological conditions during pregnancy, no statistically significant differences were found between mothers who received antenatal corticosteroids and those who did not. The sample included 74 newborns whose mothers received ACS and 76 newborns whose mothers did not receive ACS. No statistically significant differences were observed in neonatal characteristics between the ACS and non-ACS groups (Table 2). In the main sample (n = 150; magnesium data available for 146 infants), serum magnesium levels differed significantly between groups despite identical median values (0.84 mmol/L in both groups), reflecting differences in the overall distribution of values. Infants whose mothers had not received ACS had significantly higher mean ranks than those whose mothers had received ACS (84.62 vs. 62.97, respectively; Z = −3.093; p = 0.002). This finding remained unchanged in a reduced dataset excluding one infant per twin pair (N = 124; Mg data available for 121 infants; mean ranks: 71.24 vs. 51.88; Z = −3.031; p = 0.002), confirming that the result was not influenced by twin clustering (Supplementary Table S2).

Table 1. *Maternal parameters based on antenatal corticosteroid administration*

		Received antenatal corticosteroids				
		Yes		No		
		n	%	n	%	p*
Parity	1	24	40.7	25	38.5	0.801
	2+	35	59.3	40	61.5	
Type of pregnancy	Singleton	44	74.6	53	81.5	0.348
	Multiple	15	25.4	12	18.5	
Mode of conception	Natural	56	94.9	63	96.9	0.57
	In vitro fertilization	3	5.1	2	3.1	
Mode of delivery	Vaginal	27	45.8	24	36.9	0.318
	Cesarean	32	55.2	41	63.1	
Medications during pregnancy	No	26	44.1	42	64.6	0.022
	Yes	33	55.9	22	35.4	
Pathological conditions during pregnancy	No	27	45.8	34	52.3	0.467
	Yes	32	54.2	30	47.7	
Magnesium sulfate administration		2	2.7	12	15.8	0.009#

* Chi-square test, #Fisher's exact test

Yes = Received ACS; No = Did not receive ACS
Note: Data on medications and pathological conditions were unavailable for one mother in the non-ACS group (n = 64/65).

Table 2. *Neonatal parameters based on antenatal corticosteroid administration*

		Received antenatal corticosteroids				
		Yes		No		
		n	%	n	%	p*
Sex	Male	39	52.7	44	57.9	0.523
	Female	35	47.3	32	42.1	
Birth weight (grams)	<1000 g	4	5.4	3	3.9	0.616#
	1000-1999 g	45	60.8	52	68.4	
	≥2000 g	25	33.8	21	27.6	
Gestational age (weeks)	-32	28	37.8	32	42.1	0.594
	32-	46	62.2	44	57.9	
APGAR score	-7	16	21.6	19	25	0.625
	8-10	58	78.4	57	75	
Need for resuscitation in the first 24h	Yes	12	16.2	20	26.3	0.131
	No	62	83.8	56	73.7	
General condition on admission to NICU	Vital	34	45.9	39	51.3	0.06
	Mild respiratory distress	34	45.9	23	30.3	
	Critical	6	8.1	14	18.4	
Need for O2	Yes	65	87.8	69	90.8	0.558
	No	9	12.2	7	9.2	
Mechanical ventilation	Yes	41	55.4	35	46.1	0.252
	No	33	44.6	41	53.9	
Surfactant	Yes	19	25.7	18	23.7	0.777
	No	55	74.3	58	76.3	
Type of therapy	Antibiotic	3	4.1	1	1.3	0.109#
	Antibiotic + supportive therapy	29	39.2	42	55.3	
	Antibiotic + supportive therapy + inotropes	42	56.8	33	43.4	
Length of stay in the NICU (days)	-7	23	31.1	23	30.3	0.37
	7-14	32	43.2	40	52.6	
	14+	19	25.7	13	17.1	
Respiratory problems	No	26	35.1	35	46.1	0.174
	Yes	48	64.9	41	53.9	
Pathological conditions	No	3	4.1	9	11.8	0.079
	Yes	71	95.9	67	88.2	
Oral milk intake (days)	1-3	39	52.7	31	40.8	0.144
	3 and more	35	47.3	45	59.2	
Blood culture	Sterile	56	77.8	67	89.3	0.058
	Positive	16	22.2	8	10.7	
Urine culture	Sterile	51	70.8	56	74.7	0.602
	Positive	21	29.2	19	25.3	
Cranial ultrasound within the first 21 days of life	IVH grade 1-2	52	703	57	750	807
	IVH grade 3-4	13	176	11	145	
	No IVH/ultrasound not performed	9	122	8	105	
Cranial ultrasound at discharge	IVH grade 1-2	53	716	57	750	895
	IVH grade 3-4	12	162	11	145	
	No IVH/ultrasound not performed	9	122	8	105	

*Chi-square test; #Fisher's exact test

Yes = Received ACS; No = did not receive ACS
Note: Blood culture data were unavailable for two neonates in the ACS group and one in the non-ACS group, as the test was not performed (n = 72/74 and 75/76, respectively)

A significant difference in magnesium levels in the first 24 hours was therefore observed, with higher levels in the non-ACS group. This difference likely reflects more frequent use of maternal magnesium sulfate in the non-ACS group (12 mothers vs. 2 in the ACS group; p = 0.009, Table 1). No significant differences were found for other laboratory parameters. Diagnostics for normality and homogeneity of variance are provided in Table 3. For each outcome, the type of analysis applied (univariate vs. multivariable) is indicated in the corresponding table and text. Univariate tests were used for descriptive comparisons, while multivariable logistic regression models, adjusted for maternal, pregnancy-related, and neonatal variables, were used to estimate adjusted odds ratios for the six predefined neonatal outcomes. Results from the multinomial logistic regression models are presented in Table 4, comparing both the full dataset (all infants) and the reduced dataset (1 twin per pair). In both models, ACS exposure was not significantly associated with either moderate or critical illness. In contrast, maternal medications during pregnancy were consistently protective against critical illness in both samples, reaching statistical significance in the full (OR = 0.15, p = 0.020) and reduced (OR = 0.11, p = 0.013) models. Pathological maternal conditions showed a borderline significant association with increased risk of critical illness in the full model (p = 0.055), and became statistically significant in the reduced dataset (p = 0.017). No consistent or significant predictors were identified for moderate illness vs. vital status in either model. A side-by-side comparison of results from the full dataset (including all neonates) and the reduced dataset (one neonate per twin pair) showed no major deviations in effect estimates. In both models, maternal medications remained significantly protective against critical illness, and maternal pathological conditions showed consistent directionality with slightly stronger statistical significance in the reduced dataset. These findings confirm the robustness of our main conclusions despite potential bias introduced by the non-independence of twin pairs.

Additional multivariable analyses of primary neonatal outcomes

Need for mechanical ventilation

In the multivariable logistic regression model, gestational age (p = 0.001), sex (p = 0.033), and delivery mode (p = 0.040) were statistically significant predictors of the need for mechanical ventilation. Each additional week of gestation reduced the odds by 42.2% (aOR = 0.578), female sex was protective (aOR = 0.431), and cesarean/induced delivery increased the odds (aOR = 2.440). ACS exposure was not statistically significant (p = 0.106; aOR = 1.846), although it showed a non-significant trend toward higher odds. The model was statistically significant ($\chi^2(6) = 35.448$; $p < 0.001$) and explained 28.6% of the variance (Nagelkerke $R^2 = 0.286$) (Table 5, Table S3).

Intraventricular hemorrhage (IVH)

In the multivariable logistic regression model, gestational age (p = 0.002) and maternal pathological conditions during pregnancy (p = 0.013) were statistically significant predictors of IVH. Each additional week of gestation reduced the odds by 47.5% (aOR = 0.525), while the presence of maternal pathological conditions increased the odds more than fourfold (aOR = 4.270). Female sex showed a non-significant trend towards a protective effect (p = 0.066; aOR = 0.358). ACS exposure was not statistically significant (p = 0.507; aOR = 1.416), and neither was delivery mode or birth weight (both p > 0.20). The model was statistically significant ($\chi^2(6) = 28.261$; $p < 0.001$) and explained 29.7% of the variance (Nagelkerke $R^2 = 0.297$), with an overall classification accuracy of 85.0% (Table 5, Table S3).

Mortality

In the multivariable logistic regression model, none of the examined predictors was significantly associated with neonatal mortality. The model was statistically significant overall ($\chi^2(6) = 16.449$; $p = 0.012$) and explained 36.6% of the variance (Nagelkerke $R^2 = 0.366$), with an overall classification accuracy of 95.9%. Due to the very small

Table S1. *Comparison of birth weight and gestational age between ACS and non-ACS groups when analyzed as continuous variables*

	Received ACS	Did not receive ACS	p-value	Effect Size (r)	95% Confidence Interval	p-value Shapiro-Wilk test	p-value Levene's test
Birth weight (grams)	1781.51 [481.00]	1751.55 [437.87]	0.995	0	[-0.19, 0.18]	< 0.001	0.725
Gestational age (weeks)	31.93 [1.92]	31.92 [1.77]	0.796	0.02	[-0.16, 0.21]	< 0.001	0.835
Note: Values are presented as median [interquartile range]. Mann-Whitney U test was used due to violation of normality (Shapiro-Wilk p < 0.001). Effect size (r) and 95% confidence intervals (CI) are also reported.							

Table S2. *Robustness analysis of neonatal magnesium (Mg) levels by ACS exposure in the full sample and reduced twin-adjusted sample*

Sample	N (total)	N (ACS)	N (no ACS)	Median Mg (ACS)	Median Mg (no ACS)	Z	p-value
Main sample (150 infants)	146	75	71	0.84	0.84	−3.093	0.002
Reduced sample (one twin per pair)	121	64	57	0.84	0.84	−3.031	0.002
Note: p-values are from the Mann–Whitney U test. Groups: 1 = ACS received; 2 = no ACS. Mg levels in mmol/L.							

Table 3. *Laboratory parameters in newborns based on antenatal corticosteroid administration*

	Yes	No	p-value	Effect Size (r/Cohen's d)	95% Confidence Interval	p-value Shapiro-Wilk test	p-value Shapiro Yes	p-value Shapiro No	p-value Levene's test
Blood glucose on admission	3.99 [4.15]	4.48 [4.40]	0.442#	0.07 (r)	[-0.11, 0.25]	< 0.001	< 0.001	< 0.001	0.511
Lactate on admission	2.93 [1.39]	2.64 [1.23]	0.512#	-0.03 (r)	[-0.22, 0.15]	< 0.001	< 0.001	< 0.001	0.539
Red blood cells in the first 24h	4.91 [0.63]	4.98 [0.66]	0.716#	-0.03 (r)	[-0.22, 0.15]	< 0.001	< 0.001	0.113	0.275
Platelets in the first 24h	237.44 [80.60]	240.00 [64.19]	0.304#	-0.10 (r)	[-0.28, 0.09]	0.012	0.001	0.003	0.48
Calcium in the first 24h	2.15 [0.21]	2.14 [0.23]	0.611#	0.05 (r)	[-0.14, 0.23]	0.046	0.794	0.002	0.367
Magnesium in the first 24h	0.86 [0.15]	0.96 [0.27]	0.002#	0.30 (r)	[0.12, 0.46]	< 0.001	< 0.001	< 0.001	0.009
CRP on admission	2.78 [10.69]	2.62 [8.53]	0.627#	0.05 (r)	[-0.14, 0.23]	< 0.001	< 0.001	< 0.001	0.92
Highest CRP during treatment	17.49 [34.50]	38.97 [68.36]	0.287#	0.10 (r)	[-0.08, 0.28]	< 0.001	< 0.001	< 0.001	0.016
Results are presented as the median [interquartile range]; #Wilcoxon rank-sum test (equivalent to the Mann–Whitney U test) Yes = Received ACS; No = Did not receive ACS. "Shapiro Yes" and "Shapiro No" columns represent group-specific p-values from the Shapiro–Wilk test for normality.									

Table 4. *Results from the multinomial logistic regression models*

Comparison	Predictor	Odds Ratio (full sample)	p-value (full sample)	Odds Ratio (1 twin per pair)	p-value (1 twin per pair)
Moderate vs Vital	ACS (Yes)	3.09	0.136	5.1	0.062
Moderate vs Vital	$MgSO_4$ before birth (Yes)	1.56	0.49	2.07	0.323
Moderate vs Vital	Corticosteroids <24h (Yes)	0.51	0.376	0.36	0.244
Moderate vs Vital	Medications during pregnancy (Yes)	0.93	0.904	0.9	0.88
Moderate vs Vital	Pathological maternal conditions (Yes)	1.11	0.864	1.66	0.463
Moderate vs Vital	Magnesium in first 24h	0.35	0.279	0.34	0.287
Critical vs Vital	ACS (Yes)	1.6	0.688	2.42	0.477
Critical vs Vital	$MgSO_4$ before birth (Yes)	0.65	0.725	0.88	0.922
Critical vs Vital	Corticosteroids <24h (Yes)	0.32	0.363	0.27	0.323
Critical vs Vital	Medications during pregnancy (Yes)	0.15	0.02	0.11	0.012
Critical vs Vital	Pathological maternal conditions (Yes)	4.05	0.055	6.71	0.016
Critical vs Vital	Magnesium in first 24h	3.69	0.308	3.28	0.35

Table 5. *Adjusted odds ratios for ACS exposure and primary neonatal outcomes*

Outcome	aOR (ACS)	95% CI*	p-value
Mechanical ventilation	1.846	[0.88, 3.88]	0.106
IVH	1.416	[0.51, 3.96]	0.507
Mortality	1.47×10^8	not estimable	0.997
Sepsis	3.787	[0.94, 15.35]	0.062
NICU admission	0.454	[0.14, 1.48]	0.189
Critical illness	2.173	[0.98, 4.80]	0.055

*95% confidence intervals for ACS are presented here; full regression results for all predictors are shown in Supplementary Table S3.

Note: Models adjusted for gestational age, birth weight, sex, delivery mode, and maternal pathological conditions

number of mortality cases (N = 6), the model did not converge, and the estimated coefficients should be interpreted with caution. Female sex showed a non-significant trend towards a protective effect (p = 0.094; aOR = 0.137). ACS exposure was not significant (p = 0.997; aOR = 1.47×10^8), and neither gestational age, birth weight, maternal pathological conditions, nor delivery mode reached statistical significance (all p > 0.20) (Table 5, Table S3).

Sepsis

In the multivariable logistic regression model, birth weight (p = 0.046) and sex (p = 0.031) were statistically significant predictors of neonatal sepsis. Each additional gram of birth weight was associated with a slight reduction in odds (aOR = 0.997), while female sex was a significant protective factor (aOR = 0.197). ACS exposure was not statistically significant (p = 0.062; aOR = 3.787), but showed a non-significant trend toward higher odds. Gestational age, maternal pathological conditions, and delivery mode were not significant predictors (all p > 0.20). The model was statistically significant ($\chi^2(6) = 18.520$; p = 0.005) and explained 26.3% of the variance (Nagelkerke $R^2 = 0.263$), with an overall classification accuracy of 92.5% but low sensitivity for predicting sepsis cases (23.1%) (Table 5, Table S3).

NICU admission

In the multivariable logistic regression model, gestational age was the only statistically significant predictor of NICU admission (p = 0.014), with each additional week of gestation reducing the odds by 41.2% (aOR = 0.588). ACS exposure (p = 0.189; aOR = 0.454), sex, birth weight, maternal pathological conditions, and delivery mode were not statistically significant predictors (all p > 0.20), although some showed trends toward association. The model was statistically significant ($\chi^2(6) = 18.600$; p = 0.005) and explained 23.2% of the variance (Nagelkerke $R^2 = 0.232$), with an overall classification accuracy of 89.8% but low sensitivity for predicting NICU admissions (17.6%) (Table 5, Table S3).

Critical illness

In the multivariable logistic regression model, sex (p = 0.007), birth weight (p = 0.011), and maternal pathological conditions (p = 0.034) were statistically significant predictors of critical illness. Female sex was associated with a significantly lower risk (aOR = 0.316), higher birth weight was protective (aOR = 0.998 per gram), while maternal pathological conditions increased the risk more than twofold (aOR = 2.427). ACS exposure showed borderline significance (p = 0.055; aOR = 2.173), suggesting a possible trend toward increased risk. Gestational age and delivery mode were not statistically significant predictors (both p > 0.10). The model was statistically significant ($\chi^2(6) = 36.943$; p < 0.001) and explained 30.7% of the variance (Nagelkerke $R^2 = 0.307$), with an overall classification accuracy of 74.1% (sensitivity 86.5%, specificity 51.0%) (Table 5, Table S3).

DISCUSSION

Antenatal corticosteroid (ACS) administration is a well-established intervention to enhance fetal lung maturation and improve neonatal outcomes in preterm infants. This study evaluated the association between ACS exposure and early neonatal outcomes and laboratory parameters in neonates born at 28–34 weeks of gestation. Regarding maternal characteristics, no significant differences were observed in parity, type of pregnancy, mode of conception, or mode of delivery between ACS and non-ACS groups, but other medication use was higher in ACS-exposed mothers (56.7% vs. 34.4%, p = 0.022), likely reflecting a greater prevalence of pregnancy-related complications requiring pharmacologic intervention (10). ACS exposure has been associated with maternal conditions such as preeclampsia, hypertension, and gestational diabetes (11,12). However, our study found no significant differences in baseline maternal characteristics. Mode of delivery was also comparable between groups, consistent with evidence that obstetric factors, rather than ACS exposure, primarily determine delivery method (10). Similarly, no significant difference in maternal mortality has previously been observed following ACS administration, supporting the established maternal

Table S3. *Full multivariable logistic regression results for ACS exposure and neonatal outcomes*

Outcome	Predictor	B	p-value	aOR (Exp(B))
Mechanical ventilation	ACS	0.613	0.106	1.846
	Gestational age (weeks)	-0.548	0.001**	0.578
	Birth weight (g)	0	0.862	1
	Sex (female)	-0.841	0.033*	0.431
	Maternal pathology	0.607	0.13	1.835
	Delivery mode	0.892	0.040*	2.44
IVH	ACS	0.348	0.507	1.416
	Gestational age (weeks)	-0.644	0.002**	0.525
	Birth weight (g)	0	0.628	1
	Sex (female)	-1.028	0.066	0.358
	Maternal pathology	1.452	0.013*	4.27
	Delivery mode	-0.304	0.592	0.738
Mortality	ACS	18.807	0.997	1.47×10^8
	Gestational age (weeks)	0.469	0.313	1.599
	Birth weight (g)	-0.002	0.212	0.998
	Sex (female)	-1.985	0.094	0.137
	Maternal pathology	1.588	0.224	4.895
	Delivery mode	-0.363	0.746	0.696
Sepsis	ACS	1.332	0.062	3.787
	Gestational age (weeks)	0.129	0.617	1.138
	Birth weight (g)	-0.003	0.046*	0.997
	Sex (female)	-1.625	0.031*	0.197
	Maternal pathology	0.931	0.213	2.536
	Delivery mode	-0.952	0.216	0.386
NICU admission	ACS	-0.79	0.189	0.454
	Gestational age (weeks)	-0.531	0.014*	0.588
	Birth weight (g)	0	0.964	1
	Sex (female)	-0.471	0.438	0.624
	Maternal pathology	0.615	0.305	1.851
	Delivery mode	0.813	0.226	2.255
Critical illness	ACS	0.776	0.055	2.173
	Gestational age (weeks)	-0.257	0.13	0.774
	Birth weight (g)	-0.002	0.011*	0.998
	Sex (female)	-1.152	0.007**	0.316
	Maternal pathology	0.887	0.034*	2.427
	Delivery mode	0.33	0.437	1.391

*Models adjusted for gestational age, birth weight, sex, delivery mode, and maternal pathological conditions.

**p < 0.01; *p < 0.05.

safety profile of ACS in the management of preterm birth (10). Neonatal characteristics, including gender distribution, birth weight, and gestational age, were similar between groups. The absence of significant differences in birth weight and gestational age between groups, regardless of whether variables were analyzed continuously or dichotomously, supports baseline comparability (Table S1). Apgar scores, need for resuscitation at birth, and initial NICU stability showed no statistically significant differences between groups. Although the proportion of critically ill neonates upon NICU admission was lower in the ACS group (8.1% vs. 18.4%), this difference did not reach statistical significance (p = 0.060), and the study may be underpowered to detect modest effects. The need for resuscitation was comparable (16.2% vs. 26.3%, p = 0.131), but ACS may still enhance neonatal adaptation at birth [10]. Some studies suggest that neonates receiving ACS closer to delivery (from one to seven days) may require more

respiratory support due to lower gestational age (13). We found no significant difference in oxygen therapy, mechanical ventilation, or surfactant administration, which is consistent with recent studies suggesting that the universal application of antenatal corticosteroids in this gestational range may not always be justified, as they may have a limited effect in babies with relatively mature lungs (gestational age of 32 weeks and more) (11). The slightly higher ventilation rate in the ACS group may be attributed to factors such as gestational age distribution or illness severity. The effects of ACS are most pronounced at lower gestational ages (24–30 weeks), where they play a crucial role in reducing neonatal mortality, RDS, and severe IVH (10). ACS administration is linked to a significant reduction in RDS and respiratory complications (14), particularly benefiting high-risk neonates, including those with fetal growth restriction or small for gestational age (5). In babies born after 32 weeks of gestation, the effects of ACS are less pronounced, and some studies suggest that ACS may prolong neonatal hypoglycemia and potentially increase the risk of neonatal sepsis (12). However, ACS exposure has also been associated with an increased risk of neonatal pneumonia within the first six months of life (12). It significantly lowers IVH risk without affecting the incidence of NEC (15), though some evidence links ACS to an increased risk of early-life infections, including sepsis, pneumonia, and gastroenteritis [12]. Prolonged hospitalization and additional medical interventions, such as antibiotics and tocolytics, have been reported among ACS-exposed neonates (13). Although ACS has demonstrated a strong protective effect against IVH, no significant difference in IVH incidence was observed in our study. Although the proportion of severe IVH was lower in the ACS group (17.6% vs. 14.5%), this difference was not statistically significant, and the sample size may have been insufficient to detect smaller effects. There were no relevant differences in cranial ultrasound findings, neither in the first three weeks of life nor at discharge. The similar distribution of low-grade and high-grade IVH in both groups suggests ACS had no measurable effect on early neurosonographic outcomes in this gestational range. In the additional multivariable analyses, antenatal corticosteroid exposure did not emerge as an independent predictor of major neonatal outcomes. Instead, gestational age and female sex consistently showed protective effects, while lower birth weight and maternal pathological conditions increased the risk for several adverse endpoints. Cesarean or induced delivery was associated with higher odds of mechanical ventilation. A borderline association was observed between ACS and critical illness at admission ($p = 0.055$), but this did not reach statistical significance. These findings suggest that, after adjustment for key clinical factors, ACS exposure was not independently associated with the evaluated early neonatal outcomes in infants born at 28–34 weeks in our cohort. Rather, gestational maturity and baseline clinical characteristics appear to be the main drivers of neonatal prognosis, which is consistent with previous evidence that the benefit of ACS is most pronounced at lower gestational ages.

Our study identified higher magnesium levels in the non-ACS group, whereas no significant differences were observed in glucose metabolism, blood cell counts, or inflammatory markers. Because maternal magnesium sulfate administration was significantly more frequent in the non-ACS group, this finding is likely attributable to differences in maternal treatment rather than a direct effect of ACS exposure.

Maternal administration of magnesium sulfate has been shown to directly influence neonatal magnesium levels, with studies confirming a strong correlation between maternal and neonatal blood concentrations, indicating that elevated neonatal magnesium primarily reflects transplacental transfer (16). Recent studies have indicated that antenatal corticosteroid exposure may influence not only pulmonary outcomes but also broader systemic processes, including immunological and inflammatory responses. Significant alterations in neonatal immune function, such as changes in cytokine signaling and inflammatory markers, have been observed. This supports the notion that ACS may exert extensive biological effects beyond lung maturation, potentially contributing to the metabolic and immunologic differences (17). Given the complexity of high-risk pregnancies, such as those complicated by severe intrahepatic cholestasis, the administration of antenatal corticosteroids requires careful evaluation and individualized decision-making. In these cases, earlier gestational age at delivery, lower birth weight, and reduced Apgar scores have been observed, highlighting the potential systemic effects on neonates beyond pulmonary maturation and underscoring the need for customized perinatal strategies when considering ACS exposure (18). A slightly higher rate of positive blood cultures in the ACS group was not statistically significant and aligns with studies indicating that ACS does not elevate neonatal infection rates (10). These findings emphasize the need for further research to optimize ACS use in late preterm infants, balancing its potential benefits with associated metabolic effects and neonatal outcomes.

Limitations of this study

This study's retrospective design may introduce selection bias, limiting causal inferences on ACS effects. The sample size, though adequate for general comparisons, may not detect subtle differences in neonatal outcomes. The lack of standardized RDS assessment complicates comparisons with prior studies. Potential confounders, such as maternal health conditions, could have influenced findings, and the timing of ACS administration was not stratified, which may affect neonatal benefits. Future well-designed prospective studies with larger cohorts and long-term follow-up are needed to refine ACS treatment strategies and optimize neonatal outcomes. Additionally, the study did not account for the clustering effect of multiple gestations, as twins were analyzed as independent observations. This may have introduced bias due to correlated outcomes within twin pairs, potentially underestimating variability and affecting the statistical power of comparative analyses. However, the dataset reflects the actual population of preterm neonates admitted during the study period, and outcomes were evaluated per individual neonate, consistent with clinical practice. Clinical and laboratory data were available for the majority of included neonates, with missing values limited to specific parameters as detailed in table footnotes. Furthermore, this study included multiple statistical comparisons across maternal, neonatal, and laboratory variables without formal adjustment for multiple testing. This approach was chosen to preserve sensitivity in an exploratory analysis. It increases the risk of Type I error, and findings should be interpreted with this consideration in mind. While initial group comparisons were based on univariate analyses, multivariable logistic regression models were subsequently performed for six key neonatal outcomes to account for potential confounding. In the revised analysis, these models were expanded to include neonatal variables, such as gestational age, birth weight, sex, and mode of delivery, alongside maternal and pregnancy-related factors, thereby improving the robustness of the findings. Categorizing continuous variables can reduce statistical power and obscure dose–response relationships. For birth weight and gestational age, we observed no differences in results when analyzed as continuous variables (see Supplementary Table 1). NICU length of stay was not available in a continuous format and, therefore, could not be modeled accordingly. Although the proportion of critically ill neonates was lower in the ACS group, this difference was not statistically significant.

Antenatal corticosteroids (ACS) administration remains the standard approach for managing preterm birth, with well-documented benefits in improving neonatal outcomes, particularly in lower gestational ages (24–30 weeks). However, our study found no significant differences in major neonatal outcomes between ACS-exposed and non-exposed neonates born between 28 and 34 weeks of gestation. These findings suggest that the universal application of ACS in this gestational range may not always be justified and that larger prospective studies are needed before conclusions can be drawn regarding the optimal use of ACS in these populations.

Additionally, we observed significantly higher serum magnesium levels in neonates not exposed to ACS, suggesting a potential metabolic effect that might influence neuromuscular function and neonatal adaptation. However, it is important to interpret this difference with caution, as it is likely related to the more frequent administration of maternal magnesium sulfate in the non-ACS group.

While ACS has demonstrated strong protective effects in reducing RDS, IVH, and neonatal mortality at lower gestational ages, its impact at later preterm stages appears less pronounced and may be associated with prolonged neonatal hypoglycemia and an increased risk of infection. These findings highlight the need for further research to refine ACS administration strategies, optimize neonatal care, and better define its role in late preterm infants. In this study, maternal medications during pregnancy emerged as a significant protective factor against critical neonatal illness, even after adjustment for other perinatal factors. Pathological maternal conditions were also associated with an increased risk of severe neonatal outcomes. No statistically significant association was observed between ACS exposure and neonatal outcomes after adjustment, and the limited sample size restricts the ability to detect less pronounced associations. Importantly, the use of a reduced dataset excluding one twin per pair confirmed that our findings were robust to potential bias from twin clustering. These results highlight the critical role of maternal health and antenatal care in shaping early neonatal outcomes. Furthermore, a post hoc sensitivity analysis excluding one twin per pair confirmed that our findings were robust to potential intra-pair clustering.

Practical implications

In preterm infants born after 32 weeks of gestation, our findings did not demonstrate a significant association between antenatal corticosteroid (ACS) exposure and early neonatal outcomes, suggesting limited clinical benefit from routine ACS administration in this gestational age group. Early outcomes in this range appear to be more strongly

influenced by gestational maturity and baseline neonatal condition than by steroid exposure. Given the retrospective design and relatively limited sample size, these findings should be interpreted with caution. Nevertheless, they support further investigation into whether a more individualized approach to ACS administration may be appropriate in selected late-preterm populations.

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Author Contributions

Conceptualization: S.M. and M.J.R.; Methodology: S.M. and M.J.R.; Formal analysis: S.M. and M.J.R.; Investigation: S.M. and M.J.R.; Data curation: S.M. and M.J.R.; Writing – Original Draft Preparation: S.M. and M.J.R.; Writing – Review and Editing: S.M. and M.J.R. All authors have read and approved the published version of the manuscript.

Statement of Ethics

This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Institutional Ethics Committee (approval number 1305/23) on April 11, 2023. As this was a retrospective study using anonymized data, the requirement for informed consent was waived by the Institutional Ethics Committee.

Statement of Competing Interest

The authors declare no relevant 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

No generative AI was used.

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DIAGNOSTIC UTILITY OF VAGINAL PLACENTAL PROTEIN 14 AS A NOVEL BIOMARKER FOR PRETERM PRELABOR RUPTURE OF MEMBRANES

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Preterm prelabor rupture of membranes (PPROM) is a major cause of neonatal morbidity and mortality. Placental Protein 14 (PP14), a glycodelin glycoprotein, has emerged as a potential biomarker for the diagnosis of PPROM. The study aimed to evaluate the diagnostic accuracy of PP14 in detecting fetal membrane rupture. This case-control study was conducted at Al Yarmouk Teaching Hospital from January to October 2023. The sample included 45 pregnant women diagnosed with PPROM who were compared to 45 pregnant women with intact membranes. Vaginal fluid samples collected from women in both groups were tested with nitrazine paper and then sent to the laboratory for PP14c measurement using immunoassay. PPROM cases had significantly higher vaginal PP14 levels than controls (mean 0.0108 ± 0.002 ng/mL vs. 0.0056 ± 0.003 ng/mL; $p < 0.001$). ROC analysis showed an AUC of 0.852 (95% CI: 0.762–0.941; $p < 0.001$). Using a cut-off of 0.0078 ng/mL, PP14 yielded a sensitivity of 97.8% and specificity of 77.8%. The odds of elevated PP14 (≥ 0.0078 ng/mL) were significantly higher in PPROM cases (OR = 154; 95% CI: 18.8–1261.4; $p < 0.001$). In conclusion, vaginal PP14 demonstrates strong diagnostic performance for PPROM, combining high sensitivity with good specificity. PP14 is a promising, low-cost potential adjunct to conventional bedside tests and could improve early, accurate detection of membrane rupture, facilitating timely clinical management.

Keywords: PROM, PP14, nitrazine

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INTRODUCTION

Prelabor rupture of membranes (PROM) occurs when the chorioamniotic membrane ruptures before labor onset. Preterm PROM (PPROM) before 37 weeks contributes significantly to preterm births and neonatal complications, including infection, respiratory distress, and long-term neurodevelopmental impairment (1,2).

Current bedside tests (speculum exam, fern test, nitrazine paper) are rapid and inexpensive (3). Still, they can yield false positives and false negatives because vaginal pH and visual findings may be altered by blood, semen, infection, or topical agents (4).

Novel biomarkers such as PAMG-1 and IGFBP-1 improve accuracy but may be costly, variably available, or affected by contamination (5). These limitations can delay correct diagnosis or lead to unnecessary interventions.

Diagnosis typically involves clinical examination and laboratory tests, including speculum exams, the fern test (6), and the nitrazine test, though these can have false positives and negatives (4).

Novel biomarkers, such as PAMG-1 and IGFBP-1, offer enhanced diagnostic accuracy for PROM (5), while ultrasound is vital for evaluating amniotic fluid volume and overall fetal well-being (7).

Biological rationale for Placental Protein 14

Placenta Protein 14 (PP14) is an important glycoprotein in the endometrial and decidual context of pregnancy, characterized by its 42,000 MW and 162 amino acids with potential immunosuppressive properties, relevant to pregnancy maintenance (8).

PP14's immunosuppressive role supports the establishment and maintenance of pregnancy by inducing apoptosis in T cells without affecting monocytes (9). Furthermore, because it modulates T-cell activity and cytokine responses, PP14 may be linked to preterm prelabor rupture of membranes (PPROM), thus presenting a potential biomarker for PPROM diagnostics (10).

The tissue specificity and presence of PP14 in amniotic fluid make it a biologically plausible biomarker for vaginal amniotic leakage (11). Although early studies indicate that PP14 concentrations vary significantly between women with intact versus ruptured membranes (11,12), the existing evidence remains limited and heterogeneous.

Gap in knowledge and justification for this study

A critical need exists for novel diagnostic biomarkers that combine high sensitivity with acceptable specificity and affordability to prevent missed diagnoses of PPROM. Existing biomarker studies vary in population, assay method, and reported thresholds, and few have evaluated PP14 in a well-defined preterm population with standardized sample handling. Demonstrating reproducible diagnostic accuracy for PP14 would support its incorporation into clinical algorithms and could reduce diagnostic uncertainty, unnecessary interventions, and adverse outcomes. Therefore, this study aimed to evaluate the diagnostic accuracy of placental protein 14 (PP14) in detecting rupture of fetal membranes in a standardized clinical setting.

METHODS

A case-control study was conducted in the Obstetrics and Gynecology Department at Al Yarmouk Teaching Hospital from January to October 2023. The study enrolled 90 pregnant women via consecutive sampling based on eligibility criteria during the study period. The cohort comprised 45 pregnant women diagnosed with preterm PROM at gestational age ≥ 28 to < 37 weeks (cases), who were compared to 45 pregnant women at a similar gestational age with intact membranes (controls). The study used the available consecutive eligible patients during the study period.

Group allocation criteria

Cases (PPROM) were defined as pregnant women presenting with clinical evidence of amniotic fluid leakage on sterile speculum examination and a positive nitrazine test. Controls were defined as pregnant women with no observed leakage and a negative nitrazine test.

Exclusion criteria

Exclusion criteria for both groups included use of vaginal medications within 72 hours prior to the interview, recent antibiotic use (which can lead to alkaline urine), active genitourinary tract infection, sexual intercourse within the preceding 48 hours, presence of chronic diseases (e.g., hypertension and diabetes), history of trauma during the current pregnancy, and gross blood contamination in the vaginal fluid sample.

Specimen collection

Following initial history taking, each participant underwent a thorough clinical evaluation, including a sterile speculum examination. Vaginal fluid was collected from the posterior vaginal fornix using a sterile cotton swab or by direct aspiration when pooling was visible. One aliquot was used

immediately for the nitrazine test. A second aliquot was placed into a labeled sterile tube, stored at 2–8 °C, and transported daily to the laboratory for PP14 measurement.

Nitrazine test (reference standard)

The nitrazine test was performed immediately at the bedside using sterile nitrazine paper strips (pH range 4.5–7.5). Following application of the vaginal fluid sample to the strip, the color shift was evaluated against the manufacturer-provided pH color chart. A pH ranging from 7.1 to 7.3 (indicated by a dark blue color shift) was considered positive for amniotic fluid leakage (13). Women with a positive nitrazine test and visible fluid pooling were assigned to the PPROM group, while women with a negative test and no pooling were assigned to the control group. Specimens with significant blood contamination were excluded from analysis to prevent diagnostic interference.

Placental Protein 14 (PP14) ELISA (Index Test)

Concentration of PP14 in vaginal fluid was determined using a commercially available enzyme-linked immunosorbent assay (Human Placental Protein 14 ELISA Kit, Catalog No. YLA2850HU; Shanghai YL Biotech Co., Shanghai, China) according to the manufacturer's instructions.

To remove cellular debris, vaginal fluid samples were centrifuged at 1000 x g for 10 minutes. All samples were processed within 48 hours of collection, and the supernatant was collected and stored at −20 °C until analysis. The kit uses a double-antibody sandwich ELISA method. Briefly, 50 microliters (µL) of the standard, controls, or diluted samples were added to designated wells pre-coated with an anti-PP14 monoclonal antibody.

Then, a biotin-conjugated detection antibody was added, followed by the addition of a streptavidin-horseradish peroxidase (HRP) conjugate. After the incubation period, tetramethylbenzidine (TMB) was added as a chromogenic substrate. After adding sulfuric acid to halt the reaction, the optical density was measured using a microplate reader set to 450 nm.

Concentrations were interpolated from a standard curve generated with known PP14 standards supplied in the kit. All samples were assayed in duplicate, and the mean value was used for analysis. The assay sensitivity was 0.001ng/mL, and the intra- and inter-assay coefficients of variation were <10%. Results were expressed as ng/mL of PP14 in vaginal fluid. Laboratory personnel were blinded to the participants' group assignment.

Rigid attention to detail during history-taking, examination, and vaginal fluid collection was maintained, as the nitrazine test can yield false-positive or false-negative results due to contamination with alkaline urine, semen, blood, meconium, vaginitis, cervicitis, or antibiotic use.

The questionnaire

Data were collected using a structured questionnaire that included maternal demographics, such as age and smoking history, as well as obstetric history. Obstetric data comprised gravidity, parity, number of abortions, date of the last menstrual period, gestational age, and estimated date of delivery. Information on current vaginal bleeding, previous premature rupture of membranes (PROM), and prior preterm deliveries was also recorded.

Ethical considerations

The study protocol was reviewed by the Ethics Committee of Al Yarmouk Teaching Hospital (Approval No. 758; Approval Date: November 2, 2022), and administrative and ethical approvals were obtained. All participant interviews for both the PPROM group (cases – group 1) and the intact membrane group (controls – group 2) were conducted within the premises of the Obstetrics and Gynecology Department. All procedures adhered to the ethical principles of the Declaration of Helsinki. Written informed consent was obtained from all participants prior to enrolment. To maintain confidentiality, allpersonally identifiable data were anonymized, and the collected information was used exclusively for the purposes of this study.

Statistical methods

Continuous variables were reported as mean ± standard deviation (SD) or median with interquartile range (IQR) after normality assessment using the Shapiro–Wilk test and Q-Q plots. Categorical variables were presented as frequencies and percentages. Group comparisons used the independent t-test or Mann–Whitney U test for continuous variables, and the chi-square test or Fisher's exact test for categorical variables.

The diagnostic performance of PP14 was evaluated using receiver operating characteristic (ROC) curve analysis. The area under the curve (AUC) with a 95% confidence interval (CI) was reported. The optimal cut-off was selected using the Youden index, and the corresponding sensitivity and specificity with 95% CIs were calculated. The unadjusted association between elevated PP14 (dichotomized at the optimal cut-off) and PPROM was assessed using the odds ratio.

RESULTS

The mean age of women in the PPROM group was 26.6 ± 5.2 years (range: 19–37 years), while the mean age in the control group was 27.9 ± 5.1 years (range: 19–37 years), as shown in Table 1. No significant differences were observed between the two groups with regard to age, gravidity, parity, history of miscarriages, gestational age, socioeconomic status (SES), smoking, education, or employment. The majority of women in the PPROM group, 41 (91.1%), were non-smokers, while 4 (8.9%) reported a history of smoking. In the control group, smoking was reported by only 2 (4.4%) participants.

Significant differences were observed concerning the previous history of PROM and preterm delivery. Approximately 14 (31.1%) women in the PPROM group had a history of previous PROM, and another 14 (31.1%) had a history of previous preterm delivery. In contrast, no history of previous PROM was reported in the control group, and a history of previous preterm delivery was confirmed in only 4 (8.9%) control participants.

The mean PP14 level in vaginal fluids in the PPROM group was 0.0108 ± 0.002 ng/ml. while the control group was 0.0056 ± 0.003 ng/ml.

Table 1. *Baseline demographic and clinical characteristics of cases and controls*

Variables Continuous Variables (mean ± SD)	Cases (n = 45)	Control (n = 45)	p-value
Age (in years)	26.6 ± 5.20	27.9 ± 5.18	0.27 ǂ
Gravidity	3.80 ± 1.455	3.53 ± 1.05	0.323ǂ
Parity	2.56 ± 1.235	2.33 ± 1.04	0.36 ǂ
Previous history of miscarriages	0.24 ± 0.48	0.13 ± 0.34	0.213 ǂ
Gestational weeks	31.13 ± 2.17	31.33 ± 2.62	0.69 ǂ
Categorical variables, n (%)			
Smoking history			0.677 ǂ
No	41 (91.1%)	43 (95.6%)	
Yes	4 (8.9%)	2 (4.4%)	
Previous PROM			<0.001ǂ
No	31 (68.9%)	45 (100%)	
Yes	14 (31.1%)	0	
Previous history of pre-term delivery			0.008 ϯ
No	31 (68.9%)	41 (91.1%)	
Yes	14 (31.1%)	4 (8.9%)	
Total	45 (100%)	45 (100%)	

*Independent t test, ϯ Chi square test, ǂFisher's exact test

There was a highly significant difference in PP14 levels (p-value <0.001) between the two groups. As shown in Figure 1, women in the PPROM group had a higher level of PP14 compared to women in the control group.

Analysis of PP14 concentrations between gestational weeks 24 and 36 demonstrated consistently higher levels in women with PPROM compared to controls. As shown in Figure 2, PP14 values in the PPROM group remained elevated across all time points, with a pronounced increase around week 34. In contrast, the control group exhibited lower and relatively stable concentrations throughout the same gestational period. These findings suggest a potential association between elevated PP14 levels and the pathophysiological processes underlying PPROM.

Receiver operating characteristic curve and odds ratio

Receiver operating characteristic (ROC) curve analysis was performed to identify the optimal PP14 cut-off value for

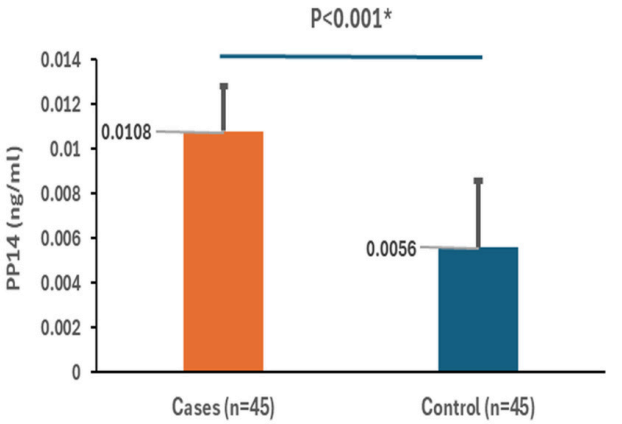

Figure 1. *Distribution of controls and cases by the PP14 level. *Mann–Whitney U test*

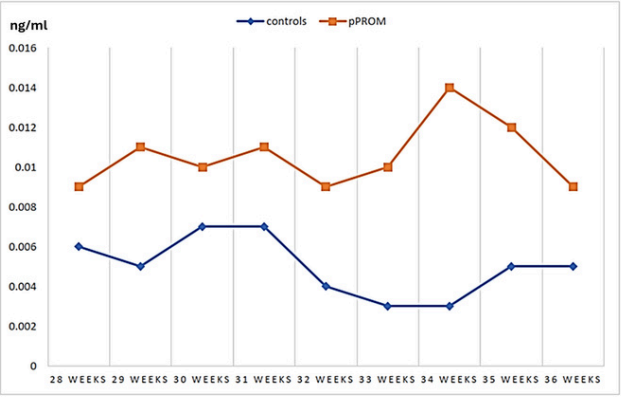

Figure 2. *Distribution of PP14 levels across gestational weeks 24 to 36, demonstrating elevated concentrations in cases relative to controls.*

diagnosing PPROM. As shown in Figure 3, the area under the curve (AUC) for PP14 was 0.852 (standard error = 0.046; p < 0.001; 95% CI: 0.762–0.941).

At the optimal cut-off threshold of 0.0078 ng/mL, PP14 demonstrated good diagnostic performance, with a sensitivity of 97.8% (95% CI: 88.2–99.9%) and a specificity of 77.8% (95% CI: 62.9–88.8%).

Table 2 depicts the distribution of cases and controls according to the vaginal fluid PP14 cut-off level. Using the optimal cut-off of ≥ 0.0078 ng/mL, 44 of 45 PPROM cases (97.8%) tested positive compared to 10 of 45 controls (22.2%).

Women with PPROM had 154 times higher odds of presenting with elevated PP14 (≥ 0.0078 ng/mL) levels than controls (odds ratio (OR) = 154.0, 95% confidence interval (CI): 18.8–1261.4; p < 0.001).

Assessment of potential circularity bias

We acknowledge that using a reference standard that includes the nitrazine test may theoretically introduce circularity bias, as PP14 could be correlated with vaginal pH. To assess this, we calculated the Spearman rank correlation between PP14 levels and nitrazine pH values (treated as a continuous variable based on color chart equivalents). The correlation was weak and not statistically significant (Spearman's ρ = 0.21, p = 0.09), indicating that PP14 provides diagnostic information largely independent of the nitrazine result. Furthermore, PP14 measurement was performed blinded to group allocation, while group assignment was based on a composite reference standard (sterile speculum pooling plus nitrazine test), not on nitrazine alone. Therefore, the risk of overestimation of diagnostic accuracy is limited.

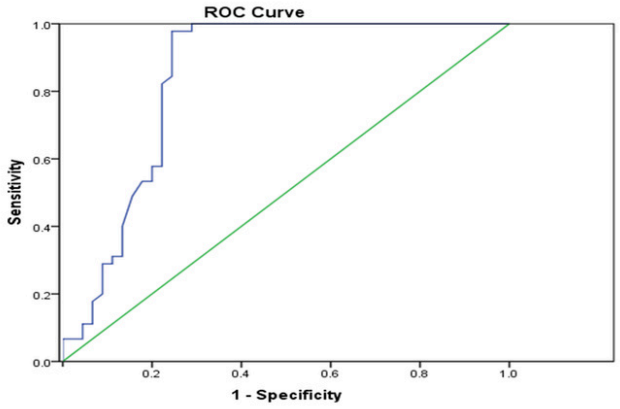

Figure 3. *Roc analysis for PP14*

Table 2. *Distribution of cases and controls according to vaginal fluid PP14 cut-off Levels*

PP14 level	Cases (n = 45)	Control (N + 45)	p-value
≥ 0.0078 ng/ml	44 (97.8%)	10 (22.2%)	p-value < 0.001
< 0.0078 ng/ml	1 (2.2%)	35 (77.7%)	

‡ Analyzed using Pearson's Chi-square test $\chi^2 = 53.519$, df =1

DISCUSSION

These results indicate that PP14 measured in vaginal fluid is a highly sensitive, reasonably specific, and low-cost biomarker that could substantially improve the diagnostic workup of PPROM, particularly in settings where conventional tests are ambiguous or where more expensive commercial immunoassays are not readily available.

Comparison with conventional diagnostic methods

Current bedside tools, including the nitrazine test, fern test, and sterile speculum examination, are rapid and inexpensive but suffer from well-documented limitations. The nitrazine test, which detects a pH shift (≥ 6.5–7.1) due to amniotic fluid, has a reported sensitivity of 70–95% and specificity of 70–90%, and they both vary widely due to contamination with blood, semen, alkaline urine, or bacterial vaginosis (1,2,14). False negatives can occur when leakage is minimal or when the fluid is diluted, while false positives are common in the presence of cervicovaginal infections.

In our study, PP14 achieved a sensitivity of 97.8%, which is at the upper bound of what is achievable with nitrazine, and a specificity that, although not perfect, is sufficient to reduce the diagnostic uncertainty that frequently leads to unnecessary hospitalization or missed intervention.

The fern test, which relies on the crystallization of amniotic fluid on a slide, is similarly operator-dependent and yields a sensitivity of 50–90% (4,15). Both conventional tests also have poor reproducibility. By contrast, PP14 measurement is an objective, laboratory-based immunoassay that eliminates observer variability.

Thus, PP14 is best viewed not as a replacement for bedside tests but as a powerful adjunct, especially when the nitrazine or fern result is equivocal or when clinical suspicion remains high despite a negative bedside test.

Comparison with other emerging biomarkers

Several protein biomarkers have been developed for the diagnosis of PPROM, most notably insulin-like growth factor

binding protein-1 (IGFBP-1) (16) and placental alpha microglobulin-1 (PAMG-1) (5). Pooled sensitivity estimates for PAMG-1 range from 87% to 99%, while pooled specificity estimates range from 88% to 100%. In contrast, the diagnostic performance of IGFBP-1 appears to be more variable, with pooled sensitivity estimates ranging from approximately 90% to 94% and specificity estimates ranging from approximately 90% to 97% (5,7).

The results for PP14 observed in our study (sensitivity 97.8%, specificity 77.8%) are comparable to those reported for PAMG-1 in terms of sensitivity but demonstrate somewhat lower specificity than that reported in some studies (5,11). However, direct comparisons should be interpreted with caution because of differences in study populations, reference standards, and disease prevalence across studies.

Importantly, the cost and accessibility of these biomarkers are what differentiate them. The PAMG-1 and IGFBP-1 tests are often offered as point-of-care assays, which, despite their convenience, incur relatively high per-test costs, sometimes exceeding 10 to 20 US dollars(17).

In our specific environment, the ELISA-based PP14 assay costs roughly $3.60 per sample and may be performed using standard laboratory equipment (18). Additionally, the requirement for a microplate reader, stable electricity, refrigeration, and trained technicians means that PP14 testing may not be immediately feasible in the most resource-constrained environments without additional investment. This is true even though the cost of each test is lower than that of some commercial point-of-care immunoassays. Furthermore, because PP14 is assessed from the same vaginal fluid sample that is acquired during a speculum examination, there is no need for any further invasive treatment to be performed.

Comparison with previous studies on PP14

Our findings are in close agreement with the initial research conducted by Wang et al. (2018), who were the first to investigate PP14 in vaginal fluid for the diagnosis of PPROM (11). The area under the curve (AUC) in that study was 0.855, the sensitivity was 90%, and the specificity was 84%, which is in striking similarity to the values that we obtained (AUC 0.852, sensitivity 97.8%, specificity 77.8%). It is possible that the modest variances can be attributed to variations in demographic characteristics (for example, the gestational age range or inclusion of term PROM in their cohort), sample handling, or the particular ELISA kit used. It is important to note that the reproducibility of PP14 as a diagnostic marker is strongly supported by the consistency observed across independent cohorts.

Notably, our research focused on patients with PPROM between 28 and 37 weeks of gestation, as this timeframe presents the highest risk. The diagnostic performance was not affected by the inclusion of both preterm and term cases of premature rupture of membranes (PROM) in the study by Wang et al. (11). Although further stratified studies are needed to confirm this finding, the results suggest that the PP14 test performs consistently across a broad range of gestational ages.

Biological plausibility

The key physiological roles of PP14 (glycodelin) include immunosuppression, which involves triggering T-cell death to increase fetal tolerance, and modulation of endometrial receptivity (10). The fetal membranes act as a barrier in the course of a normal pregnancy, thereby preventing large quantities of PP14 from entering the vagina (19). The amniotic fluid, which contains PP14 at quantities that are significantly higher than those found in cervicovaginal secretions (20), seeps into the vaginal canal when the membranes tear. The significant diagnostic signal is supported by this physiologic gradient throughout the study. Previous research has shown that PP14 is relatively stable in the presence of blood and other contaminants, which is one of its advantages over IGFBP-1 or PAMG-1(8). Although our study did not include samples with substantial blood contamination, the ability of PP14 to maintain diagnostic accuracy despite minimal contamination may be advantageous in clinical obstetric settings where vaginal bleeding is present.

Future directions

The excellent diagnostic performance of PP14, characterized by very high sensitivity (97.8%), supports further investigation of its potential as a reliable biomarker for the diagnosis of PROM. A negative PP14 test (<0.0078 ng/ml) would be a strong indicator against the presence of PPROM in clinical practice. This could help physicians avoid unnecessary interventions, such as hospitalization, corticosteroid administration, or tocolysis, thereby reducing patient stress and healthcare costs.

On the other hand, a positive result, particularly when combined with a positive nitrazine or fern test, can confirm the diagnosis with a high degree of certainty, allowing for a timely initiation of appropriate management. This management may include antibiotic prophylaxis, antenatal corticosteroids, and transfer to a tertiary center if required.

In secondary-level hospitals that have at least basic

laboratory capabilities, it may be feasible to include PP14 in standard diagnostic procedures. This is because the sample is already collected during a speculum examination, which carries a relatively low cost.

Nevertheless, in order to achieve widespread use in primary or remote institutions, the assay would need to be simplified even further, or a point-of-care format would need to be developed initially. Whenever nitrazine or fern is unable to provide a definitive answer because of limited leakage or contamination, equivocal bedside tests are performed.

Populations at high risk include women who have a history of previous preterm birth or premature preterm delivery, for whom a prompt and precise diagnosis is of the utmost importance.

Settings with limited resources are those in which costly commercial point-of-care testing is not readily available.

Several other directions for future research are warranted. To confirm the proposed cut-off value and evaluate the test's predictive performance in everyday clinical practice, it is required to conduct prospective validation through a large-scale, multicenter study with consecutive recruitment.

It would be helpful to explain the relative diagnostic accuracy and cost-effectiveness of PP14 and other established biomarkers, such as PAMG-1 and IGFBP-1, in the same population by comparing these biomarkers head-to-head. This would also help to determine the optimal clinical role for each biomarker.

Additionally, studies evaluating the stability of PP14 in vaginal fluid samples under various storage and transport conditions may provide valuable insights into its feasibility for use in remote or resource-limited settings, where rapid laboratory processing may not be readily available.

As of yet, the value of PP14 in identifying membrane rupture during the second trimester of pregnancy, the time when management decisions about fetal viability are particularly difficult, has not been well investigated and should be the subject of devoted research.

In conclusion, investigating the combined use of PP14 with other biomarkers, such as IGFBP-1, may enhance diagnostic accuracy compared with either marker alone, and thereby help determine whether a multi-marker strategy provides additional clinical value.

Limitations

While the case-control study design offers a high level of internal validity for diagnostic accuracy, it may overestimate test performance compared to a prospective cohort in which the prevalence of PPROM is considerably lower. As this was a single-center study, the findings cannot be generalized to populations with different demographic features, ethnic backgrounds, or clinical practices. It is crucial to perform multicenter validation.

The sample size was not designed to support subgroup analyses, such as those based on gestational age, the presence of bacterial vaginosis, or the extent of membrane rupture, although it provided adequate statistical power for the primary analysis. Consequently, larger studies are required to investigate the test's performance across these subgroups.

Although samples containing coarse blood were not included in the study, the impact of microscopic blood or other vaginal fluids on the concentration of PP14 was not rigorously quantified. According to the findings of prior studies, PP14 appears to maintain stability even when blood is present (8).

A final point to consider is that the research did not include women in the second trimester of pregnancy or those who had term or preterm labor; hence, the diagnostic performance of PP14 in these populations is still unknown.

Placental protein 14 (PP14) measured in vaginal fluid demonstrates strong diagnostic performance for PPROM, characterized by very high sensitivity and excellent specificity. This biomarker has the potential to complement standard bedside tests, minimize diagnostic uncertainty, and improve rapid clinical care. Furthermore, it is a promising and inexpensive biomarker. With additional prospective validation, PP14 could become a useful, easily accessible diagnostic tool for PPROM, particularly in settings with limited resources.

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Author Contributions

Conceptualization: A.A.R. and M.A.H.A.; Methodology: A.A.R. and M.A.H.A.; Formal analysis: A.A.R. and M.A.H.A.; Investigation: A.A.R. and M.A.H.A.; Writing – Original draft: A.A.R. and M.A.H.A.; Writing – review & editing: A.A.R. and M.A.H.A. All authors have read and agreed to the published version of the manuscript.

Statement of Ethics

The study protocol was reviewed and approved by the Ethics Committee of Al Yarmouk Teaching Hospital (Approval No. 758; Date: November 2, 2022). All procedures adhered to the ethical principles of the Declaration of Helsinki, and written informed consent was obtained from all participants prior to enrolment. Participant data were fully anonymized to maintain strict confidentiality.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availability

The statistical data supporting the findings of this study are included within the article. The raw datasets generated and analyzed are available from the corresponding author upon reasonable academic request.

Statement of Generative AI Use

No generative AI was used.

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ORIGINAL ARTICLE

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PERCEPTIONS OF DIGITALIZATION AND DIGITAL LITERACY AMONG HEALTHCARE EMPLOYEES: A CROSS-SECTIONAL STUDY

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This study aimed to assess employees' self-perception of digital literacy levels, participation in digital skills training, and satisfaction with the current state of digitalization within the healthcare institution. A cross-sectional study was conducted among employees in the Institute of Public Health of Vojvodina (IPHV) from December 2024 to February 2025, using an anonymous, self-administered questionnaire. The analysis included demographic characteristics, self-perceived levels of digital literacy, and perceived impact of digitalization on daily work activities. The response rate was 62%, and the research involved 156 respondents. Our findings showed that 24.4% of respondents had attended some digital skills training. Women were less likely to have attended digital courses compared to men (19.7% vs. 41.2%), while the youngest respondents (under 25 years old) were more likely to have participated in training programs. Participants with a master's degree, doctoral degree, or specialist studies were strongly associated with higher digital training attendance compared to those with a high school education ($p = 0.0001$, $p = 0.0001$, and $p = 0.0082$, respectively). A statistically significant difference ($p < 0.05$) was found in satisfaction with the current level of digitalization in the institution, with physicians reporting the highest satisfaction. The results of our study underscore the need for additional education and support in digitalization, particularly for older employees and administrative staff, to improve their job satisfaction.

Keywords: digitalization, digital literacy, healthcare institutions, job satisfaction

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INTRODUCTION

The successful implementation of digitalization in healthcare institutions largely depends on the digital literacy and adaptability of healthcare professionals (1-3).
Despite the recognized benefits of digitalization, including improved efficiency, accessibility, and workflow optimization, healthcare professionals face numerous challenges in adapting to these changes (4, 5).
Previous research has highlighted disparities in digital competencies among healthcare workers, influenced by factors such as age, education level, professional role, and work experience (6). Moreover, the perception of digital tools' usefulness varies across different professional groups, which may impact the overall effectiveness of digital transformation in healthcare settings. Some professionals view digitalization as a means to optimize workflow and reduce administrative burden, while others experience difficulties in adapting to new technologies due to inadequate training or resistance to change (7, 8).
Given the increasing reliance on digital tools in public health institutions, it is essential to assess how employees perceive digitalization and whether they have adequate training to utilize these technologies effectively (4-8).
The aim of this study was to assess, based on employees' self-perception (both medical and non-medical staff), the level of digital literacy in a healthcare institution, participation in digital skills training, as well as satisfaction with the current level of digitalization within the institution. Understanding these aspects will be crucial for optimizing workflows and enhancing overall institutional efficiency (9).

METHODS

An institution-based cross-sectional study was conducted among employees of the Institute of Public Health of Vojvodina (IPHV), from December 2024 to February 2025.
The IPHV is located in Novi Sad, the administrative center of Vojvodina, the northern province of Serbia. It is one of the largest institutions in the region dedicated to preventive healthcare, playing a key role in public health.
The research instrument was a structured questionnaire designed to assess the level of digitalization across various participant characteristics. The questionnaire was carefully developed based on a comprehensive review of the relevant literature (7-14).
The questionnaire consisted of several sections: the first assessed socio-demographic characteristics (gender, age, education, job position, and work experience); the second focused on digital literacy; the third evaluated the impact of digitalization on daily tasks; the fourth examined satisfaction with current digitalization and suggestions for future improvements. The questionnaire was distributed in paper form by a researcher during participants' working hours. A pilot version was tested on 25 employees to ensure clarity and accuracy of responses.
The study protocol was approved by the Ethics Committee of the Institute of Public Health of Vojvodina (number: 01-934/2, issued on June 13, 2024). Participation was voluntary, anonymous, and uncompensated. Participants were informed about the study's purpose and provided written consent before completing the questionnaire. Each respondent was assigned a unique code, and data were entered into a secure, password-protected database accessible only to the research team.

Statistical methods

Descriptive statistics were used to present categorical variables as frequencies and percentages, while continuous data were shown as means with standard deviation (±SD). The Chi-square test or Fisher's exact test was used to analyze categorical data. The odds ratio (OR) with confidence intervals (CI) was used to assess the strength of associations between categorical variables, with the lowest 'Yes' response category as the reference. Pareto analysis, based on the 80/20 principle, identified key digital tools and challenges in daily work. Statistical analyses were performed using SPSS version 21, with significance set at $p < 0.05$.

RESULTS

During the study period, approximately 250 questionnaires were distributed, and following multiple reminders, the response rate reached 62% (n = 156). The majority of participants were female (78.2%). The mean age of participants was 43 ± 5.2 years.
Regarding participants' digital literacy, only 24.4% reported having participated in any form of digital skills training, while approximately 30% self-reported intermediate digital skills (Table 1).
Figure 1 illustrates the utilization rates of different digital tools among respondents, along with patterns of platform usage. Viber and YouTube were the most frequently used digital tools, reported by the largest number of users (55/156; 35.3%). The cumulative curve in the graph indicates that the first few categories account for the largest share of the overall distribution, with over 80% of respondents using

Table 1. *Digital literacy of the participants*

Variables		N = 156	Percentage
Have you attended any courses or training related to digital skills?	Yes	38	24.4
	No	118	75.6
How would you rate your level of digital literacy?	Beginner	21	13.5
	Intermediate	46	29.5
	Advanced	65	41.7
	Expert	24	15.3

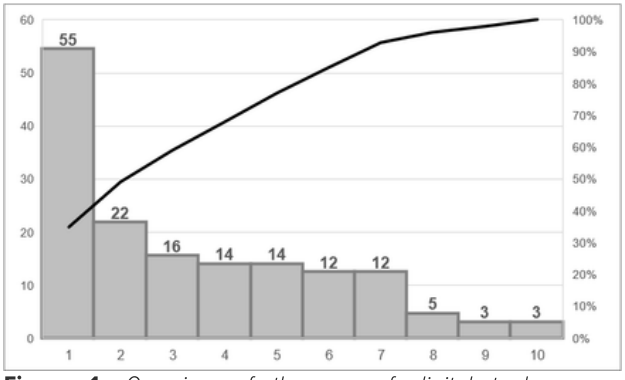

Figure 1. *Overview of the use of digital tools among participants*

Legend: 1 - Viber and YouTube; 2 - Viber and Zoom; 3 - Viber; 4 - Zoom; 5 - Viber and Skype; 6 - YouTube; 7 - Viber and YouTube and Zoom; 8 - Viber and YouTube and Zoom and Skype; 9 - Skype; 10 - Other (WeTransfer, Pinterest)

tool combinations, with Viber as the most prevalent platform.

In addition, the most frequently reported challenges included loss of personal contact and reduced quality of patient interactions (33/156; 21.2%), followed by increased administrative workload (27/156; 17.3%). Other concerns included challenges in integrating new digital solutions with existing systems, along with resistance to change and innovation (each accounting for 19%). These factors collectively account for over 70% of all reported challenges, highlighting them as key concerns in the digitalization process (Figure 2).

Females were significantly less likely to attend training than males (24/122, 19.7% vs. 14/34, 41.2%; p = 0.0116). Compared to participants aged ≥55 years, the two youngest age groups (<25 years and 25–34 years) were significantly more likely to attend training (p = 0.0001 and p = 0.0080, respectively).

Participants with a master's degree, doctoral degree, or specialist studies were strongly associated with higher training attendance compared to those with a high school education (p = 0.0001, p = 0.0001, and p = 0.0082, respectively).

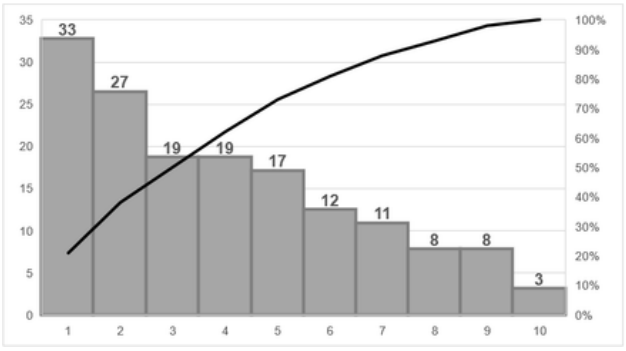

Figure 2. *Overview of the main challenges related to digitalization in the work of the participants*

Legend: 1 - Loss of personal contact – reduced quality of communication and relationships within the team; 2 - Increased administrative workload; 3 - Issues with integrating new digital solutions with existing systems or software; 4 - Resistance to changes and innovations; 5 - Lack of digital skills and training; 6 - Outdated equipment, insufficient internet connectivity, or inadequate software tools; 7 - Data security and protection; 8 - Costs of procurement, implementation, and maintenance of digital tools; 9 - Limitations in digitization due to legal regulations; 10 - System reliability – issues with frequent breakdowns, system crashes, or technical support that is not fast or efficient enough

There were no statistically significant differences in training attendance based on participants' profession (p > 0.05).

Compared to participants with ≥16 years of experience, respondents with <1 year of experience had a significantly higher probability of attendance (p = 0.0011). Similarly, participants with 1–5 years of experience (p < 0.0001) and those with 6–10 years of experience (p = 0.0077) also had a significantly higher probability of attending training (Table 2).

Table 3 presents the characteristics of participants based on their self-assessed level of digital literacy ("Beginner" or "Intermediate" or "Advanced" or "Expert").

Males comprised a higher percentage of beginners (57.1%) compared to females (42.9%), while females predominated in the expert level (91.7%) (p = 0.0002).

Age distribution was also significantly associated with self-assessed digital literacy levels (p = 0.0162). The proportion of participants aged < 25 and 25–34 years increased steadily from the beginner to expert levels (25.0% for those aged <25 years, and 33.3% for those aged 25–34). Conversely, participants aged ≥ 55 were predominantly represented among beginners (52.4%).

Education level was a strong predictor of self-assessed digital literacy (p < 0.0001). Participants with a high school education were predominantly beginners (66.7%). Advanced and expert levels were most common among

Table 2. *Characteristics of the participants in relation to whether they have attended any courses or training related to digital skills*

Characteristics		Yes (n = 38)	%	No (n = 118)	%	OR	95% CI	p-value[a]
Gender	Male	14	36.8	20	16.9	ref.		
	Female	24	63.2	98	83.1	0.35	0.15-0.79	**0.0116**
Age group (in years)	< 25	17	44.7	10	8.5	28.9	5.69-146.92	**0.0001**
	25-34	10	26.3	19	16.1	8.95	1.77-45.15	**0.008**
	35-44	5	13.2	33	28	2.58	0.47-14.22	0.2777
	45-54	4	10.5	22	18.6	3.09	0.52-18.33	0.2141
	≥ 55	2	5.3	34	28.8	ref.		
Level of education	High school	1	2.6	55	46.6	ref.		
	Higher/Vocational school	2	5.3	23	19.5	4.78	0.41-55.39	0.2105
	Specialist studies	7	18.4	21	17.8	18.33	2.13-158.15	**0.0082**
	Master's studies	8	21.1	4	3.4	110	10.88-1112.02	**0.0001**
	Doctoral studies	20	52.6	15	12.7	73.33	9.09-591.69	**0.0001**
Profession	Nurse	8	21.1	83	70.3	0.58	0.06-5.42	0.6315
	Administrative staff	14	36.8	19	16.1	4.42	0.48-40.98	0.1908
	Doctor	15	39.5	10	8.5	9	0.94-86.53	0.0571
	Other	1	2.6	6	5.1	ref.		
Years of work experience	< 1	5	13.2	4	3.4	52.5	4.86-566.95	**0.0011**
	1-5	18	47.4	9	7.6	84	9.90-712.91	**< 0.0001**
	6-10	13	34.2	32	27.1	17.06	2.12-137.31	**0.0077**
	11-15	1	2.6	31	26.3	1.35	0.08-22.51	0.8323
	≥ 16	1	2.6	42	35.6	ref.		

[a]Values that differ significantly ($p < 0.05$) are marked in bold.

Table 3. *Characteristics of the participants in relation to their self-assessment of digital literacy level*

Characteristics		Level of digital literacy								p-value[a]
		Beginner (n = 21)	%	Intermediate (n = 46)	%	Advanced (n = 65)	%	Expert (n = 24)	%	
Gender	Male	12	57.1	10	21.7	10	15.4	2	8.3	**0.0002**
	Female	9	42.9	36	78.3	55	84.6	22	91.7	
Age group (in years)	< 25	1	4.8	7	15.2	13	20	6	25	**0.0162**
	25-34	1	4.8	8	17.4	12	18.5	8	33.3	
	35-44	3	14.3	9	19.6	21	32.3	5	20.8	
	45-54	5	23.8	10	21.7	9	13.8	2	8.3	
	≥ 55	11	52.4	12	26.1	10	15.4	3	12.5	
Level of education	High school	14	66.7	27	58.7	12	18.5	3	12.5	**<0.0001**
	Higher/Vocational school	3	14.3	16	34.8	4	6.2	2	8.3	
	Specialist studies	2	9.5	2	4.3	17	26.2	7	29.2	
	Master's studies	1	4.8	1	2.2	9	13.8	1	4.2	
	Doctoral studies	1	4.8	0	0	23	35.4	11	45.8	
Profession	Nurse	15	71.4	32	69.6	41	63.1	3	12.5	**<0.0001**
	Administrative staff	3	14.3	12	26.1	14	21.5	4	16.7	
	Doctor	1	4.8	1	2.2	8	12.3	15	62.5	
	Other	2	9.5	1	2.2	2	3.1	2	8.3	
Years of work experience	< 1	1	4.8	3	6.5	3	4.6	2	8.3	**0.0002**
	1-5	4	19	13	28.3	5	7.7	5	20.8	
	6-10	8	38.1	20	43.5	8	12.3	9	37.5	
	11-15	3	14.3	5	10.9	20	30.8	4	16.7	
	≥ 16	5	23.8	5	10.9	29	44.6	4	16.7	

[a]Values that differ significantly ($p < 0.05$) are marked in bold.

participants with doctoral degrees (35.4% and 45.8%, respectively).

Professional roles were also significantly associated with digital literacy levels ($p < 0.0001$). Nurses represented the majority at the beginner (71.4%) and intermediate (69.6%) levels, while doctors made up the majority of experts (62.5%). Administrative staff were more evenly distributed across the intermediate level (26.1%).

Years of work experience were significantly associated with digital literacy levels ($p = 0.0002$). Participants with ≥16 years of experience were predominantly at the advanced level (44.6%), those with 6–10 years of experience formed the largest group at the intermediate level (43.5%), while those with 1–5 years of experience were more likely to self-assess as experts (20.8%).

Satisfaction with the current level of digitalization in the institution varied significantly between professions ($p < 0.0001$). Among nurses, 53.8% expressed partial satisfaction, 48.5% of administrative staff were satisfied, while doctors had the highest level of satisfaction (56.0%).

In contrast, the 'Other' category had the highest dissatisfaction rate (42.8%) (Table 4).

There was a significant variation across different years of work experience and various aspects of digitalization ($p = 0.0378$). Respondents with less than one year of work experience reported a higher percentage of positive responses (66.7%), while participants with more than 16 years of experience had the highest proportion of neutral responses (74.4%).

A strong association was observed ($p < 0.0001$), with respondents in the 6-10, 11-15, and ≥ 16 years of experience groups largely agreeing that further digitalization would enhance their productivity, showing higher proportions of positive responses (73.3%, 75.0%, and 76.7%, respectively). In contrast, those with less than one year of experience exhibited a lower proportion of positive responses (44.4%).

There were no significant differences regarding satisfaction with the current level of digitalization across the years of work experience ($p = 0.8007$) (Table 5).

DISCUSSION

To our knowledge, this is the first insight into the key factors influencing digital competency and adoption in public health institutions in our country, offering valuable guidance for developing targeted training programs and optimizing digital transformation strategies. The findings highlight significant differences in digital skills training participation and digital literacy levels based on gender, age, education, job position, and work experience.

Approximately 80% of participants were women, reflecting the gender distribution in the institution. Although men attended digital training more frequently, most had only beginner-level digital skills. The majority of participants (36%) had a high school education, and nurses represented 58% of the study group. Participants with 6–10 and ≥ 16 years of work experience made up 29% and 28%, respectively.

Medical personnel with access to digital devices, such as computers, smartphones, and digital medical equipment, generally exhibit high digital literacy (10, 11). This study highlights the significant impact of digital literacy and training on healthcare employees' engagement with digital tools. Despite the widespread use of platforms, only 24.4%

Table 4. *The impact of digitalization on productivity, the perception of further digitalization development, and satisfaction with the current level of digitalization according to the participants' occupation*

Variables		Profession								p-value[a]
		Nurse (n = 91)	%	Administrative staff (n = 33)	%	Doctor (n = 25)	%	Other (n = 7)	%	
How does digitalization affect your productivity?	Negative	1	1.1	4	12.1	1	4	2	28.6	**0.0013**
	Neutral	50	54.9	27	81.8	11	44	4	57.1	
	Positive	40	44	2	6.1	13	52	1	14.3	
Do you believe that further digitalization would improve your productivity?	Yes	54	59.3	19	57.6	20	80	4	57.1	0.1432
	No	12	13.2	9	27.3	2	8	2	28.6	
	Not sure	25	27.5	5	15.1	3	12	1	14.3	
Are you satisfied with the current level of digitalization in your institution?	Yes	40	44	16	48.5	14	56	2	28.6	**<0.0001**
	No	2	2.2	9	27.3	1	4	3	42.8	
	Partially	49	53.8	8	24.2	10	40	2	28.6	

[a]Values that differ significantly ($p < 0.05$) are marked in bold.

Table 5. *The impact of digitalization on productivity, the perception of further digitalization development, and satisfaction with the current level of digitalization, according to the years of work experience of the participants*

Variables		Years of work experience										p-value[a]
		< 1 (n = 9)	%	1-5 (n = 27)	%	6-10 (n = 45)	%	11-15 (n = 32)	%	≥ 16 (n = 43)	%	
How does digitalization affect your productivity?	Negative	1	11.1	1	3.7	1	2.2	2	6.3	3	7	**0.0378**
	Neutral	2	22.2	11	40.7	27	60	20	62.5	32	74.4	
	Positive	6	66.7	15	55.6	17	37.8	10	31.3	8	18.6	
Do you believe that further digitalization would improve your productivity?	Yes	4	44.4	3	11.1	33	73.3	24	75	33	76.7	**<0.0001**
	No	2	22.2	2	7.4	8	17.8	5	15.6	8	18.6	
	Not sure	3	33.3	22	81.5	4	8.9	3	9.4	2	4.7	
Are you satisfied with the current level of digitalization in your institution?	Yes	3	33.3	15	55.6	18	40	15	46.9	21	48.8	0.8007
	No	1	11.1	2	7.4	3	6.7	3	9.4	6	14	
	Partially	5	55.6	10	37	24	53.3	14	43.8	16	37.2	

[a]Values that differ significantly ($p < 0.05$) are marked in bold.

of participants reported attending formal digital skills training. This gap suggests that insufficient training may hinder the effective implementation of digitalization in healthcare institutions, limiting the optimal use of digital resources (12).

Key barriers to digitalization identified in this study include the loss of personal contact, increased administrative workload, and resistance to new technologies. These challenges, representing over 70% of reported difficulties, emphasize the need for structured integration strategies to enhance user acceptance and reduce workflow disruptions. A 2020 study among healthcare professionals in Finland found that while digitalization reduced some workloads and streamlined tasks, it also increased workload and slowed processes due to poor system usability and the continuous learning required for new digital services (13).

A study examining barriers healthcare providers perceive regarding e-health innovations identified two key psychological barriers: tradition and image. Tradition barriers refer to conflicts between the innovation and end-users' existing behaviors, norms, and experiences, while image barriers reflect negative assessments of the innovation's image, efficacy, and consequences of its use (14). Similar findings have been reported in other studies conducted in different regions (15–18).

Our findings indicate that most participants lacked formal digital literacy training, likely relying on self-directed learning or work experience. This low percentage may be due to the lack of available training programs, limited awareness of their importance, or a general perception that digital skills are not a priority in professional development. Additionally, older participants with more work experience may not have seen the need for formal training.

A study in the Portuguese public sector found that employees with higher education and professional skills were more likely to participate in digital training. Most participants also expressed interest in future training, particularly in data management, cybersecurity, and communication systems (19). Similarly, a study among hospital healthcare professionals found that those with master's degrees were 2.13 times more likely to have adequate digital literacy than those with a bachelor's degree or lower (11). This may be due to the greater use of digital technology for education and research among higher-educated health professionals (11, 19).

We found that doctors reported the highest satisfaction and confidence in digitalization improving productivity, while administrative staff showed the highest proportion of neutral or negative responses. Nurses expressed mixed perceptions, reflecting the complexity of digital adoption across different healthcare roles. These variations suggest the need for tailored digital solutions for each professional group. Another study found a significant association between digital literacy levels and attitudes toward digital health technology, concluding that a positive attitude is linked to higher digital competence (11).

Digitalization reshapes healthcare work, but managers and healthcare professionals perceive its effects differently. In developing countries, low digital literacy among healthcare workers hinders the implementation of information systems (20).

A large-scale study in Israel identified training and a positive attitude as key factors in digital adoption, while high costs remain a major barrier. Early adopters and professionals who value their impact perceive digitalization as enhancing their influence (21).

The strength of this study lies in its valuable insights into employee satisfaction with digitalization, its identification of key factors influencing digital literacy, and its emphasis on the need for targeted training initiatives. These findings provide a foundation for future research and offer practical recommendations aimed at improving digital competencies and enhancing efficiency within the healthcare sector.

Our study had some limitations. As a single-center study, its findings may not be fully generalizable. The cross-sectional design limits causal inferences, and self-reported digital skills may introduce response bias. Selection bias is also possible, as participants may have had a greater interest in digitalization. Additionally, the study does not assess the long-term impact of digitalization or the effectiveness of training programs. Small sample sizes in some subgroups may have affected the estimated precision. Despite these limitations, we believe that the main findings remain relevant, highlighting the need for further large-scale and longitudinal research.

Findings of our study indicate that younger, highly educated, and less experienced employees are more inclined toward digital skills training, while certain professional groups and older employees may require targeted interventions to bridge the digital divide.

Despite widespread digital tool usage, key barriers such as increased administrative workload, resistance to change, and insufficient formal training persist. Addressing these issues through structured training programs, institutional support, and user-friendly digital solutions could enhance the efficiency and effectiveness of digitalization efforts in healthcare settings.

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Author Contributions

Conceptualization, methodology, & investigation, formal analysis, and writing—original draft preparation: M. L.; Data curation, formal analysis, and validation: I.M.; Investigation, data curation, and visualization: K.S.; Methodology, writing—review, editing, and supervision: M.R.

Statement of Ethics

This study was approved by the Ethics Committee of the Institute of Public Health of Vojvodina (No. 01-934/2, issued on June 13, 2024). Participation was voluntary, anonymous, and uncompensated. All participants provided written informed consent prior to completing the questionnaire. Data were coded and stored in a secure, password-protected database.

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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AFMN BIOMEDICINE

ORIGINAL ARTICLE

COMPREHENSIVE MAXILLARY ANTERIOR TEETH AESTHETICS ANALYSIS WITH DEEP LEARNING IN DENTAL ANTHROPOMETRY

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The height and width of maxillary front teeth, their width-to-height ratio—W/H% (gold standard), their width ratio (gold proportion), smile width, buccal corridor, and marginal gingiva define smile form. The study examined the characteristics of dental students with complete sets of teeth and used deep learning in dental anthropometry. The study involved 88 subjects. The central incisor was measured in the subjects' mouths, while the gold proportion was photometrically established. Clinical examinations assessed smile breadth, buccal corridor visibility, and gingival contour class. The Adaptive Neuro-Fuzzy Inference System (ANFIS) was employed for deep learning analysis because of its ability to model complex, non-linear relationships in high-dimensional data. The average length of the central incisor in men amounted to 10.5 mm, whereas the value in women was 10.2 mm. The central incisor length and W/H% did not differ significantly between men and women. In the majority of subjects, the gold proportion in the width of the front teeth was established and was uniform between men and women. On average, 8 to 10 teeth were visible during smiling in the subjects. There was no significant difference in smile width between men and women. A pronounced buccal corridor was present in half of the subjects, without a statistically significant difference between the sexes. The results showed a much higher prevalence of class 1 gingiva than class 2 gingiva, with no significant gender-related difference. The results of ANFIS analyses, represented by Root Mean Square Error (RMSE) values, showed non-linear relations among anthropometric parameters. The study found similarities between anthropometric measurements obtained from the Serbian subjects and findings previously reported in the literature. Modelling complex dental anthropometric relationships using ANFIS helps improve understanding of interactions among dental parameters.

Keywords: gold standard, smile width, buccal corridor, ANFIS, deep learning

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INTRODUCTION

The teeth and dental arch, as prominent facial features, hold paramount aesthetic value. Thus, even a slight irregularity, discoloration, or tissue loss might disrupt their equilibrium. Since appearance often takes precedence over functionality, many young people see dentists to enhance their smile using less intrusive techniques. Advances in dental technology and materials have reduced constraints on tooth morphology, color, and texture, offering the precision necessary to achieve predictable aesthetic outcomes. Natural-looking teeth are generally perceived as more attractive. Despite media pressure to imitate celebrities and have white, large teeth (the “Hollywood smile”), a preference for more natural aesthetics has returned to routine dental practice (1,2). Therefore, it is crucial to understand the anthropometric proportions of natural teeth to provide a natural and attractive appearance of teeth and gums following conservative or prosthetic restorations (3).

The height and width of maxillary front teeth and their mutual ratio (gold standard), the ratio of their widths (gold proportion), the visibility of teeth—the width of the dental arch and the buccal corridor when smiling—and the condition and position of the marginal gingiva that surrounds them determine the shape of the smile (4,5). There has been no anthropometric research on these measures in a homogeneous Serbian group with a full dental arch.

The gold standard (GS) is the ratio of the width and height (W/H) values of maxillary upper incisors. The most attractive appearance of maxillary anterior teeth is obtained when W/H is 75-85% (6).

It is generally considered that individuals perceived as attractive have facial features in proportion to the mathematical formula of the gold proportion (GP) (7). The gold proportion in the dental arch is the ratio of the width of the maxillary central and lateral incisor and the mesial facet of the canine, where the lateral incisor is visible. If the maxillary lateral incisor width value is 1, the same value of the central incisor and the mesial facet of the canine amount to 1.618 and 0.618, respectively (8).

A smile's breadth is determined by the amount of exposed maxillary teeth, creating a feeling of depth via the buccal corridor or negative lateral space between the premolars and lips.

A triangular area is evident between maxillary canines, premolars, and lip corners when the mouth is slightly open. Frush and Fisher coined the term „buccal corridor” to create a more lifelike dental prosthesis (9). The breadth of the smile, maxillary dental arch, facial muscle tone, and upper premolar buccal surfaces affect the buccal corridor appearance. A "U"-shaped maxillary dental arch is excellent. While a small maxillary dental arch and a broad buccal corridor are generally considered less aesthetically pleasing, narrower buccal corridors enhance smile attractiveness (10,11).

The gingiva is visible when speaking and smiling; thus, smile reconstructions must preserve its curves to create a realistic smile. The gingival zenith is the highest point of the gingiva's exterior shape and is distal to the maxillary central incisors and canines (12). It aligns with the maxillary lateral incisors' longitudinal axes. Class 1 maxillary lateral incisors have a lower horizontal gingival contour (0.5-0.2 mm) than central incisors and canines (13). In class 2, the maxillary lateral incisor has a greater horizontal gingival contour than the central incisor and canine (14,15).

The ANFIS uses neural networks and fuzzy logic to describe complex systems more accurately and flexibly. This technique might improve the modelling of complicated interactions, prediction and optimization, individualized dental care, automation and decision assistance, and treatment quality (16-18). Using the ANFIS in dental data analysis and processing may improve accuracy, treatment customization, and decision-making. The study aimed to conduct anthropometric analysis of the proportions of maxillary anterior teeth, to demonstrate the existence of the gold standard, the gold proportion, and the buccal corridor (lateral negative space), and to investigate the potential applications of deep learning in the field of anthropometric analysis.

METHODS

Subjects

The study was conducted at the Clinic for Dental Medicine in Niš and included 88 subjects. The subjects were dental students at the Faculty of Medicine in Niš. The study included only students with a full set of teeth (with and without third molars). The exclusion criteria were front teeth destruction (caries, trauma, erosion), prosthetic and conservative restorations on front teeth, anomalies in the oral cavity, a history of orthodontic therapy, congenital or surgical facial defects, or pronounced facial asymmetry.

The subjects were familiarized with the study and signed their written informed consent. The study was approved by the Ethics Committee of the Clinic for Dental Medicine (decision No. 14/7-2019-5EO).

The anthropometric analysis of the maxillary front tooth proportions was used to evaluate the gold standard, gold proportion, and buccal corridor (lateral negative space), and to study the possible uses of deep learning in anthropometric analysis.

Anthropometric procedure

Each subject sat on the dental chair with the head supported by the backrest, so that the lower edge of the mandible was parallel to the floor. The dimensions of the maxillary central incisor were measured with a calliper directly in the mouth of the subject. The measured values involved the height and width of the teeth, and the W/H % value was calculated according to the formula:

W/H% = maximal mesiodistal width/maximal incisogingival height (mm) x 100

The subjects were photographed in the centric occlusion position from a distance of 1 meter using a Nikon D5100 (Japan) camera. Image processing was done in LightX and PhotoGrid. To determine the gold proportion of maxillary front teeth, the mesiodistal breadth of central and lateral incisors, canines, and their mesial facets was measured and compared. The measurements were expressed in millimetres.

The number of visible maxillary teeth and the presence of a clearly expressed buccal corridor during a voluntary smile were counted during the clinical examination and on the subject's maxillary dental arch image.

The gingival contour class was determined on the photograph of the subject's maxillary dental arch.

Statistical data processing

Statistical calculations of the entered data were performed using IBM SPSS Statistics 22 (IBM Corporation, Armonk, NY, USA). Standard statistical methods were used for the qualitative and quantitative assessment of the obtained results: absolute numbers, relative numbers (%), arithmetic mean, median (minimum-maximum), and standard deviation. The normality of distribution was tested using the Shapiro-Wilk test. The numerical data did not follow a normal distribution, and the difference between genders was tested using the non-parametric Mann-Whitney *U* test. The Chi-square test was used to assess the statistical significance of differences in absolute frequency among samples.

The statistical hypothesis was tested at a significance level of $\alpha = 0.05$, with differences between samples considered statistically significant when $p < 0.05$.

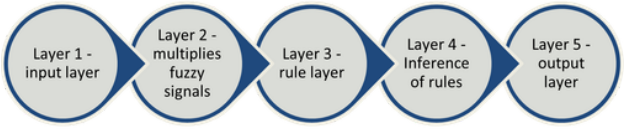

Figure 1. *ANFIS layers*

As shown in Figure 1, the ANFIS network contains five levels. The fuzzy inference system lies at the heart of the ANFIS network. Layer 1 accepts the inputs and uses membership functions to convert them to fuzzy values. The bell-shaped membership function was employed in this study because it has the best capability for non-linear data regression.

The bell-shaped membership function is defined as follows:

$$\mu(x) = bell(x; a_i, b_i, c_i) = \frac{1}{1+\left[\left(\frac{x-c_i}{a_i}\right)^2\right]^{b_i}}$$

where (a_1, b_1, c_1) is the parameter set and is the input.

The second layer multiplies the first layer's fuzzy signals and produces the rule's firing strength. The rule layers are the third layer, and they normalize all of the signals from the second layer. The fourth layer performs rule inference and converts all signals to crisp values. The last layers summarize all of the signals and provide a clean output value.

RESULTS

The study included 88 subjects: 32 males (36.4%) and 56 females (63.6%). The mean age of the subjects was 22.14 ± 1.52 years, with the youngest being 20 and the oldest 27. The age structure was uniform, 22 years (20-25) in men and 22 years (20-27) in women (U = 858.0, p = 0.736).

Maxillary central incisor dimensions

The average height of the central incisor in men was 10.5 mm, whereas the same value in women was 10.2 mm. The central incisor height (U = 724.0; p = 0.125) and W/H % (U = 891.0; p = 0.965) were not statistically significantly different between men and women (Table 1).

Table 1. *Dimensions of the maxillary central incisor of the subjects*

Parameter	Men	Women	*p*
Central incisor height (mm)	10.5±1.3, 11.0 (7.0-12.0)	10.2±1.1, 10.0 (8.0-12.1)	0.125
Central incisor width/height W/H%	83.3±8.2, 82.5 (70.0-100.0)	83.6±8.0, 82.0 (68.0-100.0)	0.965

Data are presented as mean±standard deviation, Median (Min-Max), p values were based on Mann-Whitney U test.

The gold proportion of maxillary anterior teeth

The existence of the gold proportion regarding the width of maxillary front teeth was determined in the majority of subjects. The gold proportion was present in 60.2% of men and 65.5% of women, with no statistically significant difference in frequency between genders (χ^2= 0.612, p = 0.434) (Table 2).

Obtained results revealed the existence of the gold proportion in 65.6% of male and in 57.9% of female subjects (Figure 2a).

Smile width

The smile width in men was 10.0 mm (8.0 - 10.0), whereas in women it was smaller, measuring 8.0 mm (6.0 - 12.0). The difference in smile width between genders did not reach statistical significance (U = 702.5; p = 0.061), as shown in Table 3.

Buccal corridor

A pronounced buccal corridor was present in half of the subjects, with a frequency of 53.1% in men and 48.2% in women. There was no statistically significant difference between male and female subjects (χ^2 = 0.196, p = 0.658) (Table 4) (Figure 2b).

Table 2. *Frequency of the gold proportion of maxillary anterior teeth in the subjects*

Gold proportion of maxillary anterior teeth	Total	Gender		p
		Men	Women	
	n = 88	n = 32	n = 56	
No n (%)	35 (39.8)	11 (34.4)	24 (42.9)	0.434
Yes n (%)	53 (60.2)	21 (65.5)	32 (57.1)	

Data are presented as numbers (%), p values were based on Chi-square tests.

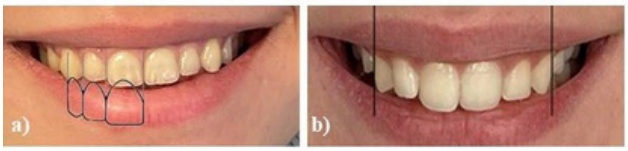

Figure 2. *a) Gold proportion on maxillary front teeth; b) The presence of buccal corridors*

Table 3. *Smile width*

Parameter	Men	Women	p
Smile width (mm)	9.2±1.0, 10.0 (8.0-10.0)	8.7±1.4, 8.0 (6.0-12.0)	0.061

Data are presented as mean±standard deviation, Median (Min-Max), p values were based on Mann-Whitney U- tests.

Table 4. *Presence of a pronounced buccal corridor regarding gender*

Presence of a pronounced buccal corridor	Total	Gender		p
		Men	Women	
	n = 88	n = 32	n = 56	
No n (%)	44 (50.0)	15 (46.9)	29 (51.8)	0.658
Yes n (%)	44 (50.0)	17 (53.1)	27 (48.2)	

Data are presented as numbers (%), p values were based on Chi-square tests.

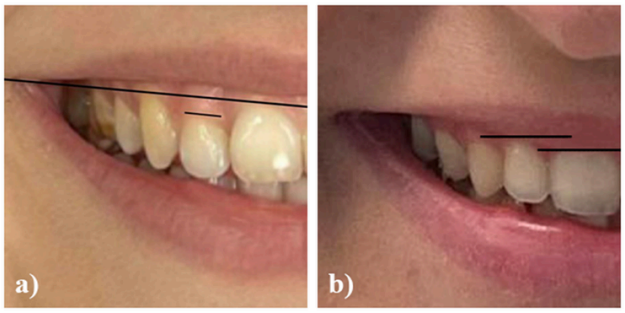

Figure 3. *a) Class 1 gingival contour; b) Class 2 gingival contour*

Table 5. *Gingival contour class—the balance of gingival heights regarding gender*

Gingival contour class	Total	Gender		p
		Men	Women	
	n = 88	n = 32	n = 56	
Class 1 n (%)	69 (78.4)	27 (84.4)	42 (75.0)	0.304
Class 2 n (%)	19 (21.6)	5 (15.6)	14 (25.0)	

Data are presented as number (%), p values were based on Chi-square tests.

Gingival contour class

The results suggest a far more frequent presence of class 1 gingival contours (Figure 3a) compared to class 2 (Figure 3b). There was no significant difference in the position of the gingival zeniths between male (15.6%) and female (25.0%) subjects (χ^2 = 1.057, p = 0.304) (Table 5).

The best predictors for different types of anthropometric parameters were chosen using the ANFIS approach. The selection is crucial, as is the preprocessing of the input parameters to eliminate irrelevant inputs. Following the command in MATLAB Software, the dataset is partitioned into a training set (odd-indexed samples) and a checking set (even-indexed samples):

>>[data] = anthropometric;
>>trn_data = data(1:2:end,:);
>>chk_data = data(2:2:end,:);

The function “exhsrch” conducts an exhaustive search of the available inputs to identify the set of inputs that have the greatest impact on anthropometric parameters. The function's first parameter defines the number of input combinations to be tested during the selection process. In essence, "exhsrch" creates an ANFIS model for each combination, trains it for one epoch, and then publishes the results. The command line below is used to find the one and two most important attributes in forecasting outputs:

>>exhsrch(1,trn_data,chk_data);

>>exhsrch(2,trn_data,chk_data);

Figures 4 and 5 show training and checking errors for the optimal one- and two-attribute anthropometric parameters. The minimal checking error occurs at the 55th epoch for both optimal combinations.

Figure 4. *Training and checking errors for optimal one-attribute for anthropometric parameters*

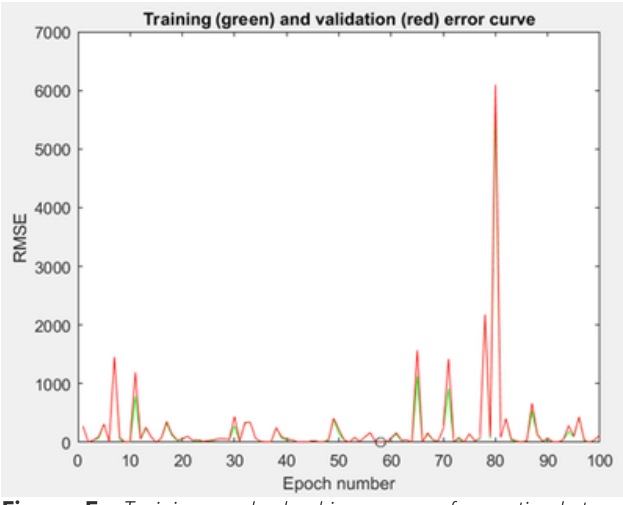

Figure 5. *Training and checking errors for optimal two-attributes for anthropometric parameters*

DISCUSSION

The size and shape of maxillary anterior teeth affect dental and facial aesthetics. The study aimed to analyze maxillary front tooth proportions, verify the existence of the gold standard, gold proportion, and buccal corridor, and identify the smile width and gingival contour class in young Serbian subjects with complete sets of teeth. The central incisor length findings corresponded favourably with those of other authors. Dietschi observed the average length of the upper central incisor anatomical crown to be 10.4 mm in women and 11.2 mm in men (19), whereas Gillen et al. reported values of 9.8 and 10.6 mm, respectively (20,21). Numerous studies have indicated that men generally have larger teeth than women and that ethnicity significantly influences tooth size (22). For instance, the average Saudi central incisor width was 8.74 mm, according to Alqahtani et al. (23). Furthermore, Asians have smaller anterior teeth than Caucasian cohorts (24). In younger patients, approximately 3.5 mm of the incisal section of the maxillary front teeth is visible when the mouth is slightly open, while mandibular teeth are scarcely visible. Maxillary teeth become less apparent with age due to falling upper lip tone and incisal edge abrasion, whereas mandibular teeth become more noticeable (19). Since maxillary front teeth are the cornerstone of an attractive smile, having a harmonic distribution of their form, size, and proportion is crucial. Reconstructing front teeth requires consideration of the tooth width-to-height ratio, as modifying a single dimension would result in inadequate aesthetics (25). The tooth width-to-height ratio varies with gender, ethnicity, and group (26). The maxillary central incisor, the most prominent tooth in the jaws, dominates in form, size, and location. The ratio of its proportions is significant (27). The gold standard for dental crown width is 75–85% of its height (28). Percent values below 60% form a tall, narrow crown, while those over 85% create a short, broad crown. This W/H percentage ratio is more attractive than tooth height or width alone (22,29). The clinical crown should be lengthened if the maxillary central incisor is too short (30). This study found a mean W/H percent of 83% in both sexes, aligning with the GS. These findings matched anthropometric data from a study by Abdallah et al. on various populations (6). Men and women also shared the gold proportion, which matches the results of Abdallah et al. (6). However, the literature does not demonstrate a preponderance of the gold proportion among maxillary front teeth across various groups (31-35). Similar to this study, dental students were tested for the gold proportion. This ratio was not common and men had

broader maxillary anterior teeth than women (33,36). Fayyad et al. identified the gold proportion between maxillary central and lateral incisors in 31.3% of men and 27.1% of women (37). Ahmed et al. did not advocate the gold proportion for front tooth repair after reviewing the research (38). The current study found no significant gender difference in smile width. When smiling, the individuals showed 8–10 maxillary teeth. These findings correspond to those of previous authors who found maxillary second premolar exposure in 60% of patients (39-42). The exposed maxillary first premolar was most common (57%) according to Tjan and Miller, whereas 3.7% of participants had first molars visible while smiling (43). Koidou et al. found that celebrities had more visible teeth and a higher proportion than dental students, demonstrating the aesthetic advantages of a broad smile (44). Other authors who studied celebrity smiles found the most common visibility to be 10 teeth, without statistically significant variations between sexes (45-47). Side teeth need cosmetic repairs since they are visible while smiling. A buccal corridor or lateral negative space is vital during smiling. Maxillary premolars are medially situated relative to the vestibular tangent of canines and molars. Without buccal corridors, prosthetic literature describes a smile as denture-like; however, orthodontists remove these "negative" areas with transverse maxillary expansion (48). Wide maxillary dental arches narrow buccal passageways (48). This study found a noticeable buccal corridor in half of the individuals, with no statistically significant difference between men and women. Previous studies demonstrated a more prevalent and noticeable buccal corridor in the smiles of celebrities who are considered attractive (45,46). However, other authors concluded that a broader smile (minimum buccal corridor) was more appealing (49). In the study by Oshagh et al., dental and art students found that men prefer narrower buccal corridors, whereas women prefer wider ones (50).

In this study, Class 1 gingival contours were more common than Class 2 (Figures 3a and 3b). Other authors reported a greater Class 1 frequency (51,52). Humagain et al. found no statistically significant variations in the gingival zenith position between sexes (53). Respecting gingival zeniths and shapes is crucial to effective periodontal surgery, extending clinical tooth crowns, gingivoplasty as a pre-prosthetic treatment for maxillary anterior tooth restoration, and implant location planning (54).

Dental anthropometry includes tooth measurements, gingival curves, and buccal corridor widths. The aesthetics and function of dental structures depend on these intricate interactions. The presented ANFIS model can represent complex interactions using non-linear and high-dimensional data (Figures 4 and 5). Deep learning with the ANFIS may reveal dental anthropometry variable patterns and dependencies (17,18,55).

When reconstructing part of a tooth or multiple teeth in the jaw, the form and ratio of the teeth can be simply replicated by following the surviving teeth. After the loss of all teeth, reconstructing the dental arch and shaping a patient's smile requires additional approaches, such as the anthropometric measurements of values and proportions in individuals with full dentition. A balance between the patient's requests and suitable dental treatment for the patient's face and smile must be made. Smiles are formed using aesthetic factors that have been researched for decades and reflect the best ratio for aesthetic satisfaction. The findings of our study demonstrate that the anthropometric measurements of Serbian individuals align with the conclusions reported in the literature. Modelling complex dental anthropometric connections using the ANFIS enhances understanding of interactions among dental parameters. Dental treatments have become more personalized, precise, and effective, improving results and patient satisfaction.

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Author Contributions

Conceptualization: M.K. and N.S.Đ.; Methodology: M.M.; Software: A.I.; Validation: M.K., M.Đ., and N.G.; Formal analysis: G.J.; Investigation: M.I.; Resources: J.P.S.; Data curation: N.G.; Writing—original draft preparation: M.K.; Writing—review and editing: M.K., M.M., and N.S.Đ.; Visualization: M.I. and M.Đ.; Supervision: G.J.; Project administration: J.P.S. and A.I. 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 the Clinic for Dental Medicine, decision No. 14/7-2019-5EO, issued on 18.01.2022.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availability

All data analyzed during this study are included in this published article and available from the corresponding author in the form of anonymized data upon reasonable request.

Statement of Generative AI Use

No generative AI was used.

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CASE REPORT

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GIANT CORONARY ARTERY ANEURYSMS MANAGED WITHOUT SURGERY: A CASE FOR CONSERVATIVE MANAGEMENT

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Giant coronary artery aneurysms are a rare and potentially life-threatening subset of coronary artery anomalies. They are most frequently detected incidentally and can pose significant management challenges due to their unclear natural history and risk of complications. Although aneurysm size often raises concern, current evidence does not uniformly support surgical intervention in all cases. We present the case of a 66-year-old asymptomatic male initially admitted for endovascular treatment of a 72 mm abdominal aortic aneurysm (AAA). Preoperative multidetector computed tomography (MDCT) angiography unexpectedly revealed two giant coronary artery aneurysms: a 75 mm aneurysm in the left anterior descending (LAD) artery and a 48 mm aneurysm in the right coronary artery (RCA). Coronary angiography showed no obstructive coronary artery disease or distal perfusion impairment. Given the absence of symptoms, preserved flow, and high surgical risk, the institutional Aortic Board opted for conservative management. Endovascular aneurysm repair (EVAR) was successfully performed for the AAA. At 12-month follow-up, the patient remained clinically stable, with no progression of coronary aneurysm size or endoleak. This case highlights the importance of individualized decision-making in patients with incidentally detected giant coronary artery aneurysms. In the absence of high-risk features, conservative management with close follow-up may be a safe and effective approach, even in cases with extremely large aneurysms.

Keywords: giant coronary artery aneurysm, endovascular aneurysm repair, conservative management

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INTRODUCTION

Aneurysmal coronary artery disease is seen in 1.5% to 5% of patients undergoing coronary angiography (1–3). Rarely are coronary artery aneurysms (CAAs) large enough to be called giant CAAs (GCAAs). A universally accepted definition of GCAAs does not exist: diameters greater than 20-50mm, or ≥1.5 times the reference vessel diameter, have been proposed, with recent literature further refining these criteria (3–5). These GCAAs are both rare and potentially fatal, predominantly occurring in males (1). They occur in about 0.02% of the general population, in which the right coronary artery (RCA) is most often affected (6). The presence of multiple CAAs located in more than one coronary artery is even more uncommon. The pathophysiology of such aneurysms remains incompletely understood, although most cases are associated with atherosclerosis, congenital heart disease, or vasculitis (7,8). Reported complications include thrombosis and distal embolization, vasospasms, compression of adjacent structures and ruptures that produce ischemia, heart failure, or arrhythmias (2). These risks may be further amplified in cases of extremely large or multiple aneurysms.

CASE PRESENTATION

We report a case of an asymptomatic 66-year-old male who was initially admitted for the surgical treatment of an abdominal aortic aneurysm (AAA) 72 mm in diameter. Routine multidetector computed tomography (MDCT) angiography unexpectedly revealed two GCAAs (Figure 1A): one in the left anterior descending (LAD) artery, measuring 75 mm (Figure 1B), and another in the RCA, measuring 48 mm (Figure 1C). To our knowledge, this represents one of the largest reported LAD aneurysms in the available literature (1–3,5).

Following the discovery, coronary angiography was conducted for further assessment. No significant stenosis of the coronary blood vessels was found. The institutional Aortic Board reviewed the case. Given the high risk associated with surgical interventions for the GCAAs and the presence of calcifications in vessel walls, it was decided to manage the patient conservatively. However, because of the large size of the AAA and the high risk of open surgery, endovascular aneurysm repair (EVAR) was performed. At the 12-month follow-up, the patient was clinically stable and asymptomatic, with no EVAR endoleak detected. There was no notable increase in the size of the GCAAs. Family screening was suggested, given the potential, albeit unclear, contribution of hereditary factors.

GCAAs are a rare subset of coronary artery anomalies, and their optimal management remains a subject of debate (2,9).

In our patient, the decision not to pursue surgical or percutaneous intervention was based on the absence of symptoms, preserved distal flow, no significant obstructive coronary artery disease, and no evidence of embolization or rupture.

However, it is important to acknowledge that aneurysms of extreme size, particularly when multiple, may carry an increased risk of thrombosis, distal embolization, and, rarely, rupture, even in the absence of symptoms. Furthermore, the coexistence of coronary artery disease may further increase the risk of adverse cardiovascular events and may influence management strategies. A case presented by Halapas et al. highlighted that coexisting GCAAs and coronary artery disease may lead to non-ST myocardial infarction necessitating urgent surgery (10).

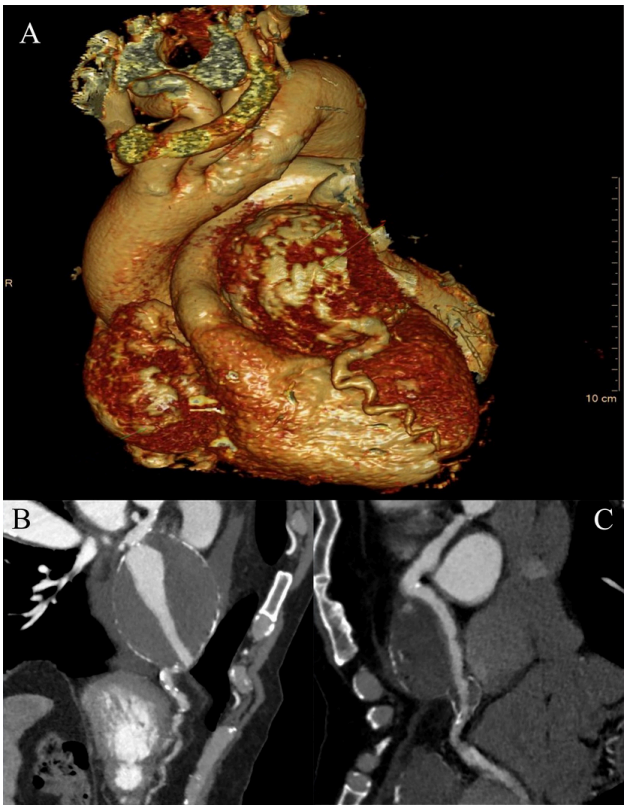

Figure 1. *Routine multidetector computed tomography (MDCT) angiography revealed two giant coronary artery aneurysms (GCAAs). (A) Overview image demonstrating the presence of both aneurysms. (B) Giant aneurysm of the left anterior descending (LAD) artery measuring 75 mm in maximum diameter. (C) Giant aneurysm of the right coronary artery (RCA) measuring 48 mm in maximum diameter.*

The CAESAR registry, the largest prospective dataset on coronary artery aneurysms to date, showed that the majority of patients with both ectasia and aneurysms were managed conservatively (3). Importantly, the study found no independent association between aneurysm size and major adverse cardiovascular events, suggesting that aneurysm morphology and clinical context are more relevant than size alone when considering invasive treatment (3). Similarly, a review by Kawsara et al. suggests that medical management may be considered in selected asymptomatic patients, noting that surgical or interventional treatment should be considered in the presence of ischemia, rupture risk, or significant coexisting stenoses (2). The authors highlight that unnecessary intervention in stable patients may expose them to procedural risks without proven long-term benefit. Keyser et al. also demonstrated good long-term outcomes with selective surgical intervention in symptomatic patients. Their findings support a case-by-case decision-making approach that balances surgical risk against the likelihood of complications such as thrombosis or rupture (7). Taken together, these findings support an individualized, case-by-case approach to management. The patient was treated with long-term antithrombotic therapy consisting of antiplatelet therapy (aspirin) in combination with oral anticoagulation, aimed at reducing the risk of thrombus formation within the aneurysmal segments and subsequent embolization. The choice of regimen was based on aneurysm size, multiplicity, and perceived thrombotic risk, with plans for indefinite therapy and periodic reassessment. Close imaging follow-up was implemented using serial MDCT angiography to monitor aneurysm stability (2).

GCAAs are rare vascular anomalies that pose diagnostic and therapeutic challenges. In asymptomatic patients without ischemia, embolization, or hemodynamic compromise, a conservative approach with antithrombotic therapy and imaging surveillance can be both safe and effective. This case highlights the importance of individualized, evidence-informed decision-making; however, careful risk assessment remains essential, particularly in patients with extremely large or multiple aneurysms.

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Author Contributions

All authors contributed equally to this work. All authors have reviewed the final version of the manuscript and agree with its content.

Statement of Ethics

Complete written informed consent was obtained from the patient for the publication of this study and accompanying images. The Institutional Ethics committee exempted this paper form ethics review.

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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CASE REPORT

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PROSTHODONTIC MANAGEMENT OF KENNEDY CLASS II/1 PARTIAL EDENTULISM IN THE LOWER JAW: A CASE REPORT

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The quality and number of remaining teeth, bone quality, feasibility of dental implant treatment, condition of the supporting tissues, and the patient's financial condition must be considered when planning prosthetic rehabilitation for partially edentulous patients. Herein, we report the prosthetic rehabilitation of a 59-year-old man with a unilaterally edentulous mandible, using a bar-and-precision-attachment system embedded in a cast partial denture. Metal-ceramic crowns were fabricated on the remaining teeth in the lower jaw, and the crowns on either side of the bounded saddle were connected using a bar. Additionally, two precision ball attachments were placed on the occlusal aspect of the bar. Distally, a precision ball attachment was placed towards the free saddle. Subsequently, cast partial dentures for the lower and upper jaws were fabricated. The patient was successfully rehabilitated, and orofacial functions were restored. At the follow-up examination 18 months after treatment, the patient had no complaints. The findings in this case show that rehabilitation of partially edentulous patients with cast partial dentures featuring an increased number of retention attachments leads to satisfactory orofacial prosthetic rehabilitation without altering the existing neutro-occlusal relationship of the teeth.

Keywords: cast partial denture, precision attachment, free saddle, edentulous space, Kennedy classification, cast denture

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INTRODUCTION

Partially edentulous patients frequently present with a unilateral free-end saddle and a bounded saddle in the posterior region on the contralateral side (1-3). In addition to the quality and number of remaining teeth in the jaw, the bone quality, the possibility of placing dental implants in the free-saddle region, the condition of the supporting tissues, and the patient's financial condition should be considered during planning the prosthetic rehabilitation of such patients. Herein, we report the prosthetic rehabilitation of a unilaterally edentulous mandible with a bar-and-precision-attachment system embedded in a cast partial denture.

CASE REPORT

A 59-year-old Caucasian man with previous experience of wearing removable partial prostheses was referred to our department from the oral rehabilitation. Anamnesis, clinical examination (inspection and palpation), and panoramic radiographic findings revealed partial edentulism in the upper and lower jaws, which resulted in other disorders of the orofacial system during swallowing, chewing, talking, facial expression, and other functions of the stomatognathic system. The edentulous spaces in the upper and lower jaws were classified as Kennedy class I and Kennedy class II/1, respectively.

The occlusal relationship of the patient's natural teeth and of the dentures he subsequently wore was Angle class I.

First, rehabilitation of the partially edentulous lower jaw was initiated. A preliminary impression of the lower jaw was made using alginate and a standard stock metal box-shaped tray, and a study model was fabricated using alabaster "soft" plaster. The study model was mounted on a semi-adjustable articulator and analyzed using a parallelometer. The patient refused implant placement in the free-end saddle in the lower jaw. Considering the patient's financial condition, the quality of the remaining solid dental tissues, and the type of partial edentulism, we determined that the best prosthetic rehabilitation for the lower jaw would be the fabrication of milled crowns on the abutment teeth connected with a bar and a cast partial denture (CPD). Next, an individual tray was fabricated on the study model for a definitive impression of the prepared teeth. The anterior teeth, premolars, and last remaining molar on the right side of the lower jaw were prepared. At the end of each preparation, demarcations were made in the form of half grooves (ISO 806 314 290 534 014 torpedo, ISO 806 314 199 534 016 and ISO 806 314 199 514 016 rotary diamond dental instruments-burs, NTI, Kahla, Germany) (4). Before making the impression, two retraction cords were placed in the sulci of all prepared teeth: a size 00 cord at the bottom of the sulcus and another size 0 cord above it (Sure-cord, knitted retraction cord, non-impregnated, Sure-endo, South Korea). The cords were moistened with Retrargin (Profarm, Beograd, Serbia) for gingival retraction (Figure 1A). Impressions of the prepared mandibular teeth and supporting tissues were made using the custom tray and addition silicone (Panasil® contact plus X-Light and Panasil putty, Kettenbach, USA). The impression was cast using plaster (HERA, Moldastone, CN), and removable dies were prepared. Wax models of fixed prostheses were constructed on the removable dies (Inlay wax Interdent, Slovenia; Red cervical wax Bego, Germany), and a milled bar (Bar Vario Soft Profilsteg, Bredent Group, Senden, Germany) was attached to bridge the saddle. Two ball-shaped precision attachments (Vario-Kugel-Snap vks-oc 1.7 Bredent Group, Senden, Germany) were placed on the occlusal surface of the bar using a parallelometer. The designed framework was milled in wax, and on the left side, an attachment matrix (Vario-Kugel-Snap vks-sg 1.7 Bredent Group, Senden, Germany, EU) was placed distally towards the free-end saddle using a parallelometer (Figure 1B). The metal skeleton was cast using an alloy (Remanium ®star, Dentaurum, EU) and returned to the master cast with dies. Thereafter, it was tried in the mouth (Figure 1C). After trying in the metal framework and taking a control bite in wax, the fixed components were sent to the laboratory for ceramic application and sintering in the esthetic zones of the restoration (Noritake Super Porcelain EX-3, Kuraray Noritake, Noritake Dental International, Japan). After ceramic firing, the fixed components were positioned on the model in the articulator (Amann Girrbach Artex Dental Semi-adjustable Dental Zinc alloy Articulator for preculate plaster model in scale, Germany, EU) and then directly tried in the patient's mouth (Figure 1D). The ceramics were then glazed in a furnace.

In the second phase, the glazed fixed framework was secured to the supporting teeth using a paste for temporary bonding (Tempbond non-eugenol temporary cement, Kerr Corporation, Orange, USA), and a pick-up impression of the partially edentulous lower jaw was made. After preparing the master cast and its duplications, a wax model of the CPD skeleton was created, invested, and cast. The cast skeleton was processed (Figure 1E), and

the skeleton and fixed framework were tried in in the patient's mouth (Figure 1F). A wax rim was created on the terminal saddle mesh, and the exact vertical dimension of occlusion was established. Thereafter, artificial teeth (Company—artificial teeth) were arranged on the skeleton, the waxed skeletal prosthesis with embedded teeth was tried in the patient's mouth (Figure 1G), and the waxed prosthesis was processed and prepared for delivery. In the delivery phase, the fixed framework was cemented (ITENA Total-Cem, UK), and the completed CPD was simultaneously inserted. At the 24-h follow-up, the patient was trained on separating the attachments and removing the lower CPD from the mouth.

After completing the rehabilitation of the lower dental arch, the treatment for restoring the partially edentulous upper jaw was initiated. An impression was made using additional silicone, and a framework was designed for the upper jaw. The exact vertical dimension of occlusion was re-established (Figure 1H, artificial teeth were set in wax (Acry-Rock RUTHINIUM, Veneto, Italy, EU), and the waxed denture was tried in. Thereafter, the wax denture was processed to obtain the upper CPD that was fitted in the patient's mouth (Figure 1I) (5).

Patient satisfaction and positive therapeutic effects were observed during the 18-month follow-up.

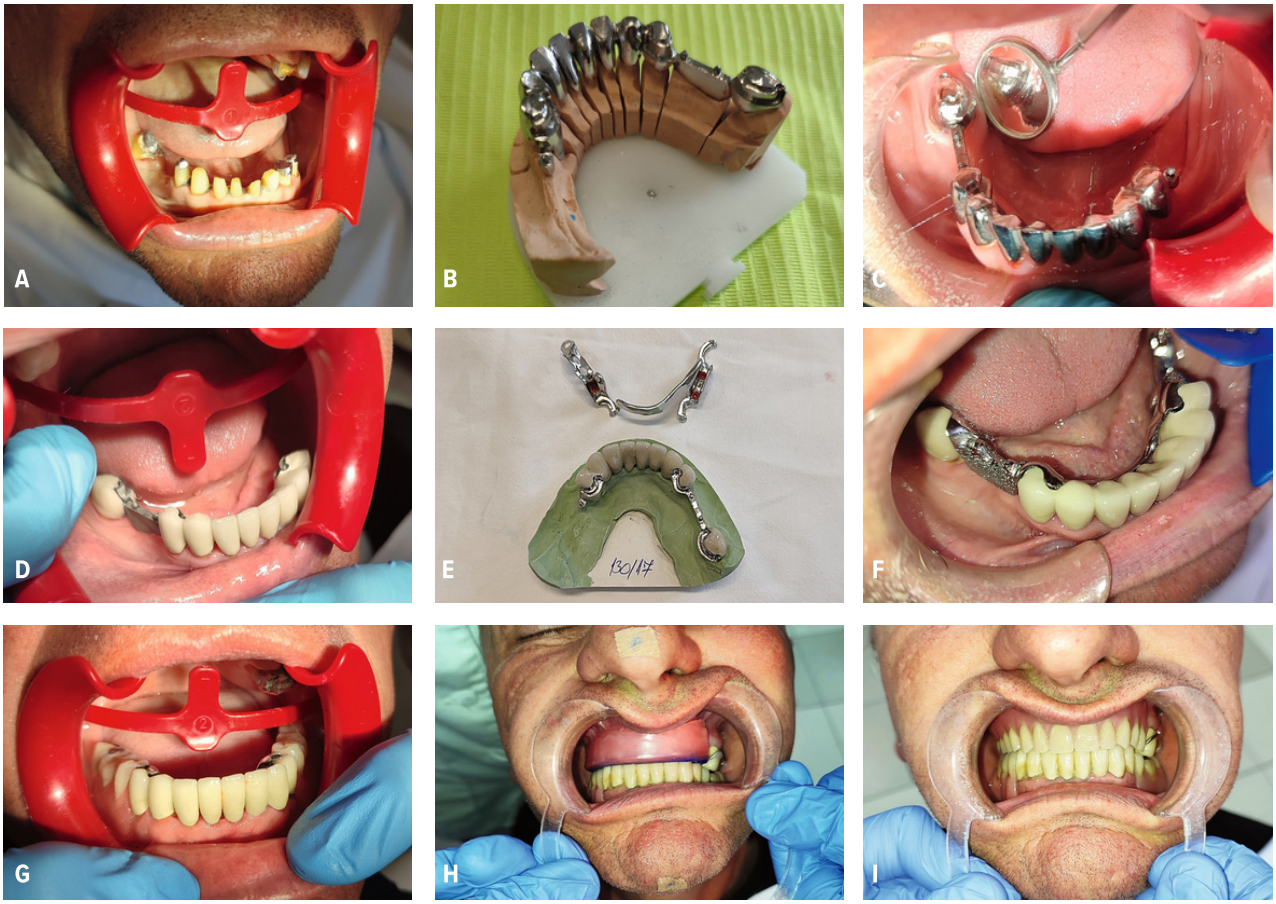

Figure 1. *A) Retraction cords in the sulci of the prepared mandibular teeth; B) Milled metal framework with matrixes of precise attachments in position; C) Try-in of the metal framework in the mouth; D) Try-in of the metal-ceramic framework in the mouth E) The metal skeleton separated from the metal-ceramic framework; F) Metal skeleton and metal-ceramic framework positioned in the mouth; G) Try-in of the waxed partial prosthesis in the mouth; H) Re-established exact vertical dimension of occlusion; I) Photograph 18 month after prosthetic rehabilitation with upper and lower cast partial dentures.*

DISCUSSION

Kennedy class II/1 partial edentulism represents a severe form of partial edentulism for which various therapeutic approaches can be considered. From a clinical perspective, this case report highlights two points (3).

First, despite the availability of advanced technology such as dental implants, the patient selected the "conventional approach" of a mobile restoration that is potentially inferior to conventional or implant-supported fixed restorations for the rehabilitation of his toothlessness.(6-10) This can be explained by the patient's poor financial condition, which did not allow him to choose implant placement in the partially edentulous jaw. Furthermore, the patient had a previous experience of wearing partial dentures, with which he was quite satisfied.

Second, some aspects of the presented prosthodontic solution for the patient's partial edentulism are debatable. In partially edentulous patients, a unilateral free saddle can lead to considerable discomfort due to insufficient cushioning or uncontrolled settling of the free denture saddle on the supporting tissue (11-14). This problem can be significantly alleviated by placing a dental implant in the edentulous region.(9,10) We attempted to significantly alleviate or eliminate the potential problem of the terminal free saddle settling on the large surface of the supporting tissues by installing two additional connecting attachments on the bar on the opposite side of the jaw (Figures 1C and 1D). Inserting a connecting attachment can strengthen the prosthesis structure and prevent settling of the free saddle of the prosthesis on the edentulous ridge (3).

However, selecting a suitable material that meets all these requirements was challenging. The hardness and strength of the alloy are two closely connected physical parameters that significantly affect the biomechanics of the terminal free saddle of the CPD (15-18). An increase in alloy strength is usually accompanied by an increase in hardness, and alloys that are too hard are usually undesirably brittle. Therefore, in this case, it was desirable to increase the alloy strength and decrease its hardness. Hence, the specified Remanium® star alloy was used to fabricate the framework of the lower partial prosthesis, as its estimated strength and hardness were satisfactory.

A skeletal prosthesis with an increased number of retention attachments achieves satisfactory prosthetic rehabilitation of the patient's orofacial system without changing the existing neutro-occlusal relationship of the teeth.

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Author Contributions

Conceptualization & investigation; Writing – original draft, review, & editing: SDP. The author has 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 the accompanying images. All procedures were performed in accordance with the ethical standards of the institutional and national research committee and the 1964 Declaration of Helsinki.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availibility

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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