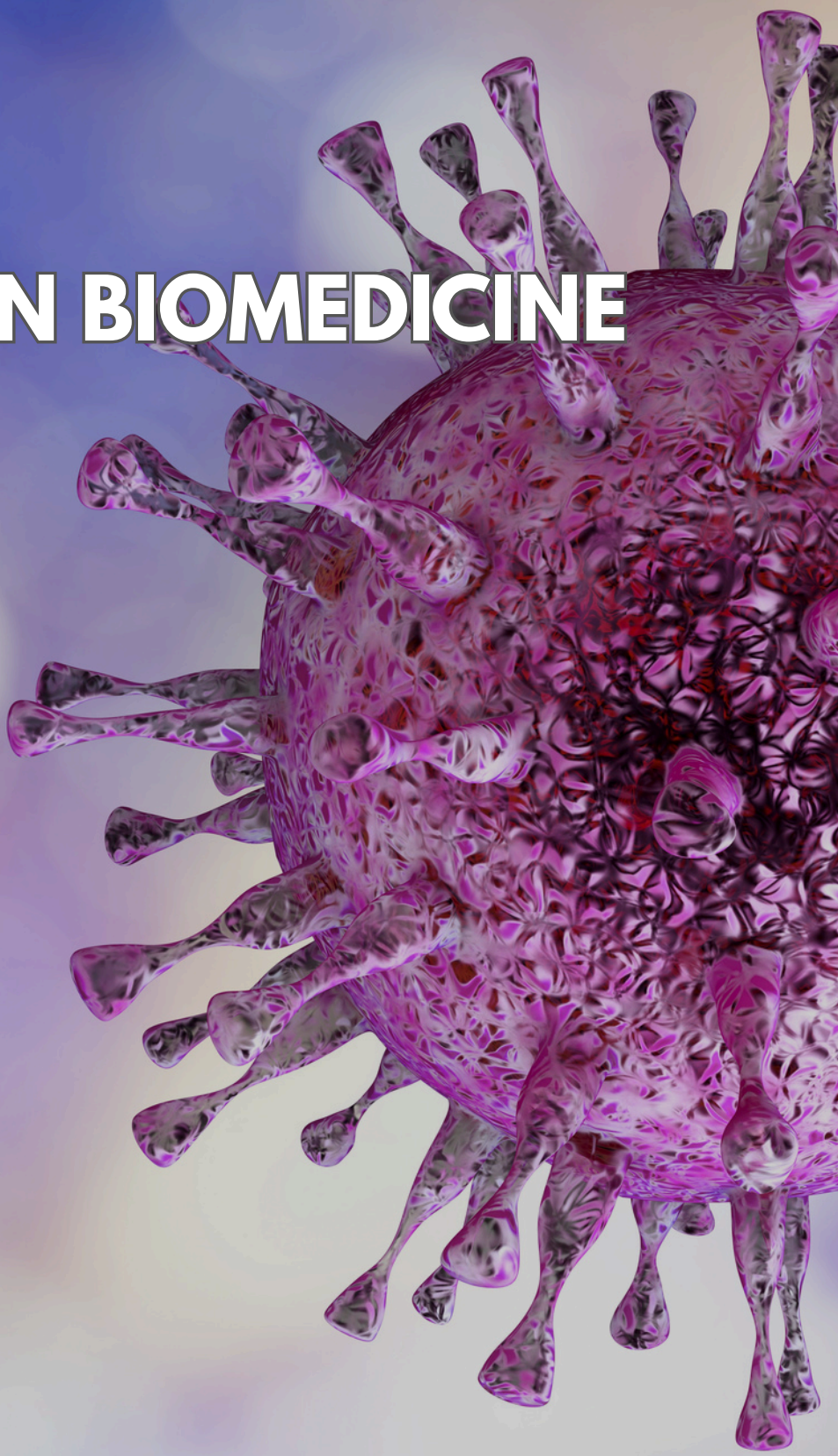
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AFMN BIOMEDICINE

CONTENTS

VOLUME 43 | NUMBER 3 | PAGES 238-375

REVIEW ARTICLE

238 Folic Acid and Related Folates as Proposed Inhibitors of Orthopoxvirus mRNA Cap Methyltransferases: A Narrative Review of Evidence and Limitations
Ali Al-Kaleel, Lubna Al-Gailani, Martha Orendu Oche Attah

249 The Use of Digital Interventions Based on Artificial Intelligence in the Psychotherapy of Adolescent Anxiety Disorders: A Preliminary Review of Potential Usability, Efficacy and Safety
Aleksandra Stojanović, Miodrag Stanković, Aleksandra Ristić

ORIGINAL ARTICLE

263 Alterations in Liver Function Biomarkers among Moderately Severe COVID-19 patients in Southeastern Serbia: A Retrospective Single-Center Study
Aleksandar Ranković, Vladimir Petrović, Maja Cvetanović, Milica Ćosić Petković, Lidija Popović Dragonjić, Jelena Spasić, Goran Radenković, Braca Kundalić, Nikola Milenković

272 Effect of Halogenated Boroxine on Hematological Parameters in Rats and on Creatine Kinase Activity *in vitro*
Safija Herenda, Elma Hasković, Muhamed Fočak, Alem Bekan, Sabina Prevljak, Nenad Vanis, Mirza Halimić, Alen Džubur, Edhem Hasković

282 Temporomandibular Disorders in Young Serbian Population: An Epidemiological Study
Slobodan Dodić, Vladimir Sinobad, Srdjan D. Poštić

289 Body Composition in Physically Active Male Students Assessed Using Multi-Frequency Bioelectrical Impedance Analysis
Valentin Leuciuc Florin, Elena Vizitiu Lakhdari, Dragoș Paul Florian, Vlad Teodor Grosu, Tatiana Dobrescu, Dalila Preljević, Saša Bubanj

299 Endothelium-Mediated Vasorelaxant Effects of Chokeberry (*Aronia melanocarpa*) Extract, Juice, and Waste: An Experimental Study in Isolated Rat Aorta
Milica Milutinović, Suzana Branković, Nada Ćujić Nikolić, Katarina Šavikin, Milica Randjelović, Bojana Miladinović, Dušanka Kitić

310 Attitudes and Practices towards COVID-19 and Seasonal Flu Vaccines and Vaccination in the General Population and among Medical Professionals
Katarina Petković, Dušanka Krajnović, Andijana Milošević-Georgijev, Brankica Filipić, Andjela Marinković, Valentina Marinković

324 Association of *Caspase-1* Gene Polymorphisms with Rheumatoid Arthritis Susceptibility and Temporomandibular Joint Involvement: An Iraqi Cohort Study
Wasan Lafta, Ali Al-Hafidh, Batool Hassan Al-Ghurabi, Abbas Fadhil Al-Hashimy

337 Clinical Significance of *Thymus serpyllum* L. Essential Oil in Patients with Syncope
Branislav Milovanović, Nikola Marković, Nada Petrović, Slobodan Petrović, Maša Petrović, Aleksa Korugić, Milovan Bojić

347 A Seven-Year Longitudinal Study of Technical Complications of Removable Dentures
Danica Popović Antić, Minja Miličić Lazić, Luka Župac, Filip Ivanjac, Slobodan Dodić, Marko Lazić, Igor Djordjević

354 Intra-Abdominal Pressure as a Modulator of Biochemical Indicators of Hepatic Function after Laparoscopic Cholecystectomy
Mladen Kasalović, Gojko Igrutinović, Saša Dimić, Nikola Miljković, Aleksandar Jakovljević, Zlatan Elek, Marija Milanović

362 The Impact of Socio-Economic Factors on the Quality of Life of Edentulous Elderly Patients in Serbia
Anika Ćuković, Danimir Jevremović, Maja Pavlović, Michal Potran, Ana Jevremović

CASE REPORT

371 Artery of Percheron Infarction Presenting as Coma and Acute-Onset Vascular Dementia: A Case Report
Ana Azanjac-Arsić, Svetlana Miletić-Drakulić

AFMN BIOMEDICINE

REVIEW ARTICLE

FOLIC ACID AND RELATED FOLATES AS PROPOSED INHIBITORS OF ORTHOPOXVIRUS MRNA CAP METHYLTRANSFERASES: A NARRATIVE REVIEW OF EVIDENCE AND LIMITATIONS

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Monkeypox virus (MPXV) replicates in the host cell cytoplasm and must therefore encode its own messenger RNA capping machinery, which makes the viral cap methyltransferases attractive antiviral targets. Folic acid has recently been proposed as an inhibitor of these enzymes and, on that basis, as a candidate treatment for MPXV. This narrative review examines that proposition and the level of evidence supporting it. PubMed, Scopus, and Google Scholar were searched through May 2026; no original experimental work was undertaken. The proposal rests almost entirely on a single virtual screening study in which folic acid emerged as a high-scoring ligand in molecular docking and molecular dynamics simulations, supported indirectly by reports that folates and folate antagonists modulate the replication of unrelated viruses. No published study demonstrates concentration-dependent inhibition of a purified orthopoxvirus methyltransferase by folic acid, and no kinetic parameters have been determined. Folate biochemistry offers no straightforward mechanism because S-adenosylmethionine, rather than folate, is the methyl donor for these enzymes and folate contributes only indirectly by supplying that pool. Furthermore, reported antiviral activity at millimolar concentrations exceeds achievable plasma exposures by approximately three orders of magnitude. By contrast, sinefungin-derived inhibitors of the MPXV 2′-O-methyltransferase VP39 have been characterized structurally, inhibit the purified enzyme at submicromolar concentrations, and suppress viral replication in cell culture, confirming that this machinery is druggable. Folic acid is therefore best described as a computationally derived hypothesis requiring enzymatic validation, rather than an established methyltransferase inhibitor or a therapy for MPXV.

Keywords: monkeypox virus, MPXV, mRNA cap methyltransferase, VP39, folic acid

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INTRODUCTION

Monkeypox virus (MPXV) is a double-stranded DNA virus of the genus *Orthopoxvirus*, family *Poxviridae*, alongside variola, vaccinia, and cowpox viruses (1–3). Two clades are recognized: clade I, comprising subclades Ia and Ib, and clade II, comprising subclades IIa and IIb (4,5). These correspond broadly to the historical Congo Basin (Central African) and West African lineages (6). Clade I infection has been associated with more severe disease and higher case fatality than clade II, whereas the 2022 global outbreak was driven predominantly by clade IIb (1,4).

Because orthopoxviruses replicate entirely in the cytoplasm, they cannot use the host nuclear capping apparatus and must encode a complete mRNA capping and cap-methylation system of their own (7,8). The 5′ cap protects viral transcripts from degradation, supports cap-dependent translation, and, through 2′-O-methylation in particular, allows viral mRNA to be read as self and so evade cytoplasmic innate immune sensors (7,9). This dependence makes the viral cap methyltransferases (MTases) an attractive antiviral target, and inhibitors of the MPXV 2′-O-MTase VP39 have already been shown to suppress viral replication in cell culture, as presented in Figure 1 (9–11).

Against this background, a 2024 virtual screening study of Middle Eastern plant-derived compounds reported that folic acid occupied a favorable position within the active site of an MPXV MTase model and achieved better docking and MM-GBSA scores than the pan-MTase reference inhibitor sinefungin (12). That observation has since been reiterated in the secondary literature, sometimes accompanied by progressively stronger claims. The purpose of the present review is therefore narrow and deliberate: to set out what is and is not established about folates as orthopoxvirus MTase ligands, to place that evidence in a defined hierarchy, and to specify the experiments that would be needed before folic acid could reasonably be described as an inhibitor, let alone a treatment.

Epidemiological Context and Clinical Management

First identified in 1958 in captive monkeys in Copenhagen, MPXV gave rise to the name monkeypox; the first human case was recognized in the Democratic Republic of the Congo in 1970 (6,13). For several decades, human infection remained largely confined to forested regions of Central and West Africa (14). In 2022, sustained human-to-human transmission of clade IIb produced a multi-country outbreak affecting more than 100 countries, which the World Health Organization (WHO) designated a public health emergency of international concern (PHEIC) in July 2022 (1,15).

The epidemiological situation has since changed in ways directly relevant to therapeutic development. A novel clade I lineage, designated clade Ib, was identified in eastern DRC in 2023–2024 and spread to neighboring and then more distant countries, prompting a second PHEIC declaration on

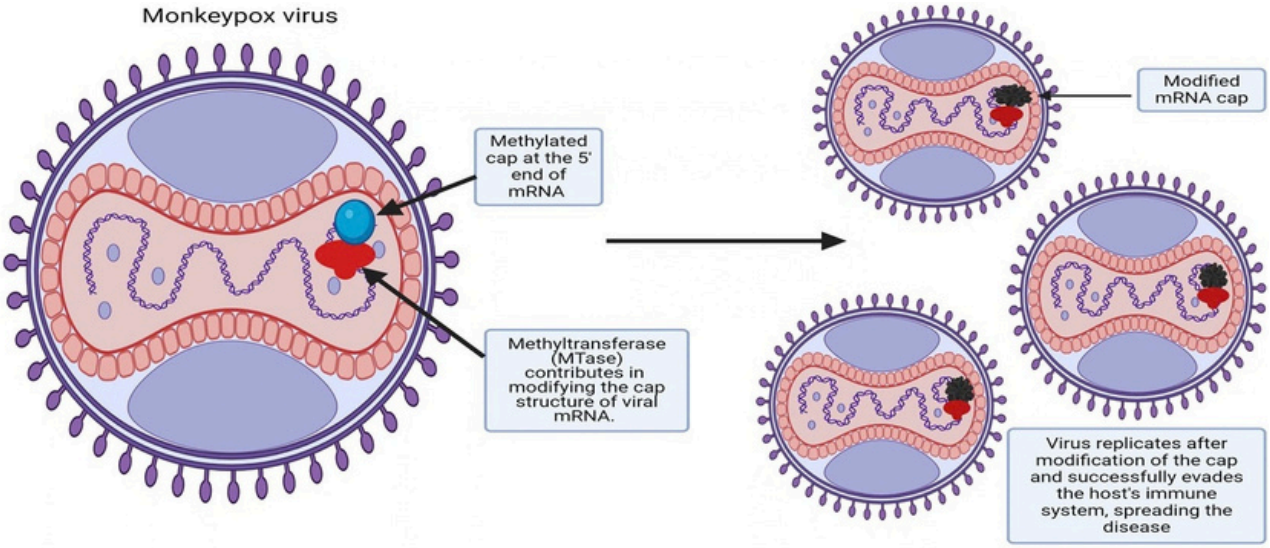


Figure 1. *Monkeypox virus structure and the role of mRNA cap methylation. Methyltransferase-mediated modification of the 5′ cap of viral mRNA supports transcript stability and cap-dependent translation. This modification allows viral mRNA to evade cytoplasmic innate immune sensing, permitting productive replication. The responsible enzymes are identified in Table 1. Image created with BioRender.com.*

14 August 2024 (5,16). That emergency was lifted on 5 September 2025 following sustained declines in case numbers, although WHO advised that this did not signal the end of the threat and extended its standing recommendations for MPXV to 20 August 2026 (16). Clades I and II and their subclades continue to co-circulate, and Africa CDC has maintained its continental emergency designation (16).

Two implications follow for the present review. First, any candidate agent must eventually be evaluated against clade Ia, Ib, and IIb isolates rather than a single laboratory strain. Second, and reassuringly for a cap-MTase strategy, the capping enzymes are among the most highly conserved orthopoxvirus proteins, so target sequence variation between clades is expected to be limited, although this expectation has not been formally tested for any of the folate-related work discussed below, and all published VP39 inhibitor studies to date have used clade IIb isolates (9,11). This is a substantive gap rather than a technicality.

Clinically, MPXV resembles a milder form of smallpox, with lymphadenopathy as a distinguishing feature (1). The incubation period is typically 7–14 days (range 5–21 days), and illness begins with fever, headache, myalgia, backache, lymphadenopathy, and fatigue (17). Rash follows within 1–3 days,progressing through macular, papular, vesicular, pustular, and crusted stages (18). Complications include secondary bacterial infection, keratitis and vision loss, pneumonitis, and encephalitis (1). Disease is usually self-limiting over 2–4 weeks, but severity depends on clade, route of infection, age, and immune status, with people with advanced HIV at particularly high risk (19). Transmission occurs through direct contact with lesions, body fluids, respiratory secretions, or contaminated materials, and, in the 2022 and clade Ib outbreaks, substantially through sexual networks (1,5).

Regarding the current clinical management of MPXV, WHO issued updated guidance on the clinical management of mpox and associated infection prevention and control in 2025 (20). The central message of that guidance is that care is primarily supportive: management of pain, of skin and mucosal lesions, of secondary bacterial infection, of hydration and nutrition, and of underlying conditions, particularly HIV.

Tecovirimat (ST-246, TPOXX), which inhibits the F13 (VP37) envelope protein and thereby blocks extracellular virion formation, was for several years the presumptive antiviral of choice based on in vitro and animal data. That position is no longer tenable. In the randomized, placebo-controlled PALM007 trial of 597 children and adults with laboratory-confirmed clade I MPXV in the DRC, tecovirimat did not reduce time to lesion resolution (median 7 days versus 8 days); overall mortality was 1.6%, substantially lower than typically reported in the region and attributed to hospitalization with high-quality supportive care (21). The STOMP trial in mild-to-moderate clade II MPXV likewise did not demonstrate clinical benefit and was stopped early. The European Medicines Agency initiated a review of tecovirimat in July 2025 in light of these data. In the United States, tecovirimat for MPXV remains investigational and is accessible through an expanded-access protocol for selected patients, rather than as an approved therapy.

Cidofovir and its lipid conjugate brincidofovir inhibit the viral DNA polymerase and have activity in animal models, but no controlled clinical efficacy data exist for MPXV; cidofovir carries significant nephrotoxicity and brincidofovir hepatic and gastrointestinal toxicity (22,23). Vaccinia immune globulin has been used in severe disease without controlled evidence. Vaccines, principally MVA-BN and LC16m8, remain the mainstay of prevention.

The correct summary of the current position is therefore that no antiviral agent has demonstrated clinical efficacy against MPXV in a randomized controlled trial. This strengthens rather than weakens the case for pursuing new mechanistic targets, but it also raises the standard of evidence that any new candidate must meet and makes premature therapeutic claims correspondingly more damaging.

Defining the Target: Orthopoxvirus mRNA Cap Methyltransferases

A recurring weakness of the earlier literature in this field is imprecision about which enzyme is being discussed. Orthopoxvirus cap maturation involves two distinct methylation reactions catalyzed by two distinct enzymes; these reactions must not be conflated (Table 1).

Cap-0 formation. The heterodimeric vaccinia capping enzyme comprises the large D1 subunit (D1R; MPXV homologue E1) and the small D12 subunit (D12L; MPXV homologue E12). The N-terminal region of D1 carries RNA 5′-triphosphatase and guanylyltransferase activities, while the C-terminal domain carries the cap (guanine-N7)-methyltransferase activity that converts GpppN-RNA to m^7GpppN-RNA (7,24). D12 is itself catalytically inactive – structurally, it appears to be a 2′-O-MTase that has degenerated – but it binds D1 and stimulates its N7-MTase activity approximately 40- to 50-fold (24). The crystal structure of the MPXV E12 subunit has been solved and used as a basis for inhibitor discovery (25).

Table 1. *Orthopoxvirus mRNA capping enzymes and cap methyltransferases relevant to antiviral targeting.*

Protein (VACV / MPXV)	Enzymatic activity	Reaction catalyzed	Methyl donor	Substrate	Evidence for essentiality	Evidence of folate interaction
D1R / E1 (C-terminal domain)	Cap (guanine-N7)-methyltransferase; N-terminal domain also RTPase + guanylyltransferase	GpppN-RNA → m7GpppN-RNA (cap-0)	SAM	5′ end of nascent viral mRNA	Essential; required for viral gene expression and early transcription termination	None reported; no enzymatic assay with folates published
D12L / E12	Catalytically inactive stimulatory subunit (degenerate 2′-O-MTase fold)	None; allosterically stimulates D1 N7-MTase ~40–50×	n/a	Binds D1	Essential as D1 partner	None reported; MPXV E12 structure available for docking
J3R (VP39) / VP39	Cap-specific 2′-O-methyltransferase; also, poly(A) polymerase processivity subunit	m7GpppN-RNA → m7GpppNm-RNA (cap-1)	SAM	Cap-0 RNA, minimum ~m7GpppGpN	Required for efficient innate immune evasion; validated inhibitor target	None reported; published inhibitors are sinefungin analogues, not folates
A27L / A29L	NOT a methyltransferase — envelope protein mediating heparan sulfate binding and virion attachment	n/a	n/a	Cell-surface glycosaminoglycans	Important for entry; vaccine and diagnostic antigen	Not applicable; earlier attribution of MTase activity to A29L was erroneous and has been withdrawn

VACV, vaccinia virus; MPXV, monkeypox virus; SAM, S-adenosyl-L-methionine; RTPase, RNA 5′-triphosphatase. Sources: (7,9,10,24–27)

Cap-1 formation. VP39 (vaccinia J3R; MPXV VP39) is a separate, monomeric, cap-specific 2′-O-methyltransferase that methylates the ribose 2′-O position of the first transcribed nucleotide, converting cap-0 to cap-1 (26). VP39 is bifunctional and serves as the processivity subunit of the viral poly(A) polymerase. Crystal structures of MPXV VP39 in complex with sinefungin and with a series of sinefungin-derived inhibitors have been reported, and several of these compounds inhibit MPXV replication in cell culture (9–11).

Both reactions use S-adenosyl-L-methionine (SAM) as the methyl donor and release S-adenosyl-L-homocysteine (SAH). Neither uses folate. The distinction between N7-guanine methylation and 2′-O-ribose methylation is not a matter of terminology: the two reactions are catalyzed by different proteins with different folds, different substrate requirements, and different consequences for innate immune evasion, and a compound active against one has no presumptive activity against the other.

At least two distinct cap-modifying methyltransferase activities are encoded in the D1/E1 C-terminal domain and in VP39. A29L is the MPXV homologue of vaccinia A27L, an envelope protein that mediates attachment to cell-surface heparan sulfate and is a well-characterized diagnostic and vaccine antigen (27); it has no described methyltransferase activity.

When evaluating the chemical role of folates within this system, precision about chemical identity is essential here, because the entities discussed in this literature differ substantially in structure, cellular handling, and pharmacology:

- Folic acid (pteroylmonoglutamic acid) — the fully oxidized synthetic form used in supplements and food fortification (Figure 2). It does not occur in appreciable quantities in unfortified foods.
- Reduced folates — dihydrofolate (DHF) and tetrahydrofolate (THF), generated from folic acid by dihydrofolate reductase; these are the metabolically active scaffolds.
- 5-methyltetrahydrofolate (5-methyl-THF) — the principal circulating and transport form of folate in human plasma.
- Folate polyglutamates — intracellular forms bearing a γ-linked polyglutamate tail, which promotes cellular retention and alters enzyme affinity; approximately 95% of intracellular folate is polyglutamated.
- Antifolates — structural analogues that inhibit folate-dependent enzymes, such as methotrexate and pemetrexed (dihydrofolate reductase, thymidylate synthase) and trimethoprim and pyrimethamine (microbial DHFR) (28).

These are not interchangeable. A compound that is added to a culture medium as folic acid is rapidly reduced and polyglutamated on entering the cell, so the species present at any intracellular target is not the species that was added. Equally, a finding that a synthetic folate analogue inhibits an enzyme is not evidence of what the vitamin does.

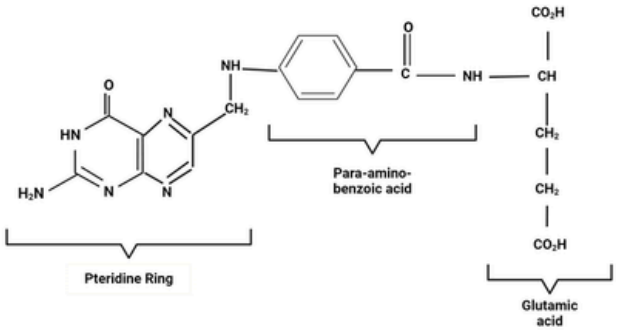


Figure 2. *Chemical structure and physiological state of folic acid (N-pteroyl-L-glutamic acid). It comprises a pteridine ring linked to p-aminobenzoic acid, which is bound via an amide bond to L-glutamic acid. At physiological pH, both glutamate carboxyl groups are ionized, resulting in a dianionic molecule with a high topological polar surface area. Image created with BioRender.com.*

SAM is the methyl donor for essentially all cellular and viral SAM-dependent methyltransferases, including every orthopoxvirus cap MTase as previously described. Folate participates in one-carbon metabolism: 5-methyl-THF donates a methyl group to homocysteine in the methionine synthase reaction (vitamin B-dependent), regenerating methionine, which methionine adenosyltransferase converts to SAM (29,30). Folate therefore contributes to methylation reactions upstream and indirectly, by supplying the SAM pool, and never by transferring a methyl group to a methyltransferase substrate directly.

If folate acted on viral cap methylation through its vitamin function, folate repletion would be expected to increase SAM availability and thereby, if anything, facilitate rather than inhibit viral cap methylation. An inhibitory effect would therefore have to arise from a mechanistically separate property, direct occupancy of the SAM-binding pocket or of the cap-RNA binding site by the intact folic acid molecule, acting as a competitive ligand rather than as a vitamin. It is precisely this second, distinct hypothesis that the docking literature addresses, and stating it explicitly clarifies what an experiment would need to demonstrate (Figure 3).

It should also be noted that folate metabolism intersects with antiviral innate immunity in ways that are neither simple nor uniformly favorable. Folate-dependent one-carbon metabolism supports nucleotide biosynthesis, on which viral genome replication depends, and folate status has been reported to modulate interferon-stimulated antiviral pathways. Antifolates such as methotrexate reduce SARS-CoV-2 replication in cell culture (31), which is the opposite polarity of intervention to folate supplementation. The direction of any net effect on an orthopoxvirus is therefore not predictable *a priori*.

Looking closely at the computational evidence gathered so

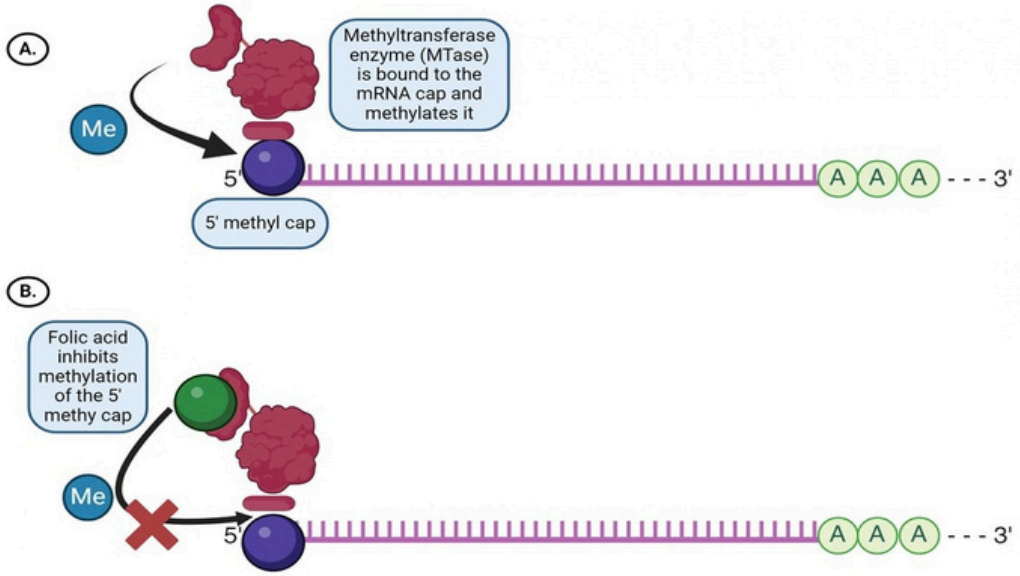


Figure 3. *Proposed mechanism of folic acid interference with orthopoxvirus mRNA cap methylation. (A) A SAM-dependent cap methyltransferase methylates the 5′ cap of viral mRNA. (B) Hypothesized mechanism in which folic acid occupies the enzyme active site, preventing methyl transfer and leaving viral transcripts susceptible to detection by cytoplasmic innate immune sensors. This figure depicts a hypothesis derived solely from molecular docking (12); it does not represent a demonstrated enzymatic result, and no biochemical assay has yet verified this mechanism. Image created with BioRender.com.*

far, the principal source for the folic acid hypothesis is the virtual screening study of Hashim and colleagues (12). In that work, 12,971 compounds from 200 Middle Eastern medicinal plants were reduced to 5,749 unique ligand conformers and screened against an MPXV methyltransferase using high-throughput virtual screening, followed by extra-precision (XP) docking and Prime MM-GBSA rescoring. Folic acid and 1,2,4,6-tetragalloylglucose emerged as the highest-scoring compounds, with MM-GBSA values of −51.87 and −60.61 kcal/mol, respectively, exceeding those of the reference compounds TO1119 (−35.71 kcal/mol) and the pan-MTase inhibitor sinefungin (−31.51 kcal/mol). In molecular dynamics simulations, folic acid was reported to be more stable in the binding site than sinefungin, and to occupy a position that would reduce enzyme interaction with the mRNA substrate.

Two points must be stated plainly. First, these are the results of that study, not of the present authors. Second, the authors of that study themselves concluded that wet-laboratory validation is necessary before any therapeutic inference can be drawn (12). The present review endorses that conclusion.

In contrast to these computational models, the biochemical and cell-based evidence reveals a stark gap: no published study reports concentration-dependent inhibition of a purified orthopoxvirus methyltransferase by folic acid, folate polyglutamates, or reduced folates. No IC_{50}, K_i or equivalent kinetic parameter has been determined for any folate against D1/E1, D12/E12, or VP39. No study demonstrates intracellular target engagement of a viral MTase by folate in infected cells. This absence is the central finding of the present review and is stated here without qualification. What can legitimately be said is more limited. Folic acid has been reported to bind the SARS-CoV-2 nucleocapsid protein in a combined computational and biochemical study (32), and folate supplementation has been proposed to restrict SARS-CoV-2 entry through effects on ACE2 (33). These are observations about a different virus and different targets; they establish that folates can act as protein ligands, which is not in dispute, but they carry no weight regarding orthopoxvirus cap methylation. Conversely, the antifolate methotrexate reduces coronavirus replication in cell culture (31), illustrating that folate-pathway modulation in either direction can affect viral replication for reasons unrelated to viral methyltransferase inhibition.

This lack of empirical proof contrasts sharply with validated inhibitors of the same target class, which provide a concrete template for what adequate evidence looks like. Silhan and colleagues determined crystal structures of MPXV VP39 in complex with sinefungin and with a series of derived inhibitors, several of which inhibit the purified enzyme at sub-micromolar concentrations, and showed that the sinefungin binding mode is conserved across unrelated viral 2′-O-MTases, including SARS-CoV-2 nsp14 (9). Skvara and colleagues defined the structural basis for RNA-cap recognition and methylation by VP39 (10). Zgarbová and colleagues then demonstrated that these VP39 inhibitors suppress MPXV replication in cell culture (11). In parallel, the MPXV E12 crystal structure has enabled inhibitor discovery against the N7-MTase heterodimer (25). This body of work establishes the orthopoxvirus cap methylation machinery as a genuinely druggable target and, importantly, sets the evidentiary standard against which folate-related claims should be judged: a defined enzyme, a purified protein preparation, a defined substrate and methyl donor, concentration–response data, structural confirmation of the binding mode, and antiviral activity in infected cells.

Limitations, Gaps, and Future Research Directions

These dynamics highlight several profound limitations of the current evidence base (Table 2). First, docking generates hypotheses about binding poses but does not establish binding affinity, enzymatic inhibition, selectivity, or cellular target engagement. Docking scores correlate only weakly with measured affinities, and end-point free energy methods such as MM-GBSA are known to be sensitive to force field, solvation model, protonation state assignment, and simulation protocol, with reported errors that frequently exceed the differences used to rank compounds (34). Highly charged ligands are handled particularly poorly, because desolvation penalties are systematically underestimated. Folic acid, bearing two ionized carboxylates at physiological pH, is precisely the class of ligand for which such scores should be treated with caution, and polar, multiply charged molecules are recognized as recurrent artefactual hits in virtual screens against SAM-binding pockets, which readily accommodate polar heterocyclic scaffolds. Two further limitations apply specifically to the study underpinning the folic acid hypothesis. Selectivity was not addressed: the human cap methyltransferase RNMT and the 2′-O-MTase CMTR1 perform analogous reactions with conserved SAM-binding architecture, and no counter-screen against them was reported. Furthermore, the identity of the target structure is not stated with sufficient precision to determine whether the N7-MTase or VP39 was modelled. This point must be resolved before the result can be interpreted at all.

Table 2. *Evidence level for compounds proposed to act on orthopoxvirus methyltransferases and for currently used mpox therapeutics.*

Candidate	Proposed target	Highest level of evidence	Experimental system	Key limitation
Folic acid	MPXV mRNA cap MTase (enzyme not specified in source)	In silico only	Glide XP docking, Prime MM-GBSA, MD simulation (2)	No enzymatic assay; no IC50/Ki; docking score is not inhibition; dianionic at physiological pH with poor expected passive permeability; target enzyme not unambiguously specified
1,2,4,6-tetragalloylglucose	MPXV mRNA cap MTase	In silico only	Same screening study (12)	As above; polyphenolic galloyl esters are recognized frequent hitters in screening assays
Sinefungin	Pan-MTase, SAM-competitive	Biochemical + structural + cell culture	Purified MPXV VP39; crystallography; MPXV-infected cells (9–11)	Poor selectivity across host and viral MTases; historical toxicity limits therapeutic use
Sinefungin analogues (e.g. TO507)	MPXV VP39 (2′-O-MTase)	Biochemical + structural + cell culture	Purified VP39; crystal structures; MPXV replication assays (9,11)	No published in vivo efficacy or pharmacokinetic data; tested against clade IIb only
E12-directed compounds	MPXV E12 / N7-MTase heterodimer	Structural + biochemical	Crystal structure of MPXV E12; inhibitor screening (25)	Early stage; antiviral activity in infected cells not established
Tecovirimat	F13 (VP37) envelope protein	Randomized controlled trials	PALM007 (clade I, n = 597); STOMP (clade II) (21)	No reduction in time to lesion resolution in either trial; efficacy not demonstrated
Cidofovir / brincidofovir	Viral DNA polymerase (E9)	Animal models; observational case series	Nonclinical models; uncontrolled clinical use (22,23)	No randomized efficacy data in mpox; nephrotoxicity (cidofovir); hepatic and GI toxicity (brincidofovir)

MTase, methyltransferase; MD, molecular dynamics; MM-GBSA, molecular mechanics generalized Born surface area; SAM, S-adenosyl-L-methionine.

Furthermore, the pharmacological plausibility of these models is highly questionable. The concentrations at which antiviral effects have been attributed to folic acid cannot be reconciled with achievable human exposures, and this deserves emphasis rather than a footnote. Physiological plasma folate concentrations lie in the range of approximately10–45 nmol/L; supplementation at 400 µg/day raises this modestly, and even therapeutic dosing at 5 mg/day yields total plasma folate in the high nanomolar to low micromolar range, constrained by saturable intestinal absorption and renal handling. Activity reported at millimolar concentrations is therefore roughly three orders of magnitude above anything attainable systemically. A compound requiring millimolar concentrations for an effect in vitro is not a viable systemic antiviral, irrespective of how favorable its safety and cost profile may appear at nutritional doses. A selectivity index of approximately 10, as reported in some of the material previously cited, sits at the absolute minimum threshold conventionally regarded to indicate a genuine antiviral effect rather than generalized cytostatic or cytotoxic activity. Typically, values of at least 10 and preferably above 100 are usually expected before a hit compound is advanced. Finally, the assumption that folic acid is intrinsically safe at any dose does not survive scrutiny. High-dose parenteral folic acid is an established and widely used method of inducing acute kidney injury and renal fibrosis in rodents (35). Folic acid also interacts with anticonvulsant therapy and with methotrexate regimens, and elevated unmetabolized folic acid at high intakes remains an area of ongoing investigation. The implication that pharmacological antiviral dosing would be free of risk was not supportable and has been removed. Even if folic acid inhibited a viral MTase in a purified system, delivering it to the cytoplasmic viral factory would be non-trivial. Folates enter cells via the reduced folate carrier, proton-coupled folate transporter, and folate receptors, all of which are saturable and subject to competition from physiological folates. Once internalized,

folic acid is reduced and polyglutamated, so the free monoglutamate species modelled in docking studies would be a minor and transient intracellular component. The relationship between an extracellular concentration and the concentration of the docked species at the target is therefore neither linear nor well characterized.

To systematically resolve these evidence gaps, several clear research directions are required. The literature reviewed above leaves the central claim untested rather than refuted, and the gaps are specific. First, no purified orthopoxvirus cap methyltransferase has been exposed to folic acid in a defined enzymatic assay, so no IC_{50} or K_i has been reported and the mode of inhibition relative to S-adenosylmethionine (SAM) and to the RNA substrate is unknown. Second, direct binding has not been demonstrated by any biophysical method independent of docking, and no experimental structure supports the predicted binding pose. Third, selectivity is entirely uncharacterized: the human cap methyltransferases RNMT and CMTR1 use the same methyl donor and a related fold, yet no comparative data exist. Fourth, where antiviral effects have been attributed to folates in cell culture, target engagement has been inferred from replication readouts rather than measured, and cap-0 and cap-1 species on viral mRNA have not been quantified. Fifth, the clade dependence of any such effect is unknown, and no in vivo data exist. Finally, the single computational study underpinning the hypothesis has not been reproduced by an independent group using different scoring functions and force fields, a step that end-point free energy methods such as MM/GBSA particularly require given their documented variability across systems (34).

Addressing these deficiencies calls for a specific sequence of experimental validations. Enzymatic characterization is the necessary first step: the recombinant MPXV E1 C-terminal domain in complex with E12, and separately purified VP39, assayed against a defined capped RNA substrate with radiolabeled or non-radiometric detection of methyl transfer, would establish whether folic acid inhibits at all. Folic acid, 5-methyl-THF, THF, representative folate polyglutamates and relevant antifolates should be tested individually across a concentration range, with sinefungin as a positive control and an inactive structural analogue as a negative control, since the folate species modelled in silico is not the species that predominates intracellularly. Parallel assays against RNMT and CMTR1 would define the selectivity window, which matters because inhibition of host cap methylation would be expected to be poorly tolerated. Direct binding would then require independent confirmation by isothermal titration calorimetry, surface plasmon resonance, or thermal shift analysis, ideally supported by a co-crystal or cryo-electron microscopy structure of the kind already obtained for sinefungin-derived VP39 inhibitors and for the E12 subunit (9,11,25). Only once inhibition and binding are established do cell-based experiments become interpretable: MPXV replication assays with paired cytotoxicity determination, EC_{50}, CC_{50} and a selectivity index, time-of-addition studies, and direct measurement of target engagement by cellular thermal shift assay or by mass-spectrometric quantification of cap-0 and cap-1 on viral transcripts. Testing across clade Ia, Ib and IIb isolates, with sequence comparison of the target enzymes, would establish generality, and established animal models of orthopoxvirus infection with accompanying pharmacokinetic data would be required before any therapeutic claim could be entertained (36,37).

Two priorities follow from this analysis. The first is that the burden of proof sits at the enzymatic level: a single well-controlled assay on a purified enzyme would either bring the hypothesis into the experimental domain or close it, and such an experiment is inexpensive relative to the attention the claim has already attracted. The second is that the folate question is best pursued within a broader program rather than in isolation. The wider and more productive direction is the systematic exploration of orthopoxvirus cap methylation as a drug target, where validated chemical matter already exists, structures are available for both the N7 and the 2′-O-methyltransferases, and the principal unmet needs are selectivity over the host enzymes, oral bioavailability, and activity across clades. Should folic acid or a reduced folate prove to inhibit these enzymes, the more informative follow-up question would concern the folate-dependent supply of SAM and whether modulating that pool alters viral cap methylation, since such a mechanism is consistent with folate biochemistry in a way that direct competitive inhibition is not. Until at least the enzymatic step has been completed, the accurate description of folic acid remains a computationally generated hypothesis, and reviews, commentaries, and public communication should characterize it as such.

Taken together, this review demonstrates that orthopoxvirus mRNA cap methyltransferases are legitimate and well-validated antiviral targets, and the case for pursuing them has strengthened now that tecovirimat has failed to demonstrate clinical efficacy in randomized trials against both clade I and clade II MPXV. Inhibitors of the MPXV 2′-O-methyltransferase VP39 have been characterized structurally, shown to inhibit the purified

enzyme, and shown to suppress viral replication in cell culture, providing proof of principle for the target class.

The status of folic acid within this landscape is much more limited. It has been identified as a high-scoring ligand in a single computational screen against an MPXV methyltransferase model, and that observation is worth following up. It has not been shown to inhibit any purified orthopoxvirus methyltransferase; no kinetic parameters have been reported; no target engagement has been demonstrated in infected cells; and the concentrations at which antiviral effects have been attributed to it exceed achievable human plasma exposures by approximately three orders of magnitude. Folate biochemistry offers no straightforward mechanistic support, since SAM rather than folate is the methyl donor for these enzymes and folate repletion would if anything be expected to increase SAM availability.

Folic acid should therefore be described as a computationally derived candidate warranting enzymatic evaluation, no more and no less. Describing it as the first small-molecule inhibitor of MPXV, or as a promising treatment for MPXV, is not supported by the available evidence. Beyond the requirements of scientific accuracy, such claims carry a practical risk: MPXV outbreaks disproportionately affect settings where an inexpensive, widely available vitamin marketed as an antiviral could readily displace supportive care and vaccination, which are the interventions currently known to help.

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Conceptualization, A.A., L.A., and M.O.O.A.; Literature search, A.A., L.A., and M.O.O.A.; Writing—original draft preparation, A.A., L.A., and M.O.O.A.; Writing—review and editing, A.A., L.A., and M.O.O.A. All authors have read and approved the published version of the manuscript.

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Statement of Competing Interest

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Statement of Data Availibility

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Statement of Generative AI Use

An AI tool was used primarily for language editing and rephrasing selected passages. The tool was not used to generate experimental data, alter raw data, fabricate results, or independently determine the scientific conclusions of the study. The authors critically reviewed, verified, and revised all AI-edited content.

Manuscript data were cross-checked against the relevant scientific literature. The authors retain full responsibility for the accuracy, integrity, interpretation, and final wording of the manuscript. Regarding privacy and compliance, no patient-level, human-subject, or personally identifiable research data were shared with the AI system; only information necessary for language editing was processed.

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REVIEW ARTICLE

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THE USE OF DIGITAL INTERVENTIONS BASED ON ARTIFICIAL INTELLIGENCE IN THE PSYCHOTHERAPY OF ADOLESCENT ANXIETY DISORDERS: A PRELIMINARY REVIEW OF POTENTIAL USABILITY, EFFICACY AND SAFETY

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Artificial intelligence (AI)-assisted digital mental health interventions are being increasingly discussed as a potential strategy for improving access to psychological support among adolescents with anxiety symptoms and anxiety disorders. Their possible advantages include flexible availability, reduced barriers to help-seeking, psychoeducation, symptom monitoring, CBT-informed exercises, conversational interaction, and personalized feedback. However, their clinical role remains insufficiently defined, particularly regarding efficacy, safety, ethical governance, and the distinction between digital support and psychotherapy in the traditional clinical sense. This narrative review examines current evidence and conceptual debates regarding AI-assisted digital interventions relevant to adolescent anxiety disorders, with a focus on usability, acceptability, preliminary efficacy, safety, ethical and legal considerations, and human clinical oversight. Available literature suggests that conversational agents and CBT-informed digital tools may provide low-threshold support and may reduce the symptoms of anxiety, depression, or general emotional distress in some users. Nevertheless, existing evidence remains heterogeneous and preliminary, often relying on young adult, mixed-age, non-clinical, or transdiagnostic samples, short follow-up periods, and self-report outcomes. Direct evidence for AI-assisted interventions specifically in adolescents with formally diagnosed anxiety disorders remains limited, and findings from broader youth, young adult, mixed-age, or transdiagnostic samples should not be directly generalized to this clinical population. It should not be considered a replacement for therapist-delivered psychotherapy, clinical assessment, or specialist child and adolescent mental health care. Their most appropriate current role is as adjunctive or supportive tools within supervised, stepped-care, or blended-care models. Future research should include adolescent-specific clinical trials, anxiety-focused outcomes, longer follow-up, systematic safety reporting, and clear evaluation of human oversight models. AI-assisted digital interventions may help reduce parts of the adolescent mental health treatment gap; however, their use must remain evidence-informed, developmentally sensitive, ethically regulated, and clinically supervised.

Keywords: artificial intelligence, adolescent anxiety, digital mental health, cognitive-behavioural therapy, conversational agents

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INTRODUCTION

Anxiety disorders are among the most common mental health problems in adolescence and are often accompanied by difficulties in emotional, social, academic, and family functioning. During this developmental period, young people face increasing demands related to peer relationships, identity formation, school achievement, autonomy, and future planning. When anxiety becomes persistent and impairing, it may affect school performance, social participation, self-esteem, family relationships, and overall quality of life. It is also frequently associated with depressive symptoms, other anxiety-related conditions, and later mental health difficulties (1-4). Early recognition and timely intervention are therefore important priorities in child and adolescent mental health care.

Cognitive-behavioral therapy (CBT) is one of the best-established psychological treatments for anxiety disorders in children and adolescents. It usually includes psychoeducation, identification and modification of maladaptive thoughts, exposure-based techniques, behavioral experiments, skills training, homework tasks, and relapse prevention. Meta-analytic evidence supports its effectiveness and acceptability in young people, although outcomes may vary depending on symptom severity, comorbidity, family involvement, treatment format, and access to trained professionals (5-8). In practice, however, many adolescents still do not receive timely psychological treatment. Limited availability of specialists, waiting lists, geographical distance, financial barriers, stigma, lack of family support, and reluctance to seek face-to-face care all contribute to this treatment gap (9,10).

Digital mental health technologies have been proposed as one way to reduce some of these barriers. Mobile applications, internet-based interventions, digital CBT programs, and conversational agents can offer flexible and low-threshold forms of support. For adolescents and young adults, these formats may be particularly acceptable because digital communication is already part of everyday life and may feel less stigmatizing than traditional service pathways (11-13). Within this broader field, artificial intelligence (AI) has attracted increasing attention because it may support personalization, automated feedback, symptom monitoring, conversational interaction, and adaptive delivery of therapeutic content (14-16).

Still, AI-assisted digital interventions should not be equated with psychotherapy in the traditional clinical sense. Psychotherapy involves a trained human therapist, individualized formulation, clinical judgment, therapeutic responsibility, supervision, and the capacity to respond to complex interpersonal and clinical dynamics. Most current AI-based tools in mental health are more accurately described as digital mental health interventions, conversational agents, CBT-informed self-help tools, or adjunctive supports rather than autonomous psychotherapeutic treatments (17-19). This distinction is particularly important in adolescent psychiatry, where developmental vulnerability, family involvement, informed assent, safeguarding, crisis escalation, and clinical oversight are essential.

The available evidence on AI-assisted interventions for adolescent anxiety remains promising but preliminary. Tools such as Woebot, Wysa, and Tess have shown potential for reducing the symptoms of anxiety, depression, or general emotional distress and for increasing access to low-threshold support (20-22). However, much of the evidence comes from short-term studies, feasibility designs, mixed-age or non-clinical samples, transdiagnostic interventions, or tools designed for general emotional well-being rather than adolescent anxiety disorders specifically (11,18,13). Conclusions about efficacy, safety, and clinical implementation therefore require caution.

The use of AI-supported interventions in adolescent mental health also raises ethical, legal, and professional questions, including privacy, transparency, informed consent and adolescent assent, parental or guardian involvement, algorithmic reliability, crisis detection, commercial influence, and professional accountability (23-27). Young users may experience conversational agents as empathic or emotionally supportive, but perceived support should not be confused with authentic empathy or a human-equivalent therapeutic alliance (17,28,29).

This narrative review examines current literature and conceptual debates on AI-assisted digital mental health interventions relevant to adolescent anxiety disorders, focusing on usability, preliminary efficacy, safety, ethical and legal considerations, and human clinical oversight. By clarifying both the potential and the limitations of these tools, the review aims to support a cautious, clinically grounded, and ethically responsible approach to their future use in adolescent mental health care.

METHODS

This paper was designed as a narrative review of current evidence and conceptual discussions on artificial intelligence (AI)-assisted digital mental health interventions relevant to adolescent anxiety disorders. A narrative

approach was chosen because the literature in this field is still heterogeneous and rapidly developing, including randomized and feasibility studies, real-world evaluations, systematic and scoping reviews, ethical analyses, conceptual papers, and clinical commentaries.

The review was guided by the following broad question: what is currently known about the usability, acceptability, preliminary efficacy, safety, ethical implications, and clinical limitations of AI-assisted digital mental health interventions for adolescents with anxiety symptoms or anxiety disorders? A targeted literature search was conducted using PubMed/MEDLINE, Google Scholar, and relevant publisher databases. Search terms included combinations of "artificial intelligence," "AI," "digital mental health," "chatbot," "conversational agent," "adolescent," "youth," "anxiety," "anxiety disorders," "cognitive behavioral therapy," "CBT," "digital psychotherapy," "AI-assisted psychotherapy," "Woebot," "Wysa," "Tess," "ethics," "safety," "privacy," "therapeutic alliance," and "clinical oversight." Particular attention was given to literature on adolescent and youth mental health, CBT-informed digital tools, conversational agents, and ethical or safeguarding issues in digital psychiatry.

The search primarily focused on publications from 2010 onward, while earlier foundational studies were included when relevant to adolescent anxiety, CBT, digital mental health, or ethical considerations. Priority was given to peer-reviewed articles, systematic or scoping reviews, meta-analyses, randomized or feasibility studies, and recent conceptual papers on responsible AI integration in mental health care. Sources were considered relevant if they addressed AI-assisted or conversational digital mental health interventions, digital CBT or CBT-informed support, adolescent or youth mental health, anxiety symptoms or anxiety disorders, usability, preliminary outcomes, safety, privacy, ethical or legal issues, or human clinical supervision.

To improve transparency while preserving the narrative character of the review, the literature identification and selection process was presented as a structured narrative search rather than as a systematic review. A total of 60 potentially relevant sources were initially considered through targeted searches and reference checking. After relevance assessment, 29 sources were excluded because they were not sufficiently aligned with the aims of the review, were replaced by more recent or more relevant sources, or addressed broader digital mental health topics without sufficient relevance to AI-assisted interventions, adolescent anxiety, usability, safety, ethics, or clinical oversight. The final synthesis included 31 sources judged to be most relevant to the aims of this narrative review. No formal PRISMA-based screening protocol, systematic risk-of-bias assessment, or quantitative evidence synthesis was performed. (Figure 1).

Given the emerging and methodologically diverse nature of the field, this review did not aim to provide a formal quantitative synthesis or risk-of-bias assessment. Instead, the evidence was narratively organized around clinically relevant themes: adolescent anxiety and treatment gaps, CBT-informed digital interventions, preliminary efficacy, usability and acceptability, safety and safeguarding, ethical and legal considerations, limitations of current evidence, and future research priorities. During the synthesis, particular caution was applied in distinguishing AI-assisted digital mental health tools from psychotherapy in the traditional clinical sense, and in separating adolescent-specific evidence from studies conducted in adult, mixed-age, non-clinical, or transdiagnostic samples.

The review was informed by literature on chatbot-based and AI-supported mental health interventions, including Woebot, Wysa, and Tess, as well as recent work on responsible AI integration, personalization, therapeutic progress, ethical governance, and limitations of AI-supported psychotherapy-

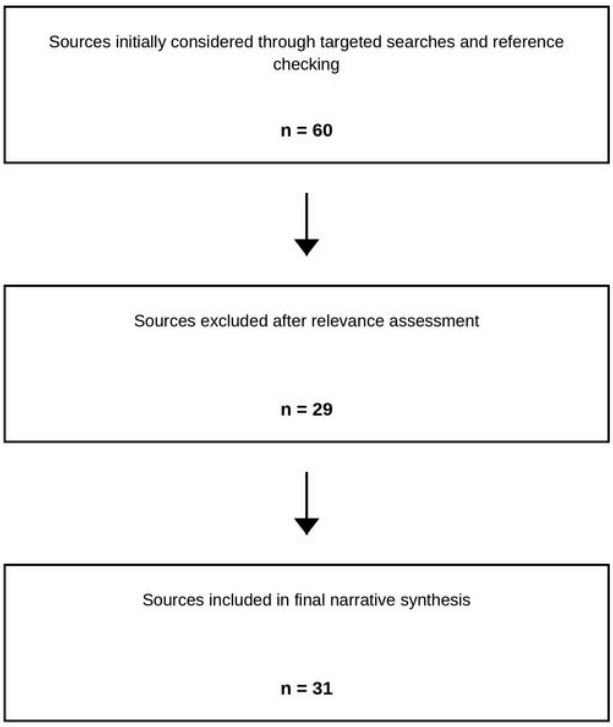


Figure 1. *Source identification and relevance-based selection*

related tools (14,30,23,20-22,18,27). Because adolescents represent a developmentally vulnerable population, special attention was given to transparency, informed consent and assent, parental or guardian involvement when appropriate, crisis escalation pathways, privacy protection, and human clinical oversight.

Adolescent anxiety disorders and the treatment gap

Adolescence is a period of rapid biological, psychological, cognitive, and social change. Young people are faced with increasingly complex peer relationships, academic expectations, identity-related questions, changing family roles, and preparation for adult life. Although fears and worries may be part of normal development, anxiety becomes clinically relevant when it is persistent, excessive, difficult to control, and associated with functional impairment.

Anxiety disorders are among the most common psychiatric disorders in children and adolescents. Epidemiological studies show that anxiety symptoms during adolescence may affect social development, academic achievement, family functioning, and overall quality of life (1-3). In many cases, anxiety does not occur in isolation, but is associated with other anxiety presentations, depressive symptoms, behavioral difficulties, substance use risk, and later mental health problems (2,4). Different forms of anxiety, including social anxiety, generalized anxiety, panic symptoms, separation anxiety, and school-related anxiety, may interfere with peer integration, autonomy, self-confidence, and academic progression.

The importance of adolescent anxiety is not limited to current distress. When untreated, anxiety disorders may become chronic or recurrent and may increase vulnerability to depression, functional impairment, and difficulties in later psychosocial adjustment (1,4). For this reason, adolescence can be seen not only as a period of vulnerability, but also as a window of opportunity. Timely intervention may reduce current impairment and potentially influence longer-term mental health trajectories.

Despite the availability of evidence-based psychological treatments, many adolescents with anxiety symptoms or anxiety disorders do not receive adequate or timely care. This treatment gap remains one of the major challenges in youth mental health. Barriers include limited availability of child and adolescent mental health professionals, waiting lists, geographical distance from specialized services, financial constraints, stigma, parental hesitation, limited information about treatment options, and adolescents' reluctance to disclose emotional difficulties in face-to-face settings (9,11,10). These barriers may be even more pronounced in underserved regions and in health systems with limited specialized resources.

Digital mental health interventions have been proposed as one way to reduce parts of this gap. Internet-based and mobile interventions may offer flexibility, easier access, and a greater sense of privacy than traditional service pathways. For some adolescents, digitally delivered support may also be more acceptable because it fits their usual ways of communication and information seeking (11,12). Still, accessibility alone is not enough. In adolescent mental health care, digital access has to be balanced with developmental sensitivity, clinical safety, family context, professional responsibility, and clear procedures for escalation when risk is identified.

AI-assisted digital tools may further expand the possibilities of digital mental health care through automated interaction, personalization, symptom monitoring, and adaptive delivery of CBT-informed strategies. However, these tools should be viewed as potential adjuncts or supportive interventions within a broader care framework, not as independent replacements for clinical assessment or therapist-delivered psychotherapy. This distinction is especially important in adolescent anxiety disorders, where treatment often requires not only symptom reduction, but also developmental support, family involvement, assessment of comorbidity, and safeguarding in situations of clinical risk.

CBT-informed digital and AI-assisted interventions

Cognitive-behavioral therapy is a structured and evidence-based approach commonly used in the treatment of anxiety disorders in children and adolescents. In therapist-delivered CBT, treatment usually includes psychoeducation, identification and modification of maladaptive thoughts, exposure-based techniques, behavioral experiments, skills training, homework tasks, relapse prevention, and, when appropriate, involvement of parents or caregivers. These elements are delivered within a therapeutic relationship that allows the therapist to individualize treatment, monitor emotional responses, adapt interventions to developmental level, and respond to comorbidity or clinical risk (5-8).

Digital CBT and CBT-informed interventions translate some of these components into online, mobile, or application-based formats. They may include psychoeducational modules, symptom monitoring, mood tracking, guided exercises, relaxation techniques, exposure planning, cognitive restructuring worksheets, behavioral activation tasks, reminders, and feedback based on user input. Some programs are self-guided, while others include therapist

guidance, coaching, parental involvement, or professional monitoring. In adolescent anxiety, such formats may be useful because they can reduce practical barriers to care and provide support in a form that many young people perceive as familiar and accessible (11,12).

Artificial intelligence may add further functions to these interventions, such as conversational interaction, personalization of content, automated feedback, adaptive intervention pathways, and monitoring of symptom changes over time. Conversational agents such as Woebot, Wysa, and Tess have been studied as examples of chatbot-based mental health support using CBT-informed or related psychological strategies. These tools may offer psychoeducation, emotional support, self-reflection prompts, mood tracking, behavioral suggestions, and structured exercises aimed at reducing anxiety, depression, or general emotional distress (20-22,18).

At the same time, CBT-informed digital support should not be confused with psychotherapy in the classical clinical sense. A chatbot or AI-based conversational tool does not independently provide comprehensive psychiatric assessment, individualized case formulation, therapeutic responsibility, or a human therapeutic alliance. Although some users may experience conversational agents as supportive, current AI systems are more accurately understood as digital mental health tools, self-help supports, or adjunctive interventions rather than replacements for therapist-delivered psychotherapy (17,19). This distinction is particularly important in adolescent psychiatry, where clinical work often includes developmental assessment, family context, school functioning, comorbidity evaluation, safeguarding, and crisis management.

Recent literature suggests that AI-supported psychotherapy-related tools may contribute to personalization, engagement, and perceived therapeutic progress, but their implementation should remain clinically cautious and ethically governed. Beg and colleagues emphasize both the potential of AI-driven psychotherapy-related systems and the challenges related to usability, personalization, therapeutic boundaries, clinical validity, and responsible integration into mental health care (14,30,15). In this sense, AI should not be framed as a simple technological replacement for psychotherapy, but as a developing set of tools whose value depends on evidence, transparency, safety procedures, and human oversight.

In adolescent anxiety disorders, AI-assisted digital interventions are best understood within stepped-care or blended-care models. In lower-risk situations, they may support psychoeducation, symptom monitoring, CBT-informed exercises, or work between clinical contacts. In more complex presentations, including comorbidity, severe impairment, family dysfunction, school refusal, trauma-related symptoms, or elevated clinical risk, they should not be used as stand-alone interventions. Their more appropriate role is as an adjunct to therapist-delivered care, with clear procedures for supervision, referral, escalation, data protection, and communication with caregivers when clinically and legally appropriate.

The central question is therefore not whether AI can replace psychotherapy, but whether carefully designed and validated AI-assisted tools can safely extend the reach of evidence-informed psychological support. Their potential lies in improving access, engagement, continuity, and personalization, while their limitations require cautious implementation, especially in vulnerable adolescent populations.

Current evidence for preliminary efficacy

The evidence base for AI-assisted digital mental health interventions in adolescent anxiety is growing but remains methodologically heterogeneous and clinically preliminary. Existing studies suggest that conversational agents and CBT-informed digital tools may reduce symptoms of anxiety, depression, or general emotional distress in some users. However, the strength and specificity of this evidence vary considerably across interventions, populations, study designs, and outcome measures.

Several frequently cited chatbot-based interventions, including Woebot, Wysa, and Tess, have shown promising short-term outcomes in reducing psychological distress. Woebot has been evaluated in young adults with symptoms of depression and anxiety, with reductions in self-reported symptoms over a brief intervention period (20). Tess has been examined as a psychological AI system for symptoms of depression and anxiety. At the same time, Wysa has been evaluated using real-world data as a conversational agent for digital mental well-being (21,22). These studies support the potential relevance of conversational tools for low-threshold emotional support and CBT-informed self-help.

However, findings from these interventions should be applied to adolescent anxiety disorders with caution. Many studies include young adults, university students, mixed-age samples, nonclinical users, or participants with subclinical symptoms rather than adolescents with formally diagnosed anxiety disorders. Several tools also target emotional distress, depression, stress, or general well-being rather

than anxiety disorders specifically. Outcomes are commonly based on self-report scales and short follow-up periods, while evidence on diagnostic recovery, long-term maintenance, relapse prevention, functional improvement, and safety in clinically complex adolescent populations remains limited (11,18,13).

Another important distinction concerns the level of human involvement. Fully automated conversational agents, self-guided digital interventions, therapist-guided programs, coaching models, and blended-care approaches are not directly comparable. In adolescent psychiatry, human oversight may strongly influence safety, engagement, adherence, risk detection, family communication, and referral when symptoms worsen. For this reason, evidence from supervised digital interventions should not be interpreted as evidence for fully autonomous AI-based care.

Evidence for digital mental health interventions in young people is broader than evidence for AI-specific interventions in adolescent anxiety. Reviews suggest that online and mobile interventions may be acceptable and may improve symptoms, particularly when they are structured, theory-based, and supported by some degree of guidance (11,12). AI-assisted tools are a newer and narrower category within this field, and their mechanisms of action, safety profile, sustained engagement, and comparative effectiveness remain insufficiently established.

Current evidence is therefore best described as promising but preliminary. AI-assisted conversational tools may have value as accessible, low-threshold, CBT-informed supports for adolescents with mild symptoms, psychoeducational needs, or barriers to traditional care. At present, however, the literature does not justify their use as stand-alone replacements for clinical assessment, therapist-delivered CBT, or specialist child and adolescent mental health care.

Overall, the quality of evidence was interpreted as preliminary and heterogeneous rather than definitive. Randomized controlled trials provide the strongest available evidence for selected conversational agents. Still, many were conducted in young adult, university, mixed-age, or non-clinical samples and used short follow-up periods and self-report outcomes. Real-world evaluations provide useful information on engagement and ecological use, but are limited by selection bias and lack of control groups. Reviews and conceptual papers are useful for mapping the field and identifying ethical or implementation issues, but they do not provide direct evidence of clinical efficacy. For this reason, the present review interprets the available evidence cautiously and avoids conclusions that would imply equivalence between AI-assisted tools and therapist-delivered psychotherapy.

Taken together, the overall certainty of evidence was judged to be low for AI-assisted interventions specifically targeting adolescents with diagnosed anxiety disorders. Evidence was considered somewhat stronger for broader digital CBT and CBT-informed interventions in youth, and for selected conversational agents in young adult or mixed-age samples, but this evidence remains indirect for the specific population and clinical focus of the present review. The main factors limiting certainty were population indirectness, intervention heterogeneity, short follow-up periods, reliance on self-report measures, limited diagnostic assessment, and insufficient safety reporting.

To clarify the heterogeneity of the available evidence, Table 1 summarizes selected AI-assisted and conversational digital mental health interventions relevant to anxiety, emotional distress, and adolescent mental health. Because the available sources include randomized controlled trials, feasibility studies, real-world evaluations, longitudinal studies, reviews, and conceptual papers, the table also distinguishes the level and interpretation of evidence for each source. As shown in Table 1, these interventions should currently be interpreted mainly as adjunctive, supervised, or low-threshold forms of digital support rather than stand-alone replacements for therapist-delivered CBT.

Usability and acceptability among adolescents

Usability and acceptability are important for AI-assisted digital mental health interventions in adolescents. Even when an intervention is clinically well designed, its value depends on whether young people are willing to use it, understand its purpose, find the interface appropriate, and can fit it into everyday routines.

Adolescents and young adults commonly use digital technologies for information, communication, validation, and peer support. This may increase the acceptability of digital mental health tools, especially when traditional pathways involve waiting lists, parental mediation, geographical barriers, or stigma (11-13). For some adolescents, a mobile or web-based tool may feel more private, less intimidating, and easier to access than an initial face-to-face meeting with a mental health professional.

Features such as immediate access, flexible timing, conversational interaction, brief exercises, reminders, personalized feedback, symptom tracking, and use outside clinical settings may improve usability. In anxiety disorders, where avoidance, fear of evaluation, shame, or difficulty initiating help-seeking may delay treatment, low-threshold digital support may help some adolescents begin with psychoeducation and basic CBT-informed strategies earlier

Table 1. *Selected AI-assisted and conversational digital mental health interventions relevant to anxiety, emotional distress, and adolescent mental health*

Intervention /tool	Modality and therapeutic orientation	Population/ age group	Clinical focus	Study design/evidence type	Main reported findings	Safety and methodological considerations	Evidence level / interpretation
Woebot	Fully automated conversational agent delivering CBT-informed self-help content	Young adults/college students	Symptoms of depression and anxiety	Randomized controlled trial	Short-term reductions in self-reported depressive symptoms and feasibility of chatbot-delivered CBT-informed support were reported; anxiety-related outcomes were also assessed	Not adolescent-specific; short intervention period; self-selected sample; symptom reduction rather than diagnostic recovery; should not be interpreted as evidence of autonomous psychotherapy for adolescent anxiety disorders (20)	Randomized controlled trial; preliminary evidence in young adults, not adolescent-specific clinical anxiety evidence.
Tess	Psychological AI / conversational agent using integrative therapeutic strategies, including CBT-informed elements	Adults / students and mixed user groups in available studies	Symptoms of depression and anxiety	Randomized controlled trial / feasibility-oriented evaluation	Reported reductions in self-identified symptoms of depression and anxiety, suggesting potential for low-threshold digital support	Limited adolescent-specific evidence; intervention should be framed as AI-assisted support rather than classical psychotherapy; longer-term efficacy, safety, and clinical supervision require further evaluation (21)	Randomized controlled trial / feasibility-oriented evidence; preliminary and limited by sample characteristics and follow-up.
Wysa	AI-enabled text-based conversational mental well-being application using supportive and CBT-informed techniques	Real-world users; primarily self-selected app users	Depression symptoms, emotional distress, general well-being; anxiety-related support may be included	Real-world data evaluation / mixed-methods study	Preliminary evidence suggested user engagement and improvement in self-reported symptoms among users with depressive symptoms	Real-world design limits causal inference; self-selection bias; not specific to adolescents or diagnosed anxiety disorders; safety and crisis escalation require explicit reporting (22)	Observational real-world evidence; useful for engagement and ecological use, but limited causal inference.
Kai.ai	Automated conversational agent delivering ACT-based self-help tools	Adolescents	Well-being and emotional support	Longitudinal study	Findings suggested potential usefulness of an AI-powered mobile tool for supporting adolescent well-being	Focus was well-being rather than diagnosed anxiety disorders; not CBT-specific; evidence is preliminary and does not establish efficacy for adolescent anxiety treatment (13)	Longitudinal youth-focused evidence; preliminary and not equivalent to disorder-specific anxiety treatment evidence.
AI-assisted online social therapy / youth mental health platforms	Digital platform with AI-supported or technology-assisted components, usually embedded in broader psychosocial support	Young people / youth mental health populations	Social functioning, emotional distress, youth mental health support	Conceptual and early implementation evidence	May support engagement, social connectedness, and access to structured online therapeutic environments	Often not fully automated and not equivalent to stand-alone chatbot intervention; requires moderation, human oversight, safeguarding, and integration into clinical care (12)	Conceptual and early implementation evidence; relevant for youth digital mental health support, but not direct efficacy evidence.

Digital CBT and internet-based interventions for youth	Online or mobile CBT-informed interventions; may be self-guided, guided, or blended	Children, adolescents, and young people	Anxiety, depression, and broader emotional symptoms	Systematic reviews and meta-analytic evidence across digital interventions	Evidence suggests that structured digital mental health interventions may be acceptable and may reduce symptoms, particularly when guided or clinically supported	This evidence is broader than AI-specific evidence; intervention heterogeneity is high; adolescent anxiety-specific AI evidence remains limited (11,6,8)	Review and conceptual evidence; useful for interpretation, personalization, and governance, but not direct clinical efficacy evidence.
Emerging AI-driven psychotherapy-related systems	AI-supported personalization, automated feedback, conversational interaction, monitoring, and therapeutic content delivery	Mostly adult, mixed-age, or conceptual literature	Various mental health conditions; anxiety and emotional distress included in broader discussions	Narrative reviews, conceptual papers, qualitative and implementation-oriented literature	Recent literature highlights potential for personalization, perceived therapeutic progress, usability, and expanded access	Evidence remains preliminary; responsible integration requires transparency, ethical governance, data protection, clinical validation, and human oversight (14,30,15,23)	Ethical and governance evidence; relevant to safety, accountability, and regulation, but not efficacy evidence.

(11,10). Digital tools may also support continuity between clinical contacts through symptom monitoring, homework reminders, and reinforcement of coping strategies.

However, acceptability should not be confused with clinical adequacy. Adolescents may initially be attracted by novelty, accessibility, or anonymity, but sustained use depends on perceived usefulness, trust, privacy, personalization, emotional relevance, and user experience. Digital interventions often face attrition and declining engagement, which is especially relevant in anxiety disorders, where avoidance may also interfere with therapeutic tasks.

The conversational format may increase engagement by creating a sense of responsiveness and emotional availability. Some users experience chatbots as supportive, non-judgmental, and easier to disclose to than human professionals (28,18). Still, simulated empathy is not equivalent to human empathy, clinical intuition, or a therapeutic alliance (17,29).

Personalization may further improve acceptability, particularly when tools adapt to user input, symptom patterns, engagement history, or preferred communication style (15). In adolescents, however, personalization must be balanced with privacy, transparency, developmental differences, family context, comorbidity, and digital literacy.

Acceptability also depends on parents, caregivers, schools, and clinicians. Adolescents may value privacy and autonomy, while adults remain responsible for safety, consent, monitoring, and escalation when risk is present. Therefore, usability should be evaluated not only through satisfaction or frequency of use, but also through developmental appropriateness, sustained engagement, clinical relevance, privacy, safety, and integration with professional care.

Safety, safeguarding, and human clinical oversight

Safety is a central requirement for the responsible use of AI-assisted digital interventions in adolescent mental health care. Adolescents are a developmentally vulnerable population, and anxiety symptoms may coexist with depression, self-harm risk, substance use, trauma-related symptoms, family conflict, school refusal, neurodevelopmental difficulties, or emerging severe mental disorders. For this reason, AI-assisted tools should not be implemented only because they are accessible, convenient, or acceptable to users. Their use requires clear safety procedures, defined limits, and human clinical oversight.

A major concern is that an AI-assisted tool may fail to recognize clinical complexity or deterioration. Anxiety in adolescents may present through avoidance, irritability, somatic complaints, sleep problems, concentration difficulties, social withdrawal, school refusal, or family conflict. Automated systems may not reliably distinguish mild anxiety from clinically significant anxiety disorders, comorbid depression, trauma-related symptoms, or situations requiring urgent clinical evaluation. AI-supported interventions should therefore include screening procedures, limits for self-guided use, and referral pathways when symptoms exceed the intended scope of the tool.

Safeguarding is particularly important when digital tools are used with minors. Responsible implementation requires

transparent procedures for parental or guardian consent, adolescent assent, confidentiality, data sharing, and crisis escalation. Adolescents should know that they are interacting with a digital tool or conversational agent rather than a human therapist, what support it can provide, its limitations, how data are stored and used, and when information may need to be shared for safety reasons (23,27).

Crisis detection and escalation remain especially sensitive. AI-assisted systems should have predefined procedures for identifying signs of acute distress or elevated clinical risk and directing users toward immediate human support. However, automated detection is not sufficient on its own. Missed risk signals, false reassurance, inappropriate generic responses, or delayed referral remain possible. AI tools should therefore not be presented as crisis services, and adolescents, families, and clinicians should be informed about emergency pathways and available professional support.

Human oversight is essential because AI systems do not possess clinical judgment, professional responsibility, or the ability to integrate developmental, family, and contextual information in the way a trained clinician can. AI-assisted tools may support monitoring, psychoeducation, or between-session practice, but they should not independently make diagnostic decisions, determine treatment suitability, or manage clinically high-risk situations (17-19).

The required level of oversight should depend on clinical risk and intervention purpose. In mild cases, AI-assisted tools may be used as low-threshold psychoeducational support. In moderate or complex presentations, they should be adjuncts to professional care. In severe or high-risk situations, they should not be used as stand-alone interventions. Overall, AI-assisted interventions appear safest within stepped-care or blended-care models, where digital tools support psychoeducation, symptom tracking, CBT-informed exercises, and continuity of care, while human professionals remain responsible for assessment, formulation, treatment planning, risk management, and safeguarding.

Ethical, legal, and data governance considerations

The use of AI-assisted digital interventions in adolescent mental health raises ethical, legal, and data governance concerns because these tools may collect highly personal psychological information, including mood states, anxiety symptoms, family and school difficulties, interpersonal problems, and risk-related content. Ethical implementation therefore requires attention to autonomy, beneficence, non-maleficence, justice, transparency, accountability, privacy, and safeguarding (23,25,27).

Autonomy is complex in adolescent care. Young people may value privacy and may disclose emotional difficulties more easily to a digital tool than to adults around them. At the same time, minors usually require some level of parental or guardian involvement, depending on age, legal context, clinical indication, and risk. AI-assisted interventions should therefore provide developmentally appropriate information about the nature and limits of the tool, data use, confidentiality, human oversight, and circumstances in which confidentiality may be limited for safety reasons. Informed consent and adolescent assent should be treated as a process of understanding, not as a formality.

Transparency is equally important. Adolescents and caregivers should know when they are interacting with an AI system rather than a human therapist. Conversational agents may use empathic language and may be experienced as supportive, but simulated empathy is not equivalent to human empathy, clinical judgment, or a therapeutic alliance (17,29). Clear communication about the non-human nature of the intervention may reduce unrealistic expectations and inappropriate reliance on automated support.

Non-maleficence requires attention to possible harms, including inaccurate responses, failure to recognize deterioration, excessive reassurance, delayed help-seeking, privacy breaches, algorithmic errors, overdependence, or use in situations for which the tool was not designed. These risks are particularly relevant in adolescents with comorbidity, severe impairment, family conflict, trauma-related symptoms, or elevated clinical risk. AI-assisted tools therefore require safety boundaries, crisis escalation procedures, mechanisms for human review, and guidance on when professional help is needed (18,19).

Beneficence and justice should also guide implementation. Digital tools may support psychoeducation, self-monitoring, skills practice, and low-threshold access, but their value should be interpreted cautiously because evidence in adolescent anxiety disorders remains preliminary (11,12). They may improve access in underserved areas or reduce stigma-related barriers, but may also reproduce inequalities if access depends on smartphones, stable internet, privacy at home, digital literacy, language, or culturally adapted content.

Data governance, accountability, and commercialization require explicit regulation. Psychological data may reveal

intimate information about emotions, relationships, family functioning, and risk, raising questions about ownership, secondary use, anonymization, withdrawal of consent, and security (24,27). If an AI-assisted tool gives inappropriate guidance, fails to detect risk, or contributes to delayed care, responsibility cannot remain unclear. Roles of developers, institutions, clinicians, schools, regulators, and commercial providers should be defined before implementation (23,26). Because many tools are commercially developed, claims about efficacy, safety, and personalization should be evidence-based, transparent, and proportionate, particularly when intended users are adolescents (31).

Overall, a clinically acceptable model should include transparent communication, meaningful consent and assent, appropriate parental or guardian involvement, privacy protection, human oversight, crisis escalation pathways, evidence-based claims, and clear accountability.

Limitations of current evidence

Although AI-assisted digital mental health interventions have attracted considerable interest, the current evidence base remains limited, especially for adolescent anxiety disorders. This is important because adolescence involves developmental vulnerability, family and school context, diagnostic complexity, and safeguarding responsibilities.

A major limitation is that much of the evidence is not specific to adolescents with diagnosed anxiety disorders. Frequently cited studies of conversational agents and AI-supported tools often include young adults, university students, mixed-age users, non-clinical samples, or participants with general psychological distress rather than adolescents who have undergone formal assessment for anxiety disorders (20-22,18). These findings therefore cannot be directly generalized to clinical adolescent populations.

Many interventions are also transdiagnostic or aimed at general emotional well-being rather than anxiety disorders specifically. Reductions in self-reported distress, depressive symptoms, stress, or general anxiety symptoms are clinically relevant, but they are not the same as evidence of effective treatment for adolescent anxiety disorders. Few studies distinguish symptom improvement from functional recovery, diagnostic remission, relapse prevention, or long-term outcome. This is important because evidence-based anxiety treatment usually requires structured assessment, formulation, exposure-based work, monitoring of avoidance, and adaptation to comorbidity and developmental context (6-8).

The available studies are often short-term and feasibility-oriented. They may show initial acceptability, symptom change, or user satisfaction, but provide less information about durability of effects, adherence, relapse prevention, adverse events, or outcomes after discontinuation (11,12). Methodological limitations are also common, including small samples, self-selected users, high attrition, limited blinding, lack of active control conditions, reliance on self-report measures, incomplete adherence data, and insufficient assessment of adverse events (11,18).

Another source of uncertainty is the level of human involvement. Fully automated conversational agents guided digital CBT programs, therapist-supported online interventions, school-based tools, and blended-care models differ substantially in intensity, safety procedures, adherence, and clinical responsibility. In adolescent anxiety, human oversight may be essential for risk management, differential diagnosis, family communication, and treatment adaptation (17,19).

Safety reporting remains insufficient. Many studies focus on usability, symptom reduction, or engagement, while less attention is given to adverse events, symptom deterioration, crisis-related responses, inappropriate recommendations, privacy concerns, or escalation procedures. Tools intended for adolescent mental health support should be evaluated not only for benefit, but also for their ability to recognize situations outside their intended scope and direct users toward human support (23,25,27).

Finally, evidence on mechanisms and implementation remains limited. It is still unclear whether improvements result mainly from CBT-informed content, self-monitoring, psychoeducation, perceived support, reminders, novelty, or the structure provided by the tool. The role of perceived empathy and alliance-like experiences is also unresolved and should not be equated with a human therapeutic alliance (17,28,29). Overall, AI-assisted digital interventions should be described as promising but preliminary, with a possible role as supportive or adjunctive tools rather than replacements for comprehensive clinical assessment or therapist-delivered evidence-based treatment.

This review also has limitations. As a narrative review, it did not use a formal systematic review protocol, PRISMA flow diagram, or standardized risk-of-bias assessment. The selection of literature was guided by relevance to the review aims, which may introduce selection bias. The heterogeneity of study designs, populations, interventions, and outcomes also limited direct comparison between sources. In addition, because AI-assisted mental health care is developing rapidly, some very recent tools or studies may not have been included. Publication bias is also possible, as

positive findings and technologically novel interventions may be more likely to be published than negative or inconclusive results.

Future research and clinical implementation priorities

Future research on AI-assisted digital interventions for adolescent anxiety disorders should move beyond feasibility, novelty, and user satisfaction toward stronger clinical evaluation. Studies need clearly defined adolescent samples, standardized diagnostic procedures, anxiety-specific outcomes, and transparent reporting of symptom severity, comorbidity, medication use, previous treatment, family involvement, and functional impairment. Without this detail, it remains difficult to determine whether observed improvements represent clinically meaningful change or only short-term reductions in general emotional distress.

Randomized controlled trials with adequate sample sizes, active comparison groups, and longer follow-up periods are needed. Future studies should compare AI-assisted tools with established digital CBT programs, therapist-guided interventions, blended-care models, and standard face-to-face CBT where appropriate. Outcomes should include not only symptom reduction, but also functional improvement, adherence, dropout, relapse prevention, safety, and user satisfaction (6-8).

Future work should also describe interventions more precisely. Fully automated conversational agents, CBT-informed self-help tools, therapist-supported digital programs, school-based interventions, and blended-care models should not be treated as equivalent. They differ in therapeutic intensity, personalization, clinical responsibility, safety procedures, and expected outcomes. Clear reporting is needed on the role of AI, the degree of human oversight, therapeutic components, intended users, and contraindications for use (18,19).

Safety and implementation should be evaluated together. Studies should report adverse events, symptom deterioration, crisis-related interactions, inappropriate responses, privacy concerns, dropout related to negative user experience, and referral or escalation outcomes. In adolescents, this also includes parental or guardian involvement, adolescent assent, safeguarding, emergency referral, and communication with clinicians or responsible adults when risk is identified (23,25,27).

Implementation research should clarify how these tools could be responsibly used in specialist services, schools, primary care, and stepped-care pathways. Clinicians should be able to evaluate whether a tool is evidence-based, safe, developmentally appropriate, and suitable for a particular adolescent, not only whether it is attractive or easy to use (23,10,12). User-centered research remains important. Adolescents, caregivers, clinicians, school psychologists, and other stakeholders should be involved in design and evaluation. Qualitative studies may clarify how young users experience trust, personalization, privacy, usefulness, and the boundaries of interaction with conversational agents (15,28).

The rapid development of generative AI and large language model-based applications represents an additional challenge for future research and regulation. These systems may allow more flexible, naturalistic, and personalized interaction than earlier rule-based or scripted chatbots, but they also introduce new risks, including inconsistent responses, hallucinated or inaccurate content, reduced transparency, over-reliance by users, and difficulties in clinical accountability. In adolescent mental health, these risks are particularly relevant because conversational fluency may increase perceived trust and emotional reliance. Therefore, future studies should evaluate generative AI and large language model-based tools separately from earlier chatbot interventions, with specific attention to safety, explainability, clinical validation, age-appropriate design, and human oversight (32). Finally, future studies should examine mechanisms of change and ethical regulation. It remains unclear whether benefits result from CBT-informed exercises, psychoeducation, self-monitoring, perceived support, personalization, novelty, or the structure of the tool. Implementation should be guided by standards for transparency, privacy protection, accountability, crisis escalation, evidence-based claims, and professional responsibility (17,24,29,26,31). The goal should not be to replace human psychotherapy, but to determine how AI-assisted tools can support evidence-based care while preserving the central role of clinicians in assessment, formulation, risk management, and treatment planning.

AI-assisted digital mental health interventions represent a promising but still preliminary approach to improving access to support for adolescents with anxiety symptoms and anxiety disorders. Their potential advantages include flexible access, reduced barriers to help-seeking, psychoeducation, symptom monitoring, CBT-informed exercises, and personalized feedback. These features may be relevant for adolescents who face barriers to traditional care or prefer more accessible digital forms of support. However, current evidence does not support the use of AI-assisted tools as replacements for therapist-delivered psychotherapy, specialist clinical assessment, or child and adolescent mental health care. Available studies are hetero-

geneous and often include mixed-age, non-clinical, or transdiagnostic samples, short follow-up periods, self-report outcomes, and limited safety reporting. Evidence specific to adolescents with diagnosed anxiety disorders remains insufficient. Therefore, the current clinical applicability of AI-assisted tools for adolescents with diagnosed anxiety disorders remains limited and should be interpreted with caution. At present, these interventions are best understood as adjunctive or supportive tools rather than autonomous psychotherapeutic treatments. In adolescent psychiatry, implementation must account for developmental vulnerability, comorbidity, family context, informed consent and adolescent assent, privacy, safeguarding, crisis escalation, and professional responsibility. Conversational agents may be perceived as supportive, but simulated empathy should not be equated with human empathy, clinical judgment, or therapeutic alliance. The most appropriate current role of AI-assisted tools appears to be within supervised, stepped-care, or blended-care models. In such frameworks, digital tools may support psychoeducation, self-monitoring, skills practice, and continuity between clinical contacts, while human professionals remain responsible for diagnosis, formulation, treatment planning, risk management, and ethical decision-making. Their future value will depend on rigorous adolescent-specific research, systematic safety reporting, ethical regulation, and responsible integration into mental health care systems.

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Conceptualization, A.S. and M.S.; Investigation, A.S. and A.R.; Writing – original draft preparation, A.S.; Writing – review and editing, M.S. and A.R.; Visualization, A.S. and A.R.; Supervision, M.S.; Project Administration, A.S. All authors have read and approved the published version of the manuscript.

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Not applicable.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availability

Data sharing is not applicable to this article as no new datasets were generated or analyzed during the preparation of this narrative review.

Statement of Generative AI Use

During manuscript preparation, AI-assisted tools were used for language editing, formatting support, and improvement of clarity. All AI-assisted outputs were critically reviewed, revised, and verified by the authors. The authors take full responsibility for the final content of the manuscript.

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ALTERATIONS IN LIVER FUNCTION BIOMARKERS AMONG MODERATELY SEVERE COVID-19 PATIENTS IN SOUTHEASTERN SERBIA: A RETROSPECTIVE SINGLE-CENTER STUDY

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Liver biochemical abnormalities have been reported in nearly half of patients with SARS-CoV-2 infection. The study aimed to determine the prevalence of altered liver biomarkers (alanine transaminase—ALT, aspartate aminotransferase—AST, lactate dehydrogenase—LDH, gamma-glutamyl transferase—GGT, and albumin) in COVID-19 patients with a moderately severe clinical picture on hospital admission, and to assess the presence of altered liver-related biomarkers in the absence of a severe clinical presentation. Research material consisted of medical histories of patients with a moderately severe clinical picture of COVID-19 who were hospitalized at the Clinic for Infectious Diseases of the University Clinical Centre Niš. The study included 655 patients (425 male and 230 female) who were divided into three groups: patients without comorbidities (NC), patients with arterial hypertension (HTA), and patients previously diagnosed with diabetes mellitus (DM). Additionally, within each research group, patients were divided into three age subgroups: patients aged 20-40 years, 40-60 years, and over 60 years. AST, ALT, and LDH showed statistically significant differences across the defined groups, while GGT did not. Logistic regression confirmed increased odds for elevated AST and LDH values in all older and comorbid groups, with the most pronounced effects for LDH. For ALT, significantly increased odds were observed among patients with HTA (40–60 years) and DM (40–60 years). The most pronounced changes were observed in elderly patients and those with hypertension and diabetes, with LDH emerging as the most sensitive, although non-specific biomarker, likely reflecting systemic inflammation and disease severity in addition to possible hepatic involvement.

Keywords: COVID-19, aspartate aminotransaminase (AST), alanine aminotransaminase (ALT), gamma-glutamyltransferase (GGT), lactate dehydrogenase (LDH), albumins

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INTRODUCTION

COVID-19 is a multisystem disease caused by SARS-CoV-2 that affects the respiratory, cardiovascular, central nervous system, liver, and kidneys (1). The virus enters the body by binding to the angiotensin-converting enzyme 2 (ACE2) receptor, which it uses to enter cells. ACE2 receptors are found in type 2 alveolar cells in the epithelium of the nose, mouth, lungs, in glandular cells of the stomach, in epithelial cells of enterocytes, in the ileum and colon, in most cholangiocytes, and in less than 3.0% of hepatocytes (2,3).

The reports indicate that almost half of patients with SARS-CoV-2 had liver biochemical abnormalities, including elevated aminotransferase levels (AST, ALT), gamma-glutamyltransferase (GGT), bilirubin, and alkaline phosphatase (3-5). Possible causes of liver function disorders during COVID-19 include direct damage by the virus itself, a severe inflammatory response in the sense of the appearance of a cytokine storm, damage due to hypoxia, the occurrence of microthrombosis of the sinusoid of the liver resulting from hypercoagulability of the blood, the toxic effect of drugs administered during treatment or a combination of several associated factors (6,7). Coagulation disorders and microthrombosis, that are commonly seen in COVID-19, may compromise liver perfusion, leading to hepatocyte damage and increased AST levels (8,9). Cholangiocyte damage index alkaline phosphatase is less susceptible to major changes in COVID-19, whereas aminotransferases and gamma-glutamyltransferase, as indicators of hepatocyte damage, were more commonly elevated and almost always changed in severe patients (10,11).

In pathohistological liver samples, the most common findings were preserved biliary and vascular elements without significant damage, along with pronounced Kupffer cell activation and marked regenerative changes in hepatocytes. No obvious viral inclusions were identified in liver tissue (12-14).

Several risk factors are associated with a more severe clinical course of COVID-19, the most prominent being male gender, older age, and the presence of previous comorbidities. Epidemiological data indicate that approximately 75% of hospitalized patients have at least one chronic disease, among which the most common are hypertension, diabetes, malignancies, neurodegenerative and cardiovascular diseases, obesity and kidney diseases (15). The frequency of individual comorbidities is further supported by findings indicating that arterial hypertension (15.8%), cardiovascular and cerebrovascular diseases (11.7%), and diabetes (9.4%) are the most frequently diagnosed comorbidities (16,17). However, little is known about the prevalence and pattern of liver laboratory abnormalities in moderately severe, non-ICU patients at the time of admission.

The study aimed to determine the prevalence of altered liver biomarkers (ALT, AST, LDH, GGT, and albumin) in COVID-19 patients with a moderately severe clinical picture on admission for hospital treatment, and to assess the presence of biochemical abnormalities potentially associated with hepatic involvement in the absence of a severe clinical presentation.

METHODS

The study included medical histories of patients with COVID-19 who were hospitalized at the Clinic for Infectious Diseases of the University Clinical Centre Niš from March to November 2020. A total of 655 patients' medical histories were analyzed, of whom 425 were male, and 230 were female. All patients presented with a moderately severe clinical picture.

For the study, laboratory findings of liver parameters of non-ICU patients with a moderately severe clinical picture of COVID-19 were collected. According to the national guide for the treatment of COVID-19 of the Republic of Serbia, a moderately severe clinical picture of COVID-19 in patients without comorbidities is defined by the development of hypoxia (saturation <90% on room air), rapid breathing with more than 30 breaths per minute, persistent fever and radiographic changes in the lungs that involve more than 50% of the parenchyma (or corresponding findings on CT). In patients with comorbidities, a moderately severe form of the disease was defined by the following parameters: signs of lower respiratory tract disease and oxygen saturation between 90% and 94%. In all patients, the nasopharyngeal swab for SARS-CoV-2 (PCR or antigen test) was positive.

The most common comorbidities associated with COVID-19, according to the literature, were arterial hypertension (HTA) and diabetes mellitus (DM). HTA and DM were therefore selected because they were the most prevalent comorbidities in our study population and had sufficient sample sizes to allow statistical comparisons, while other chronic conditions were either infrequent or heterogeneous and were therefore excluded from the study.

All patients were classified into three groups: patients without comorbidities (NC), patients with arterial hypertension (HTA), and patients with previously diagnosed diabetes mellitus (DM). Additionally, within each research group, patients were further stratified into three age categories: 20 to 40 years, 40

Table 1. *Classification of patients by research group, age and sex*

	NC			HTA		DM	
Age (in years)	20-40	40-60	60+	40-60	60+	40-60	60+
Male	43	114	84	38	77	18	51
Female	28	37	55	22	53	3	32
Total	71	151	139	60	130	21	83

NC – no comorbidities, HTA – arterial hypertension, DM – diabetes mellitus.

to 60 years, and over 60 years. Patients aged 20–40 years with comorbidities were not included due to their small number. The distribution of patients by age, sex, and comorbidity group is presented in Table 1.

Exclusion criteria for the study were: intensive care unit patients, previously diagnosed liver conditions, vascular and autoimmune disorders, previous hepatobiliary surgeries, and chronic conditions other than arterial hypertension and diabetes mellitus.

Data selection and statistical analysis

In this study, we analyzed the levels of the following liver function biomarkers in patients with COVID-19: ALT (alanine aminotransferase), AST (aspartate aminotransferase), LDH (lactate dehydrogenase), GGT (gamma-glutamyl transferase), and albumin. Biomarker values obtained upon patient admission to the Clinic for Infectious Diseases at the University Clinical Centre Niš were used to determine the percentage of patients in different age and comorbidity groups who had already developed COVID-19-related biochemical abnormalities potentially associated with hepatic involvement. All blood analyses of the patients included in the study were performed in the Central Laboratory of the University Clinical Centre Niš. For the examined biomarkers, the reference ranges were: ALT - 10-35 U/L, AST - 10-31 U/L, LDH - 220-450 U/L, GGT - 0-38 U/L, albumin - 62-81 g/L.

Descriptive and χ^2 analyses

Gender structure analyses were performed using a Pearson's Chi-square (χ^2) test. Initially, we compared the gender distribution of men versus women in the clinical groups (NC, HTA, and DM), and then we performed separate χ^2 tests in each group to evaluate the changes in the gender distribution by age group (20–40, 40–60, and >60 years).

The χ^2 test was also applied to evaluate differences in the prevalence of elevated liver enzyme levels (AST, ALT, LDH, GGT) between groups defined by age and comorbidity status, and to assess their association with arterial hypertension and diabetes mellitus across age categories.

Logistic regression

A binary variable (normal vs. elevated) was created for each enzyme based on standard clinical reference values. Binary logistic regression was conducted to assess the association of defined groups with elevated values of individual enzymes. A separate model was built for each enzyme, with the group included as an independent variable with seven levels. The NC 20–40 group was chosen as the reference category because it represents the least metabolically affected population and enables consistent comparison with older and comorbid groups. Results are presented as odds ratios (OR) with 95% confidence intervals (CI), both in tabular form and as a forest plot using a logarithmic scale.

All statistical tests were performed using R software (R Core Team, 2026) within RStudio (RStudio Team, 2026). Statistical significance was defined as $p < 0.05$.

This study was approved by the Ethical Board of the University Clinical Centre Niš, Republic of Serbia, number: 34794/3.

RESULTS

This study comprised the analysis of liver biomarkers in 655 patients upon admission to the Clinic for Infectious Diseases of the University Clinical Centre Niš.

Our results show that, in the total sample, men were overrepresented compared with women ($p < 0.0001$). Moreover, the gender distribution was statistically similar

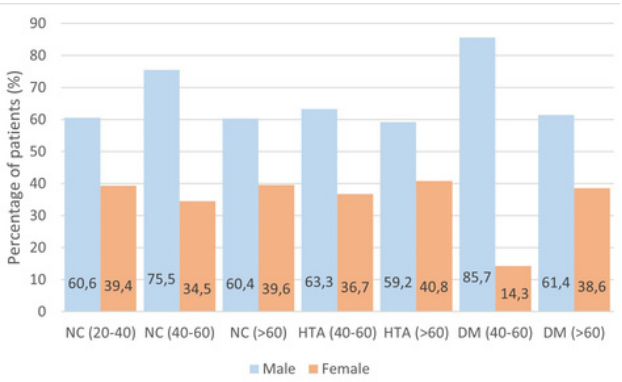


Figure 1. *Distribution of male and female patients per percentage across clinical groups and age categories. Bar chart showing the number of male (blue) and female (orange) patients within each age category (20–40, 40–60, >60 years) across the NC, HTA and DM groups.*

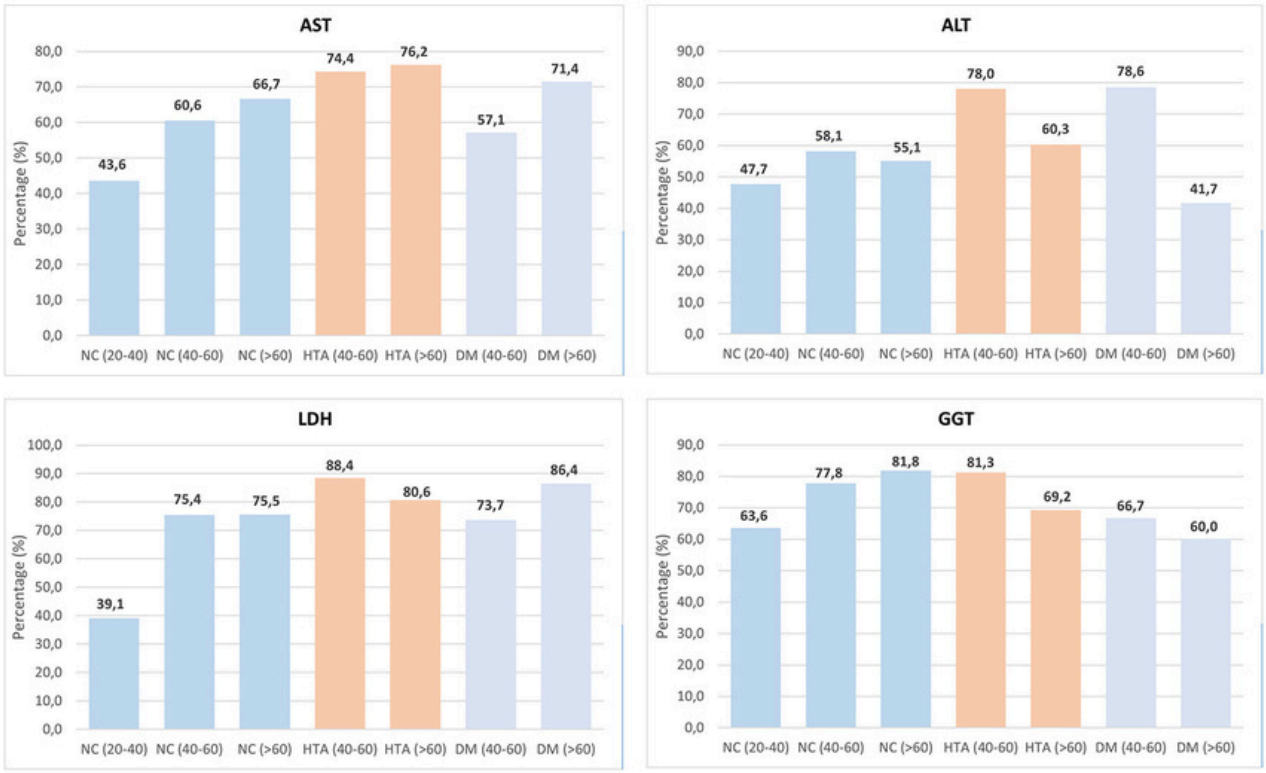


Figure 2. *Percentage of patients with elevated biomarker values in clinical (NC, HTA, DM) and age subgroups. The bar graph shows the percentage of patients with values above the reference range for each subgroup.*

across the clinical groups (χ^2 = 2.24, df = 2, p = 0.33), with a predominance of male patients in the NC, HTA, and DM groups. In the analysis by age categories, however, in the NC (χ^2 = 8.93, df = 2, p = 0.012) and DM (χ^2 = 6.94, df = 1, p = 0.008) groups, a statistically significant difference can be observed in the gender distribution between the age categories, but this is not observed in the HTA group (χ^2 = 0.004, df = 1 p = 0.95) (Figure 1).

The percentage of patients with elevated values of each examined biomarker per group is presented in Figure 2.

In the observed cohort, differences in the prevalence of elevated AST, ALT, and LDH values were observed between the defined groups, whereas no such differences were observed for GGT. The global χ^2 test showed statistically significant differences for AST (χ^2 = 22.9, p = 0.0008), ALT (χ^2 = 14.7, p = 0.022) and LDH (χ^2 = 34.6, p < 0.00001), while no significant difference was observed between groups for GGT (χ^2 = 4.9, p = 0.55).

For each enzyme, a binary logistic regression was performed using seven age-comorbidity categories, with the NC 20–40 group as the reference. Results are presented as odds ratios (ORs) with 95% confidence intervals (Figure 3, Tables 2 and 3).

Table 2. *Odds ratios (OR) with 95% CI for elevated AST and ALT across age- and comorbidity-defined groups, using NC 20–40 as the reference category*

Group	AST OR (95% CI)	p	ALT OR (95% CI)	p
NC 40–60	1.99 (0.95–4.24)	0.067	1.52 (0.75–3.10)	0.245
NC >60	2.59 (1.20–5.69)	0.015	1.34 (0.65–2.78)	0.42
HTA 40–60	3.75 (1.47–10.11)	0.006	3.89 (1.55–10.44)	0.004
HTA >60	4.14 (1.87–9.45)	<0.001	1.24 (0.59–2.58)	0.56
DM 40–60	1.73 (0.51–6.16)	0.38	4.02 (1.08–19.59)	0.047
DM >60	3.24 (1.39–7.78)	0.007	0.78 (0.36–1.71)	0.54

Table 3. *Odds ratios (OR) with 95% CI for elevated LDH and GGT across age- and comorbidity-defined groups, using NC 20–40 as the reference category*

Group	LDH OR (95% CI)	p	GGT OR (95% CI)	p
NC 40–60	4.77 (2.34–10.02)	<0.001	2.00 (0.45–8.13)	0.36
NC >60	4.79 (2.30–10.26)	<0.001	3.43 (0.61–21.54)	0.16
HTA 40–60	11.82 (4.20–39.52)	<0.001	2.48 (0.43–15.86)	0.31
HTA >60	6.47 (3.02–14.34)	<0.001	1.29 (0.27–5.63)	0.76
DM 40–60	4.36 (1.41–15.44)	0.013	1.14 (0.20–6.62)	0.88
DM >60	9.92 (3.98–27.13)	<0.001	0.86 (0.16–4.27)	0.85

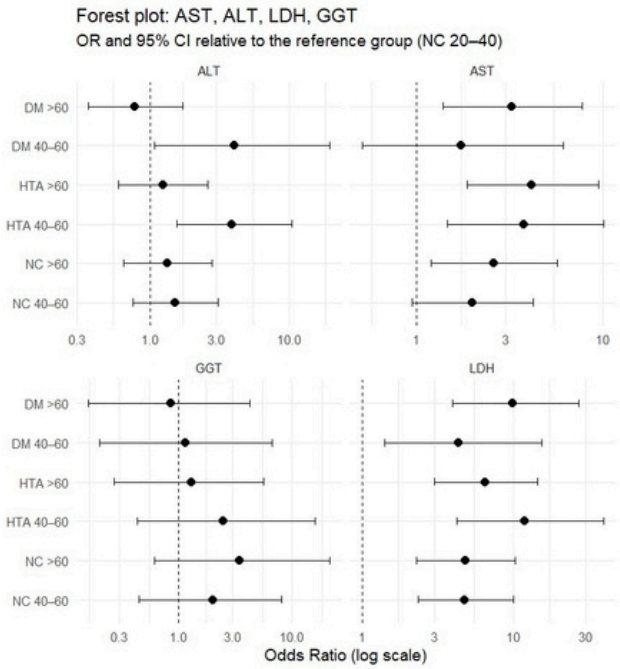


Figure 3. *Forest plot of odds ratios for elevated liver biomarkers (ALT, AST, LDH, GGT) across age- and comorbidity-defined groups. Forest plot showing odds ratios (OR) with 95% confidence intervals for elevated ALT, AST, LDH, and GGT levels in patients with arterial hypertension (HTA) and diabetes mellitus (DM) across two age categories (40–60 and >60 years), as well as in older NC groups (40–60 and >60 years), compared with the reference group (NC 20–40). OR values are displayed on a logarithmic scale.*

In AST, older NC categories, as well as all HTA and DM groups, had higher odds of elevated values than the reference group. The most pronounced risk was observed in the groups HTA 40–60 (OR 3.75, 95% CI 1.47–10.11) and HTA >60 (OR 4.14, 95% CI 1.87–9.45), while the group DM >60 also had significantly increased odds (OR 3.24, 95% CI 1.39–7.78).

For ALT, the groups HTA 40–60 (OR 3.89, 95% CI 1.55–10.44) and DM 40–60 (OR 4.02, 95% CI 1.08–19.59) had statistically significantly increased prospects, while the other categories did not show significant differences compared to NC 20–40.

LDH showed the greatest differences among the groups. All groups older than the reference (NC 20–40), including those with arterial hypertension and diabetes mellitus, had significantly increased odds of elevated LDH values. The highest risk was observed in the HTA 40–60 group (OR 11.82, 95% CI 4.20–39.52), while the DM >60 group also had significantly elevated odds (OR 9.92, 95% CI 3.98–27.13). Older NC categories showed a moderate but statistically significant increase in risk (OR 4.77–4.79). These findings indicate that LDH is the most sensitive marker among the analyzed enzymes.

For GGT, no statistically significant difference was found between age-comorbidity categories and elevated enzyme values. All OR estimates had wide confidence intervals that included 1, indicating no clear effect.

DISCUSSION

The results of our research show that hepatic biochemical markers—primarily AST, ALT, LDH, and GGT—are elevated in a significant number of patients with COVID-19 upon admission to the hospital, even in the absence of a severe clinical picture and without the need for intensive care. This finding is consistent with previous studies indicating that liver biochemical abnormalities occur in approximately 15-53% of hospitalized patients (5). The exact pathophysiological mechanisms of abnormal liver biochemistry in COVID-19 are still not completely elucidated, however, it is considered that the contributing factors might be the direct cytopathic effect of the virus, hypoxia, immunologically mediated damage, iatrogenic causes such as drugs and ventilation, as well as the exacerbation of underlying liver disease (6,18,19).

The AST and ALT levels were most frequently elevated in older patients and in those with hypertension or diabetes. These findings are consistent with reports that metabolic comorbidities, especially diabetes and hypertension, are associated with an increased risk of altered liver-related biomarkers during COVID-19 (20-23). Younger patients without comorbidities had the lowest prevalence of elevated transaminase values, suggesting that age-related metabolic and inflammatory changes play an important role in early liver involvement. Our findings indicate that a significant number of non-ICU patients with moderately severe COVID-19 have increased levels of aminotransferases upon admission to the hospital. Previous studies report a wide range of aminotransferase abnormalities (3.8–80%) (21,24–29), and that the degree of biochemical abnormalities potentially associated with hepatic involvement correlates with the severity of the clinical picture (30–32).

It is important to note that none of the patients in our study had decreased albumin levels, which is expected, given that all were non-ICU patients evaluated upon admission. Hypoalbuminemia is strongly associated with severe and fatal outcomes in the literature, but usually occurs later during hospitalization, which might explain the absence of this finding in our population (33-36).

LDH emerged as the most sensitive biomarker in this study, with prevalence ranging from 39.1% to as high as 88.4% across subgroups. This finding is consistent with meta-analyses that identify LDH as one of the most reliable predictors of disease severity and mortality (32,37). Its elevated values across nearly all older groups and among those with comorbidities are consistent with studies identifying LDH as an indicator of hepatocellular damage, systemic inflammation, hypoxia, and damage to other tissues, which are characteristic of COVID-19 (38,39). Multiple studies have found LDH to be a predictor of worse outcomes in hospitalized patients (40,41). It is particularly significant that the highest LDH values were observed in groups with comorbidities (HTA and DM), which is consistent with findings that patients with metabolic diseases exhibit a more pronounced inflammatory response and a more severe course of COVID-19 infection (38).

In our study, GGT was elevated in most patients (60–81,8%), but there was no clear association with age or comorbidities. This finding is consistent with the literature indicating that GGT may be elevated in patients with COVID-19, but its interpretation is complex, as it may also reflect drug-induced hepatotoxicity, inflammation, or cholangiocellular stress (21,29). Increased GGT levels were found in up to 72% of patients with a severe clinical picture (42,43). Given that our patients were evaluated on admission, before the administration of therapy, it is more likely that elevated GGT levels reflect direct or indirect effects of SARS-CoV-2 on the liver. ACE2 receptors are highly expressed in cholangiocytes, which may explain the relatively high prevalence of elevated GGT (29,44).

Logistic regression additionally confirmed that older age and the presence of hypertension or diabetes increase the likelihood of elevated AST and LDH values. The greatest effects were recorded in the HTA 40-60 and DM >60 groups. ALT showed a more selective pattern, with a significant increase only in the HTA 40-60 and DM 40-60 groups. In contrast to these markers, GGT did not show an independent association with age or comorbidities, indicating that its increase probably depends on other factors.

Although our results show that LDH was the most sensitive and consistent indicator in our study, it is important to emphasize that, unlike aminotransferases, LDH is not liver-specific and is released from a wide range of tissues. Therefore, the marked elevation of LDH observed in our cohort, particularly among older and comorbid patients, should not be interpreted as direct evidence of hepatocellular damage, but rather as a reflection of the overall systemic inflammatory state, tissue hypoxia, and disease severity characteristic of COVID-19 (37-39). Consequently, while LDH proved to be the most sensitive biomarker in our analysis, its elevation likely reflects a composite of pulmonary, cardiac, and systemic inflammatory injury in addition to any hepatic component and should be interpreted with this limitation in mind.

Different studies indicate that the most common comorbidities associated with hospitalized COVID-19 patients were arterial hypertension and diabetes mellitus (45). Our results clearly show that elderly patients and patients with comorbidities had a higher percentage of elevated liver biomarkers. This is in line with a large number of studies that indicate that hypertension and diabetes might significantly increase the risk of a more severe form of COVID-19 and of altered liver-related biomarkers (45-49). It is particularly interesting that patients without comorbidities in the 20-40-year age group had the lowest prevalence of elevated biomarkers, except for GGT, which confirms that the younger population without chronic diseases is less likely to present with biochemical signs of hepatic involvement on admission. Although elevated liver biomarkers are usually associated with a severe clinical picture and worse disease progression, some reports indicate that liver enzyme levels do not differ significantly between the mild/moderately severe and severe clinical pictures of COVID-19 (50).

Several biological mechanisms could contribute to the more pronounced hepatic biomarker alterations observed in older and comorbid patients (18,19). Aging has been associated with reduced hepatic blood flow, decreased hepatocyte regenerative capacity, and a degree of chronic low-grade inflammation, which could lower the threshold for liver involvement during an acute infection with COVID-19, which is in line with the generally more severe clinical course reported in elderly patients (46,50). In patients with arterial hypertension, pre-existing endothelial dysfunction and altered microvascular regulation may increase the susceptibility of the liver sinusoids to hypoperfusion or microthrombosis during the hypercoagulable state associated with COVID-19 (9,45). Similarly, in patients with diabetes mellitus, underlying insulin resistance and a heightened baseline inflammatory state may act together with the COVID-19-related immune response to increase hepatocellular vulnerability (23,47). These age- and comorbidity-related factors might act together with the direct and indirect hepatic effects of SARS-CoV-2 to produce the more pronounced biomarker elevations observed in these subgroups.

Overall, our results show that altered liver-related biomarkersare present even in COVID-19 patients with a moderately severe form of disease at the time of admission. This suggests that hepatic involvement may occur early and should not be interpreted exclusively as a marker of advanced disease. Routine assessment of liver biomarkers, particularly LDH and AST, may help identify patients at increased risk of clinical deterioration.

The key strength of our study is that all biomarker measurements were obtained at the time of hospital admission, before the initiation of any COVID-19-specific hospital therapy, allowing the observed alterations in AST, ALT, LDH, and GGT to be attributed more confidently to the disease process itself rather than to therapeutic interventions. However, this cross-sectional design at a single time point also represents a limitation, since we did not assess the potential influence of subsequently administered therapy on liver biomarkers, nor did we follow the evolution of these biomarkers or of the clinical picture throughout hospitalization. In addition, our findings are limited to non-ICU patients with a moderately severe clinical presentation and therefore cannot be directly extrapolated to patients with severe or critical disease.

Our results confirm that biochemical abnormalities of the liver are common in patients with moderately severe COVID-19 already on admission. The most pronounced changes were observed in elderly patients and those with hypertension and diabetes, which is in accordance with the existing literature. Our results indicate that AST and ALT are primarily associated with comorbidities, LDH predominantly with age, while GGT shows a specific pattern that requires additional research. These findings emphasize the importance of early liver function screening even in patients who do not yet present with a severe clinical picture. Routine monitoring of these biomarkers could provide insight into the underlying inflammatory state and guide more personalized therapeutic approaches.

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Author Contributions

Conceptualization, A.R. and V.P.; Methodology, A.R. and B.K.; Formal analysis, A.R., V.P., and B.K.; Data curation, M.C., M.Ć.P., L.P.D., J.S., and N.M.; Writing—original draft preparation, A.R. and V.P.; Writing—review and editing, G.R. and L.P.D. All authors have read and agreed to the published version of the manuscript.

Statement of Ethics

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University Clinical Centre Niš, Republic of Serbia (Approval No. 34794/3, dated October 1, 2019). The Ethics Committee waived the informed consent requirement because we analyzed only anonymized biochemical data, devoid of personal or identifiable patient information.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availability

The data analyzed during this study are included in the published article. Additional data are available from the corresponding author upon reasonable request.

Statement of Generative AI Use

No generative AI was used.

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ORIGINAL ARTICLE

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EFFECT OF HALOGENATED BOROXINE ON HEMATOLOGICAL PARAMETERS IN RATS AND ON CREATINE KINASE ACTIVITY *IN VITRO*

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Halogenated boroxine is an important enzyme inhibitor with potential applications in cancer therapy. This study aimed to evaluate its effects on hematological parameters in rats *in vivo* and creatine kinase activity *in vitro*. Halogenated boroxine, administered either intraperitoneally (IP) or orally, induced route-dependent changes in hematological parameters compared with controls. IP administration significantly increased neutrophils, mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), while decreasing hematocrit (HCT). In contrast, oral administration significantly decreased lymphocytes and increased basophils, while other hematological parameters remained unchanged. These effects were more pronounced following IP administration. *In vitro*, halogenated boroxine exhibited competitive inhibition of creatine kinase without pre-incubation, whereas after a 30-minute pre-incubation, the inhibition pattern indicated a non-competitive mechanism. Further analysis showed a time-dependent shift in creatine kinase inhibition mechanism following pre-incubation, characterized by a decrease in maximum reaction velocity (V_{max}), while the Michaelis constant (K_m) remained unaltered. These findings show that halogenated boroxine modulates specific hematological parameters and inhibits creatine kinase activity, with effects depending on the route of administration and experimental conditions.

Keywords: halogenated boroxine, hematological parameters, spectrophotometry, creatine kinase, inhibition

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INTRODUCTION

Boroxins, which are cyclic oxides of boron, represent a class of compounds characterized by a stable core structure and the ability to form various coordination complexes. Their chemical modification, particularly through halogenation, changes their electronic and steric properties, often resulting in new biological activities. Consequently, boron-containing compounds have attracted considerable attention in biomedical research because of their antimicrobial, enzyme-inhibitory, and antitumor properties (1,2). Halogenated boroxine $K_2[B_3O_3F_4OH]$ is a specific boron-containing compound with pronounced antitumor potential that has been patented as a therapeutic agent for benign and malignant skin lesions (Figure 1). Previous studies have demonstrated significant antiproliferative effects of halogenated boroxine (HB) across several tumor cell lines, as well as distinct antitumor activity in experimental animal models. Notably, these effects are more pronounced in tumor cells than in normal cells. Recent investigations have shown that halogenated boroxine induces deregulation of genes involved in apoptosis and autophagy, thereby contributing to its selective antitumor activity. The core structure of boroxine, consisting of three boron atoms interconnected through oxygen bridges with fluorine substitutions on the ring, provides both the structural stability and chemical and biological reactivity necessary to interact in different cellular systems (3,4).

Evaluation of hematological parameters in experimental animals is a standard approach in toxicological and pharmacological research. Hematological indices, including erythrocyte, leukocyte, and platelet counts, together with hemoglobin concentration and hematocrit values, are sensitive indicators of systemic toxicity, inflammatory processes, immune responses, and bone marrow function. Previous studies have demonstrated that halogenated boroxine exhibits selective genotoxic and cytotoxic effects in malignant hematopoietic and tumor cells, whereas normal peripheral blood cells show substantially lower sensitivity. These findings suggest the potential utility of halogenated boroxine in treating hematological malignancies, while emphasizing the necessity of further *in vivo* safety evaluations. Although numerous studies have investigated the antitumor activity and toxicity profile of halogenated boroxine, data concerning its influence on hematological parameters *in vivo* remain scarce (3,5-7).

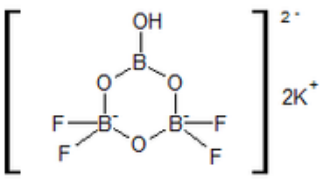


Figure 1. *Structure of dipotassium trioxohydroxytetrafluorotriborate, $K_2[B_3O_3F_4OH]$*

Since *in vitro* studies cannot fully reflect the complex interactions occurring within a living organism, an *in vivo* assessment of hematological parameters was included to evaluate the systemic biological effects of halogenated boroxine (HB). The selected parameters included white blood cell count (WBC), lymphocyte count (LYM) and granulocyte count (GRAN) as indicators of immune status and inflammatory response; platelet count (PLT) as a marker of coagulation and bone marrow activity; red blood cell count (RBC), hemoglobin concentration (Hb), and hematocrit value (HCT) as indicators of oxygen transport capacity; and mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) as indices of erythrocyte morphology and quality. These parameters provide a broader and more clinically relevant assessment of biological activity than enzyme testing alone (6).

In addition to hematological evaluation, investigation of the effects of halogenated boroxine on creatine kinase (CK) activity provides insight into its interaction with enzyme systems. Boron-containing compounds are known to act as enzyme inhibitors due to their ability to interact with nucleophilic groups and metal-containing active sites. Notably, $K_2[B_3O_3F_4OH]$ has previously been identified as a potent inhibitor of several human carbonic anhydrase isoenzymes, suggesting that its biological effects may partly arise from enzyme modulation. Therefore, it is reasonable to hypothesize that halogenated boroxine may also interact with other enzymes, including creatine kinase (2). Creatine kinase is a key enzyme in cellular energy metabolism responsible for maintaining adenosine triphosphate (ATP) homeostasis in tissues with high energy demand, particularly skeletal muscle. Elevated CK activity in serum is widely recognized as a biomarker of muscle injury, cellular damage, and drug-induced toxicity. Changes in CK activity may therefore indicate direct interactions between halogenated boroxine and cellular enzyme systems or reveal potential cytotoxic effects. Through spectrophotometric analysis, this study seeks to clarify the possible inhibitory action of halogenated boroxine on creatine kinase activity and to characterize the type of enzyme inhibition involved (8).

The study aimed to investigate the effects of dipotassium trioxohydroxytetrafluorotriborate, $K_2[B_3O_3F_4OH]$, on hematological parameters following intraperitoneal and oral administration, as well as its effect on creatine kinase activity *in vitro*. The obtained results may contribute to a better understanding of the biological relevance, safety profile, and potential therapeutic applications of this boron-containing compound in medicine and pharmacy.

METHODS

Our *in vivo* studies were done on a total of 24 Wistar rats bred in the vivarium of the Department of Biology, Faculty of Science, University of Sarajevo. Housing conditions met standard regulatory criteria for experimental animals: temperatures of 20–24 °C, a standard light/dark cycle, and isolation from excessive noise. The animals were housed in stainless steel cages featuring solid, transparent, hard-plastic floors covered with suitable wood litter and equipped with a feeder and a waterer providing food and water ad libitum. Following acclimatization, 90-day-old rats weighing 180 ±12.31 g were randomly assigned to three groups: a control group (n = 6) and two experimental treatment groups (n = 9 each). Halogenated boroxine was dissolved in redistilled water and administered at a dose of 10 mg/kg of body weight. The first experimental group received halogenated boroxine intraperitoneally for 5 days at the same time, while the second experimental group received it orally (*per os*) for five days at the same time. Intraperitoneal application of the appropriate dose of halogenated boroxine was performed once daily for 5 days at a consistent time using a syringe and needle. The second experimental group received the same dose orally (*per os*) once daily for 5 days using a metal gavage probe with a rounded tip to minimize the risk of esophageal injury. Control animals were maintained under the same housing and dietary conditions as the experimental groups and received only an equivalent amount of redistilled water for 5 days without halogenated boroxine.

Animals in the control and experimental groups were of the same age. The control group provided baseline values for the examined hematological parameters, which were compared against the values from both experimental groups. Dosing criteria and exposure times were determined based on previous research by Haverić *et al*. (9). All animal care procedures adhered to the Principles of Laboratory Animal Care, established by the Universal Declaration of Animal Welfare, the UNESCO Declaration on the Rights of Animals, and the European Parliament's Directive 2010/63/EU on the protection of animals used for scientific purposes. This *in vivo* research was governed by the Law on the Protection and Welfare of Animals (Official Gazette of BiH, No. 25/2009 and 9/2018) and approved by the Ethics Committee of the Faculty of Science, University of Sarajevo (Approval No. 01/01-1945/2-2025, issued on October 23, 2025).

Halogenated boroxine, $K_2[B_3O_3F_4OH]$, is a water-soluble, white, inorganic boron powder (7.1% solubility at 20 °C) with a purity of 99.99%. It was synthesized by reacting potassium hydrofluoride (KHF_2) with boric acid at a 2:3 molar ratio at room temperature, as reported in the literature (10).

Before a cardiac puncture, an intraperitoneal dosage of xylazine (20 mg/kg) and ketamine (100 mg/kg) was administered, and a total of 4 mL of blood was taken 24 hours after the treatment. Blood sampling was performed as a terminal procedure. The collected blood was immediately mixed with EDTA for hematological analysis. The examined hematological parameters included WBC, LYM, GRAN, PLT, RBC, Hb, HCT, MCV, MCH, and MCHC. These parameters were determined using a Mythic 5VET PRO hematology analyzer, which utilizes multi-dimensional laser light cell classification for WBC differentials, impedance for WBC, RBC, and PLT counts, and spectrophotometry for Hb determination.

For *in vitro* studies, a spectrophotometric method was used with measurements made at 340 nm and a temperature of 37 °C, using a commercial assay kit (Creatine Kinase Activity Assay Kit, MAK116; Sigma-Aldrich, USA) according to the manufacturer's instructions. The concentration of halogenated boroxine used was 10 mg/mL (11).

Statistical methods

Data were analyzed using descriptive statistics and presented as mean ± standard deviation ($\bar{X}$ ± SD). Differences between groups were analyzed using Student's t-test for independent samples and one-way ANOVA. Comparisons were made between the control group and each experimental group, as well as between the two experimental groups. Differences were considered statistically significant at $p < 0.05$. Statistical analysis was performed using SPSS software.

RESULTS

In vivo

The control group consisted of 6 rats, and their basic hematological parameters and differential blood count were analyzed. Individual values, calculated mean values ($\bar{X}$), and standard deviations (SD) for the analyzed parameters are shown in Table 1. The parameters evaluated included total leukocyte count (WBC), differential leukocyte formulas (LYM%, MON%, NEU%, EOS%, BASO%), erythrocyte indicators (RBC, Hb, HCT, MCV, MCH, MCHC), and platelet count (PLT). The average value of leukocyte count (WBC) in the control group was 6.95 × 10^9/L, with a standard deviation of 1.66 × 10^9/L. Within the differential leukocyte formula, the average values were: lymphocytes 58.68%, monocytes 14.49%, neutrophils 22.69%, eosinophils 4.08%, and basophils 0.05%. Erythrocyte parameters of the control group showed an average erythrocyte count (RBC) of 8.30 ×10^{12}/L. The average hemoglobin concentration (Hb) was 147.90 g/L, while the hematocrit (HCT) value was 0.43 L/L. The erythrocyte indices values were: MCV 51.85 fL, MCH 17.91 pg, and MCHC 347.07 g/L. The average platelet count (PLT) in the control group was 413.00 ×10^9/L. All obtained control values were consistent with established baseline reference intervals for Wistar rats shown in Table 2 (12,13). The greatest variability between individual animals was recorded in platelet values (SD = 100.88 ×10^9/L), MCV (SD = 5.06 fL), total leukocyte count (SD = 1.66 ×10^9/L), and erythrocyte count (SD = 1.01 ×10^{12}/L). In contrast, smaller variations were recorded in the differential leukocyte formula parameters, especially basophil values. Overall, the control group established a baseline range of individual values, mean values, and standard deviations for all analyzed hematological indicators.

The first experimental group consisted of 9 Wistar rats that received halogenated boroxine intraperitoneally at a dosage of 10 mg/kg of body weight for five consecutive days. The results of hematological analyses are shown in Table 3. Analysis of the hematological parameters indicated that certain values deviated from the control. The average WBC value in the IP group was lower than that of the control group, but this difference was not statistically significant ($p > 0.05$). Analysis of the differential leukocyte formula showed a statistically significant increase in the proportion of neutrophils in the IP group compared to the control (28.62 ± 2.33% vs. 22.69 ± 4.87%, $p < 0.05$). Lymphocyte, monocyte, eosinophil, and basophil values did not exhibit statistically significant differences compared to the control group. Regarding erythrocyte parameters, both the erythrocyte count and hemoglobin concentration were slightly lower in the IP group, but these differences were not statistically significant ($p > 0.05$). However, the hematocrit value was significantly lower compared to the control group (0.36 ± 0.07 vs. 0.43 ± 0.03 L/L, $p < 0.05$). The value of MCV showed no significant changes, while the values of MCH and MCHC were significantly increased in the IP group ($p < 0.05$). The platelet count was slightly higher in the IP group compared to the control, but this difference was not statistically significant ($p > 0.05$).

Table 1. *Values of hematological parameters of the control group without halogenated boroxine*

	WBC 10^9/L	LYM%	MON%	NEU%	EOS%	BASO%	RBC10^{12}/L	Hb g/L	HCT L/L	MCV fL	MCH pg	MCHC g/L	PLT10^9/L
Countrol group	3.94	60.09	17.37	13.16	9.38	0	8.25	152	0.457	55.5	18.4	332	236
	8.8	53.53	17.05	26.89	2.39	0.14	10.28	157	0.437	42.6	15.2	359	494
	7.13	60.12	13.46	23.23	3.03	0.16	7.75	141	0.395	51	18.1	356	524
	6.56	58.4	13.9	23.34	436	0	7.98	146	0.41	51.4	1,829	356	423
	7.38	58.75	12.74	25.38	3.11	0	7.43	143	0.4	53.83	19.24	357.5	389
	7.88	61.24	12.41	24.13	2.22	0	8.13	148.4	0.461	56.76	18.25	321.9	412
$\bar{X}$	6.95	58.68	14.49	22.69	4.08	0.05	8.3	147.9	0.43	51.85	17.91	347.07	413
Sd	1.66	2.73	2.17	4.87	2.7	0.08	1.01	5.92	0.03	5.06	1.39	15.95	100.88

Table 2. *Reference values for hematological parameters of Wistar rats (12,13)*

WBC 10^9/L	LYM %	MON %	NEU %	EOS %	BASO %	RBC 10^{12}/L	Hb g/L	HCT %	MCV fL	MCH pg	MCHC g/L	PLT 10^9/L
3.7-5.8	54.9-65.3	7.0-8.3	23.4-40.5	0.3-3.4	0–0.8	6.1–8.5	118-162	32.6-46	51-62	17.7-20.0	327-362	315-512

Table 3. *Hematological parameters of experimental group 1 which received halogenated boroxine intraperitoneally (IP) 10 mg/kg*

	WBC 10^9/L	LYM%	MON%	NEU%	EOS%	BASO%	RBC 10^{12}/L	Hb g/L	HCT L/L	MCV fL	MCH pg	MCHC g/L	PLT 10^9/L
Experimental group 1 (IP)	2.79	55.71	8.13	28.69	7.47	0	3.65	75	0.186	51.2	20.5	403	459
	5.95	66.59	0.67	27.48	5.26	0	6.95	135	0.367	52.9	19.4	367	467
	4.76	41.82	22.6	28.75	6.51	0.32	7.82	147	0.423	54.1	18.7	347	469
	7.82	45.51	20.9	25.94	7.61	0.04	7.27	134	0.368	50.7	18.4	364	487
	5.21	49.91	13.49	33.1	3.09	0.41	7.36	137	0.385	52.4	18.6	355	461
	6.11	56.34	14.61	24.59	4.46	0	6.56	134	0.37	52.9	19.4	367	455
	5.84	48.78	15.98	30.48	4.45	0.31	6.24	131	0.31	49.6	20.99	422.5	463
	4.87	55.17	10.18	29.87	4.54	0.24	7.23	140	0.35	49.79	19.36	400	424
	5.12	56.22	10.88	26.31	6.45	0.14	7.78	142	0.38	48.88	18.25	373.68	476
$\bar{X}$	5.66	52.78	11.94	28.62	5.6	0.16	7.03	134.3	0.36	51.9	19.6	378.9	465.4
Sd	1.47	7.46	6.93	2.33	1.47	0.14	1.42	22.1	0.07	1.7	0.8	19.7	19.6

The second experimental group consisted of 9 male Wistar rats to which halogenated boroxine was administered orally (*per os*) at a dose of 10 mg/kg of body weight for 5 consecutive days. The obtained hematological parameters are presented in Table 4.

The average value of WBC in the OG group was lower compared to the control group, but the difference was not statistically significant ($p > 0.05$). Analysis of the differential leukocyte formula showed a statistically significant decrease in the percentage of lymphocytes compared to the control group (53.58 ± 4.63% vs. 58.68 ± 2.73%, $p < 0.05$). The proportion of monocytes and neutrophils did not show statistically significant differences compared to the control group ($p > 0.05$). A statistically significant increase in basophils was recorded in the OG group compared to the control group (0.50 ± 0.25% vs. 0.05 ± 0.08%, $p < 0.05$) and compared to the IP group ($p < 0.05$). Eosinophil values showed individual variations, but without statistically significant differences ($p > 0.05$). Erythrocyte parameters did not show statistically significant differences compared to the control group. The RBC count, Hb concentration, and HCT values remained relatively preserved. Erythrocyte indices (MCV, MCH and MCHC) did not show significant changes. The platelet count showed an increase in mean value in the OG group compared to the control, but the difference was not statistically significant ($p > 0.05$).

Comparative statistical analysis given in Table 5 revealed that halogenated boroxine administration induced selective changes in certain hematological parameters, highly dependent upon the route of administration.

The total leukocyte count (WBC) remained statistically unaltered among the control, intraperitoneal (IP), and oral (OG) groups ($p > 0.05$). However, differences were observed in the differential leukocyte formula. The IP group showed a significant elevation in neutrophil percentage relative to the control group (28.62 ± 2.33% vs. 22.69 ± 4.87%, $p < 0.05$). Conversely, the OG group showed a significant decline in lymphocyte percentage compared to controls (53.58 ± 4.63% vs. 58.68 ± 2.73%, $p < 0.05$), alongside a significant increase in basophil proportions compared to both the control and IP cohorts ($p < 0.05$). IP administration significantly reduced hematocrit (HCT) levels compared to the control group (0.36 ± 0.07 vs. 0.43 ± 0.03 L/L, $p < 0.05$) while significantly elevating MCH and MCHC values ($p< 0.05$). No statistically significant differences were found in the number of erythrocytes, hemoglobin concentration, and MCV values among the groups. Platelet values showed an increasing trend in both treated groups compared to the control, but these differences were not statistically significant ($p > 0.05$).

In vitro

Halogenated boroxine was evaluated at a concentration of 10 mg/mL, both with and without a 30-minute pre-incubation step. Enzyme activity was assessed before and after incubation to determine whether the incubation period modulated changes in enzymatic activity. The Lineweaver–Burk plot obtained without pre-incubation demonstrates that halogenated boroxine competitively binds to the enzyme, as shown in Figure 2. The obtained kinetic values of maximum velocity (V_{max}) remained constant regardless of the halogenated boroxine addition at 0.0003 mM s^{-1}. Conversely, the Michaelis constant (K_m) shifted significantly, increasing from 0.0004 mM in the absence of the inhibitor to 0.007 mM upon the addition of halogenated boroxine. Measurements of creatine kinase activity prior to incubation confirmed that halogenated boroxine induces significant enzyme inhibition at a concentration of 10 mg/mL, yielding an IC_{50} value of 42%.

Table 4. *Values of hematological parameters of experimental group 2, halogenated boroxine administered orally (OG)*

	WBC 10^9/L	LYM%	MON%	NEU%	EOS%	BASO%	RBC 10^{12}/L	Hb g/L	HCT L/L	MCV fL	MCH pg	MCHC g/L	PLT10^9/L
Experimental group 2 (OG)	5.57	58.47	15.87	22.35	3.14	0.17	8.36	154	0.436	52.2	18.4	353	680
	7.93	60.53	15.19	21.58	2.06	0.64	7.88	139	0.401	50.9	17.6	346	445
	3.81	50.37	12.24	26, 79	10.14	0.46	5.48	105	0.274	50	19.1	383	359
	3.76	46.32	20.92	30.1	2.08	0.58	6.3	119	0.318	50.6	18.8	374	710
	5.08	52.42	14.26	29.5	3.41	0.41	10.77	186	0.557	51.8	17.2	333	445
	4.88	48.23	19.25	29.66	2.45	0.41	4.98	119	0.25	50.2	23.89	476	550
	4.56	55.12	14.32	26.34	3.12	0.96	5.9	128	0.29	49.15	21.69	441	414
	6.78	54.34	15.11	27.22	2.98	0.44	6.68	146	0.31	46.4	21.85	470	489
	6.23	52.45	13.89	24.65	3.56	0.38	7.76	147	0.4	51.54	18.94	367	507
$\bar{X}$	5.62	53.58	15.45	26.44	3.67	0.5	7.13	138.1	0.37	50.54	19.59	385.4	511
Sd	1.42	4.63	2.87	3.01	2.49	0.25	1.77	24.1	0.09	1.74	2.12	47.8	118

Table 5. *Comparative presentation of statistical analysis of the examined parameters*

Parametar	Control $\bar{X} \pm Sd$	IP $\bar{X} \pm Sd$	OG $\bar{X} \pm Sd$	T test IP vs Control	T test OG vs Control	T test IP vs OG
WBC (10^9/L)	6.95 ± 1.66	5.66 ± 1.47	5.62 ± 1.42	0.154	0.14	0.954
LYM (%)	58.68 ± 2.73	52.78 ± 7.46	53.58 ± 4.63	0.054	0.019*	0.789
MON (%)	14.49 ± 2.17	11.94 ± 6.93	15.45 ± 2.87	0.327	0.475	0.189
NEU (%)	22.69 ± 4.87	28.62 ± 2.33	26.44 ± 3.01	0.029*	0.133	0.106
EOS (%)	4.08 ± 2.70	5.60 ± 1.47	3.67 ± 2.49	0.248	0.772	0.067
BASO (%)	0.05 ± 0.08	0.16 ± 0.14	0.50 ± 0.25	0.076	<0.001*	0.004 *
RBC (10^{12}/L)	8.30 ± 1.01	7.03 ± 1.42	7.13 ± 1.77	0.064	0.128	0.897
Hb (g/L)	147.90 ± 5.92	134.30 ± 22.10	138.10 ± 24.10	0.111	0.272	0.732
HCT (L/L)	0.43 ± 0.03	0.36 ± 0.07	0.37 ± 0.09	0.021*	0.093	0.796
MCV (fL)	51.85 ± 5.06	51.90 ± 1.70	50.54 ± 1.74	0.982	0.565	0.113
MCH (pg)	17.91 ± 1.39	19.60 ± 0.80	19.59 ± 2.12	0.030*	0.087	0.99
MCHC (g/L)	347.07 ± 15.95	378.90 ± 19.70	385.40 ± 47.80	0.005 *	0.049*	0.713
PLT (10^9/L)	413.00 ± 100.88	465.40 ± 19.60	511.00 ± 118.00	0.262	0.111	0.284

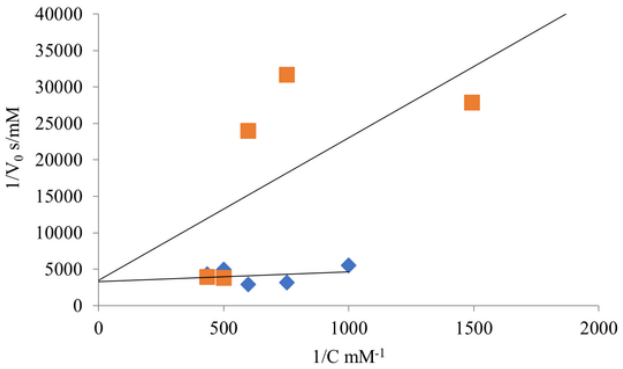


Figure 2. *Lineweaver-Burk plot for determining V_{max} and K_m in the absence or presence of various concentrations of halogenated boroxine; ◆ 0 mg/mL; ◆ 10 mg/mL (without incubation)*

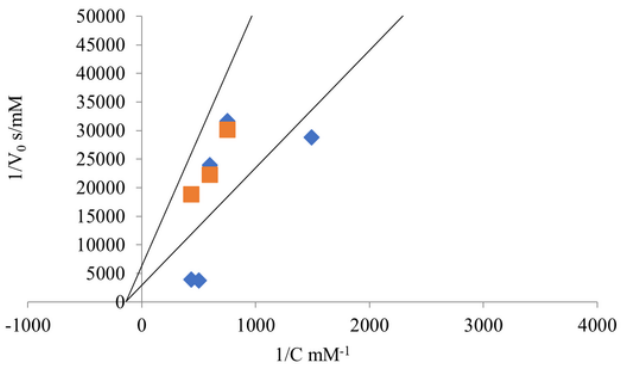


Figure 3. *Lineweaver-Burk plot for determining V_{max} and K_m in the absence or presence of different concentrations of halogenated boroxine; ◆ 0 mg/mL; ◆ 10 mg/mL (with incubation)*

Analysis of the Lineweaver–Burk plot following a 30-minute pre-incubation with halogenated boroxine revealed a non-competitive mechanism of inhibition, as shown in Figure 3. In the presence of halogenated boroxine, the maximum reaction velocity (V_{max}) decreased to 0.0001 mM s^{-1}, whereas the Michaelis constant (K_m) remained unaltered at 0.007 mM. In comparison, control measurements in the absence of halogenated boroxine yielded a $V_{max}$of 0.0003 mM s^{-1}, while the K_m value remained the same, amounting to 0.007 mM.

DISCUSSION

In vivo

The hematological profile of the control group fell within established reference limits for Wistar rats, confirming the validity of the experimental model. Erythrocyte parameters, including RBC, hemoglobin, hematocrit (HCT) values, and erythrocyte indices, as well as platelet (PLT) counts, were entirely consistent with physiological baseline values reported for laboratory rats. Minor variations observed within the differential leukocyte formula reflect expected biological variability. These variations align with the previous literature demonstrating that hematological parameters in laboratory animals are dynamically influenced by genetic strain, housing environments, and experimental conditions (14–16).

Following intraperitoneal (IP) administration of potassium hydrofluoroboroxine ($K_2[B_3O_3F_4OH]$) at a dose of 10 mg/kg of body weight, the majority of hematological parameters remained unaltered relative to the control group. The absence of significant shifts in overall leukocyte (WBC), erythrocyte (RBC), hemoglobin (Hb), and platelet (PLT) counts indicates that the 5-day administration of the tested compound neither caused general hematological suppression nor disruption of basic hematopoietic functions.

The most significant change in the IP group involved an elevated neutrophil percentage with a simultaneous decrease in hematocrit. An increase in neutrophils, occurring independently of any increase in the total number of leukocytes, represents a shift in the relative proportions of leukocyte subsets and potentially signifies a mild activation of the innate immune response. Similar shifts in the differential leukocyte formula have been described in laboratory animals after exposure to various chemical substances and most often represent a transient adaptive response, especially when they are not accompanied by other indicators of inflammation (15,17). Furthermore, because the HCT reduction in the IP group was not accompanied by declines in either RBC counts or Hb concentrations, nor by shifts in MCV, it cannot be linked to erythroid pathologies or the development of anemia. This pattern indicates an isolated change in the HCT value without any evidence of a disorder of erythropoiesis. Similarly, the isolated elevation in MCH, occurring while all other erythrocytic markers remained preserved, may indicate a localized shift in hemoglobin content per cell, though it lacks apparent pathological relevance. Upon oral administration of $K_2[B_3O_3F_4OH]$, most hematological parameters also did not show significant changes compared to the control group. The stability of erythrocyte parameters and platelets indicated the preserved function of the erythrocyte and platelet lineages after systemic absorption of the compound. However, the OG group was uniquely characterized by a statistically significant contraction in lymphocyte percentages alongside an increase in the percentage of circulating basophils.

A decrease in the relative share of lymphocytes, without a change in the total number of leukocytes and without pronounced neutrophilia, does not indicate a systemic stress response, but rather a change in the ratio of different leukocyte forms. Similarly, the observed basophilia must be interpreted with caution given the naturally low abundance of basophils in the peripheral blood of healthy rats. In the absence of other hematological deviations, these changes may indicate mild immunomodulatory activity, but not the development of a pathological immune process.

A comparison of the two routes of administration shows that the hematological response depended on the route of administration. Intraperitoneal administration was associated with a more pronounced change in the neutrophil population, while oral administration led to changes in the lymphocytic and basophilic components of the differential leukocyte formula. These differences may be due to different routes of absorption, distribution, and interaction of the compounds with the immune system. It is known that the route of administration significantly affects the intensity of the biological response after exposure to chemical substances in experimental animals (18).

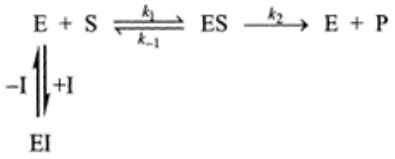
The obtained results should be viewed in the context of biological significance, and not just the statistical significance of individual changes. Although certain parameters showed statistically significant differences between the groups, their values mostly remained within the reference physiological limits, without simultaneous changes in other hematological indicators that would indicate toxic effects. Boron compounds represent a group

of substances whose biological effects depend on their chemical structure, dose, and time of exposure. Previous research indicates that boron compounds can participate in the regulation of various cellular processes and exhibit biological activity without the necessary development of toxic effects at moderate doses (19-21).

Accordingly, the changes in this study, limited primarily to the differential leukocyte formula, can be interpreted as a mild adaptive immune response after administration of $K_2[B_3O_3F_4OH]$. Overall, administration of dipotassium trioxohydroxytetrafluorotriborate ($K_2[B_3O_3F_4OH]$) at a dose of 10 mg/kg body weight for 5 consecutive days in rats did not cause marked hematological disorders 24 hours after the last application. The observed changes were selective, dependent on the method of administration, and mostly limited to individual components of the differential leukocyte formula and erythrocyte indices. Preservation of basic hematological parameters indicates the absence of significant hematological toxicity at the examined dose and period of exposure.

In vitro

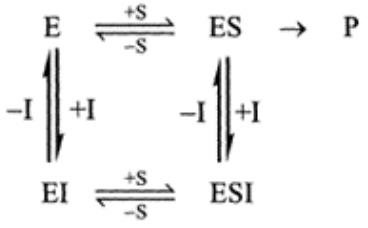
Halogenated boroxine showed a dual mechanism of creatine kinase inhibition, depending on the time of contact with the enzyme. Without prior incubation, Lineweaver–Burk analysis indicated competitive inhibition, which was confirmed by an unchanged V_{max} value (0.0003 mM s^{-1}) and a significant increase in K_m from 0.0004 to 0.007 mM. An increase in K_m with a constant V_{max} is a characteristic indicator of competitive inhibition, in which the inhibitor and substrate compete for the same or overlapping binding site on the enzyme, according to the following mechanism:



This pattern indicates that halogenated boroxine reduces the affinity of the enzyme toward the substrate, but does not affect the maximum catalytic capacity of the enzyme when the substrate concentration is high enough. This finding is in accordance with the known properties of boronic compounds, which, due to the electron-deficient boron atom, can achieve specific interactions with the catalytic centers of enzymes and act as reversible competitive inhibitors (22,23). Additionally, the measured inhibition of creatine kinase activity at 42% at a halogenated boroxine concentration of 10 mg/mL confirms that this compound possesses a significant ability to modulate enzyme activity. Although IC_{50} was not fully reached at the tested concentration, the degree of recorded inhibition indicates a pronounced interaction between the inhibitor and the enzyme.

Similar inhibitory effects of halogenated boroxine were previously suggested for other enzymes, including human catalase and carbonic anhydrase, where molecular docking experiments showed binding of the compound within the active sites of the enzymes and potential inhibition of their activity (22).

Notably, after a 30-minute pre-incubation, the change of type of inhibition from competitive to non-competitive occurred. In this case, K_m value remained practically unchanged, while V_{max} decreased from 0.0003 to 0.0001 mM s^{-1}. Such a kinetic pattern indicates that the inhibitor no longer acts primarily on substrate binding, but instead affects the catalytic function of the enzyme by forming a complex with the enzyme or an enzyme–substrate complex at an allosteric site, according to the following mechanism:



An unchanged K_m suggests that the affinity of the enzyme for the substrate remains the same, while a decrease in V_{max} reflects a decrease in the number of functionally active enzyme molecules. This result indicates that a certain time of interaction between halogenated boroxine and the enzyme is required to achieve a full inhibitory effect (24). The change in the mechanism of inhibition after incubation can be explained by the gradual formation of a more stable complex between the inhibitor and the enzyme. In the case of numerous boron derivatives, time-dependent inhibition of enzymes caused by the formation of reversible covalent or very strong non-covalent interactions has been described. Such inhibitors often show initial competitive binding, which over time develops into a complex of higher stability, which results in a drop in V_{max} and the appearance of kinetic characteristics of non-competitive or mixed inhibition (24,25).

The obtained results indicate that the mechanism of action of halogenated boroxine is complex and probably involves multiple sites of interaction with creatine kinase. Such behavior is not unusual for boron-containing compounds, as they can act as analogs of the transition state of the reaction and form stable complexes with amino acid residues inside

or outside the active site of the enzyme. It is known that boron compounds have the ability to reversibly bind to enzymes and can significantly change their kinetic parameters (2,4,24). Overall, the results of this research show that halogenated boroxine at a concentration of 10 mg/mL acts as an efficient creatine kinase inhibitor. Without prior incubation, the inhibitor behaves as a competitive inhibitor, while after 30 minutes of incubation, it shows the properties of non-competitive inhibition. This time-dependent change in the mechanism of inhibition suggests the gradual establishment of more stable interactions between halogenated boroxine and the enzyme, which may be of importance for understanding its biological action and potential pharmacological application.

The findings of this study demonstrate that halogenated boroxine modulates specific hematological profiles, depending on the method of application and experimental conditions. Under *in vitro* conditions, halogenated boroxine inhibited creatine kinase activity. Furthermore, enzyme kinetic analysis revealed a distinct, time-dependent shift in the mechanism of inhibition following a 30-minute pre-incubation period, characterized by a significant decrease in the maximum reaction velocity, $V_{max,}$, while the Michaelis constant, K_m, remains unaltered.

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Author Contributions

Conceptualization, S.H., E.H. (First author), and E.H. (Second author); Formal analysis, E.H. (First author), M.F., and A.B.; Investigation, E.H. (First author), M.F., and A.B.; Methodology, S.H., E.H. (First author), and E.H. (Second author); Supervision, S.P., and M.H.; Writing – original draft, S.H., E.H. (First author), M.F., A.B., S.P., M.H., and A.D.Z.; Writing – review & editing, S.H., N.V., and A.D.Z. 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 University of Sarajevo Faculty of Science (approval number 01/01-1945/2-2025, issued on October 23, 2025.

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 included in the article.

Statement of Generative AI Use

No generative AI was used.

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TEMPOROMANDIBULAR DISORDERS IN YOUNG SERBIAN POPULATION: AN EPIDEMIOLOGICAL STUDY

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Temporomandibular disorders are a common problem in everyday dental practice. This study aimed to evaluate the prevalence of signs and symptoms of temporomandibular disorders (TMDs) in the young Serbian population. The study comprised 500 participants between 18 and 25 years of age from various regions of Serbia. Among this sample, 325 persons were males, and 175 were females. All participants were subject to a clinical examination: assessment of mandibular mobility and temporomandibular joint (TMJ) function, and recording any tenderness or pain during mandibular movements or palpation. The presence of signs and symptoms of TMDs was confirmed using the Helkimo's dysfunction index (Di). According to the values of the Helkimo's Di, the presence of signs or symptoms of TMD was confirmed in 327 (64.5%) examined subjects. The most prevalent signs were: disturbed mandibular kinematics (restricted mandibular opening, deviation or deflection at mouth opening) found in 74.5 % of the examined subjects; TMJ sounds during mandibular opening/ closing were found in 38.5% of subjects; sensitivity of masticatory muscles was confirmed in 10.4 %. Pain as the most severe symptom of TMD was found in only 5.2 % of the examined subjects. The signs and symptoms of TMDs were more prevalent in females than in males, the ratio being 3:1. The recorded signs and symptoms of TMD in this population group were of a mild nature. The severe symptoms of TMD (pain) were found in only 5.2 % of the examined persons.

Keywords: Helkimo's index, prevalence, signs, symptoms, temporomandibular disorders

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INTRODUCTION

The term temporomandibular disorders (TMDs) refers to various functional disturbances involving the orofacial muscles, the temporomandibular (TM) joints or both (1).

The essential symptoms and signs of these disturbances are pain and tenderness around the TM joints or chewing muscles, impaired mandibular mobility and TM sounds during mandibular movements. The etiology of TMD remains a subject of controversy and is generally considered to be multifactorial. Thus, the influence of occlusal condition in the onset of TMDs has been intensely debated for many years. Until the 1980s, occlusal factors, such as the presence of uncured malocclusions, discrepancies between the intercuspal and retruded contact position of more than two millimetres, retrusive and non-working side interferences and loss of posterior teeth were considered to be the primary causes of TMD. Many researchers supported this hypothesis (1-7).

However, contemporary scientific and clinical evidence has not confirmed any direct correlation between occlusal factors and TMDs symptoms. In fact, numerous scientists deny any influence of occlusal factors in the onset of TMDs. It appears that many individuals can adapt to occlusal disturbances and function within tolerable limits without the onset of TMD signs and symptoms (8-12).

Between these opposing camps are those who believe that occlusion does not play a primary role in the etiology of TMD but has a secondary influence and can exacerbate symptoms caused by other sources after the onset of TMD, such as trauma, congenital deformities, systemic diseases or emotional stress (13-17).

In order to determine the prevalence of TMDs in different population groups, numerous epidemiologic studies from different nations were conducted starting in the 1980s, and a number of indices and criteria were created (22–25). It is challenging to determine the true prevalence of TMDs in the general population due to the many approaches and interpretations of the signs and symptoms employed in these studies.

Opinions concerning the treatment needs of TMD patients vary greatly in the relevant literature. Contrary to earlier studies, which estimated that 20%–25% of the general population had severe signs of dysfunction (26), according to recent studies, the demand for active treatment seems to decrease to about 10% (27). The presence of malocclusion and craniofacial deformities may provoke the signs and symptoms of TMDs (28), which tend to increase with age, according to various reports. It was found that TMDs were more prevalent in females than in males (29).

To date, there have been only a few studies dealing with the prevalence of TMDs in the Serbian population (30, 31). The purpose of this study was therefore to determine the prevalence of signs and symptoms of TMDs in a young Serbian adult population and the possible connection between the present occlusal discrepancies and the presence of signs and symptoms of TMDs.

METHODS

The investigation included 325 males and 175 females between 18 and 25 years of age, from various regions of the Republic of Serbia (101 from Vojvodina, 193 from central Serbia, and 206 from Belgrade). Trained physicians from the Clinic for Dental Prosthetics conducted the study in Belgrade and Novi Sad as part of the project of the Ministry of Science and Technology of the Republic of Serbia No. 1549.

For data collection, the special questionnaire according to Helkimo's (21) was used and adapted for digital evaluation of the Helkimo's dysfunction index (Di), anamnestic index, and occlusal index (Oi) (26). The anamnestic examination was based on the symptoms reported by the subjects and classification according to the anamnestic dysfunction index (Ai) as 0, I or II. The subjects were grouped according to the criteria of the clinical dysfunction index, which included the following variables: range of mandibular movements, function of TM joints, muscle pain on palpation, TM joint pain on palpation and pain on movements of the mandible. Each finding was judged according to a three-point scale of severity as 0, 1 or 5 points. No symptom was registered as 0, a mild symptom as 1, and a severe symptom as 5 points. The scores awarded for the five signs/ symptoms of TMD were added together to give a total dysfunction score, which formed the basis for classification into the clinical dysfunction index group. The dysfunction index Di > 0 denoted the presence of signs/symptoms of TMD (Di I denoted mild dysfunction, Di II indicated moderate dysfunction, and dysfunction index Di III pointed to severe dysfunction). The dysfunction index Di = 0 denoted a clinically symptom-free subject.

The Helkimo's occlusal index (Oi) was calculated from data obtained by clinical examination of the occlusal complex, which included the following variables: reduced number of natural teeth, reduced number of teeth in contact, the presence of the RCP-ICP slide and the presence of protrusive, laterotrusive and mediotrusive interferences. The severity of occlusal discrepancies was judged on a

three-point scale as 0, 1 or 5. The awarded scores formed the basis for calculating the occlusal index Oi according to Helkimo's. The occlusal index Oi > 0 pointed to the presence of certain occlusal discrepancies, and Oi = 0 denoted discrepancy-free subjects (26). All data were input into a personal computer, which enabled the calculation and statistical evaluation of the obtained values. The statistical software was SPSS 15.0.

RESULTS

The prevalence of signs and symptoms of TMD according to the Helkimo's dysfunction index (Di)

According to the Helkimo's dysfunction index (Di), the values calculated for the complete sample and selected regions of Serbia, signs/symptoms of TMD were present in 72.3% of subjects from Vojvodina, 56.3% from Belgrade, and 71.5% from central Serbia. An average of 65.4% of young adults in the Republic of Serbia had at least one sign or symptom of TMD (Di > 0), and 34.6% of subjects were symptom-free (Di = 0) (Figure 1).

The most prevalent signs/symptoms of TMD found in 327 (65.4%) of subjects with the positive dysfunction index (Di > 0) were:

- Limitations of mandibular movement and deviations during mandibular opening in 245/327 (74.9%) subjects.
- TMJ sounds in 126/327 (38.5%) subjects.
- Pain on mandibular movements in 17/327(5.2%) subjects.
- Muscle sensitivity to palpation in 34/327 (10.4%) subjects.
- TMJ sensitivity to palpation in 19/327(5.8%) subjects.

The prevalence of symptoms of TMD according to the Helkimo's anamnestic index (Ai)

According to the Helkimo's anamnestic index (Ai) values calculated for the complete sample (500) and selected regions of Serbia, the presence of TMD symptoms was confirmed in 17.8% of subjects in Vojvodina, 37.9% in Belgrade, and 15.5% in central Serbia. On average, 25.2% of young adults in the Republic of Serbia had at least one symptom of TMD/Ai > 0/, whereas 74.8% of subjects were symptom-free (Ai = 0) (Figure 2).

The prevalence of occlusal disturbances according to the Helkimo's occlusal index (Oi)

According to the values of the Helkimo's occlusal index (Oi > 0), occlusal disturbances were confirmed in 58/271 (57.4%) subjects from Vojvodina, 93/271 (45.1%) subjects from Belgrade and 120/271 (62.1%) subjects from central Serbia. Evaluation of the Helkimo's occlusal index (Oi) in the complete sample confirmed the presence of occlusal disharmonies in 229 (45.5%) young adults (Figure 3).

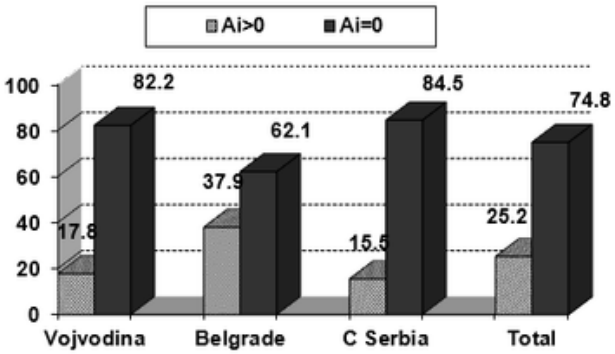


Figure 2. *The prevalence of signs and symptoms of TMDs in the young population*

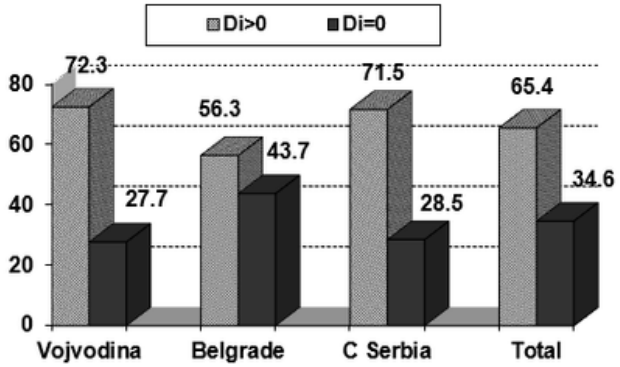


Figure 1. *The prevalence of signs and symptoms of TMDs in young adults of the Republic of Serbia, and selected regions according to the Helkimo's dysfunction index*

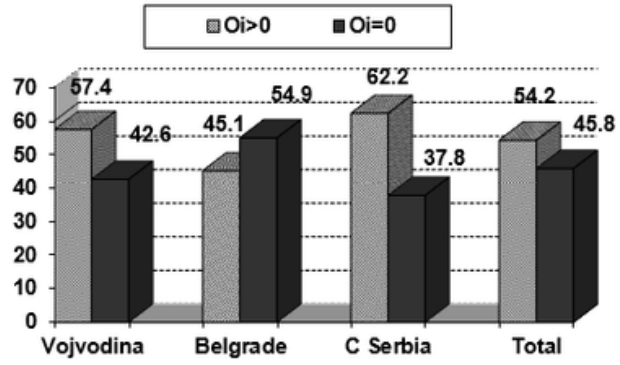


Figure 3. *The prevalence of occlusal disharmonies in young adults of the Republic of Serbia according to Helkimo's occlusal index (Oi)*

Analysis of the occlusal parameters needed to calculate the Helkimo's occlusal index showed that 17.8% of young adults from Serbia had fewer natural teeth, 28.4% of subjects had fewer teeth in contact with the antagonists, 23.4% had an outstanding RCP-ICP slide, and 28.6% of subjects had mild or severe protrusive, laterotrusive or mediotrusive interferences during mandibular movements (Table 1).

DISCUSSION

A significant number of epidemiological studies demonstrate that the prevalence of signs and symptoms of TMDs is exceedingly high. The prevalence of signs and symptoms of TMD in the population ranges from 60% to 70% among individuals aged 20 to 40 years, with a significantly higher prevalence in women than in men (25). The prevalence of signs and symptoms of TMD in the community varies from 60% to 70% among individuals aged 20 to 40 years, with a markedly higher incidence in women compared to men (25,26, 32-37).

In the present study, conducted on 500 young adults from various regions of Serbia, the special questionnaire adopted for the calculation of the Helkimo's dysfunction and occlusal indexes was used to establish the prevalence of TMDs in this population group and to evaluate the possible influence of the occlusal condition on the onset of TMDs. Comparable research conducted by Helkimo, Dworkin and Magnuson used the identical methodology (25-27).

The signs and symptoms of TMD were confirmed in 65.4% of subjects, with the highest prevalence in the region of Vojvodina, where 72.3% of subjects had at least one clinical sign/symptom of TMD. These results are in line with the results of previous studies examining the prevalence of TM dysfunction in adolescents and young adults, particularly when the severity of signs/symptoms is concerned (32-37, 39-41).

According to the Helkimo's dysfunction index scale (Di I, II, III), mild dysfunction signs (disturbed mandibular kinematics, TMJ sounds, Di I) were the most prevalent signs, found in 74.9% and 38.5% of the subjects in this study. Severe symptoms (tender to muscle palpation, tender to TMJ palpation, corresponding to DiII) were confirmed in 10.4% and 5.8% of subjects, respectively, and pain on mandibular movements, categorized as Di III, was confirmed in only 5.2% of subjects. These results can be explained by the fact that the sample consisted of healthy young adults, presumably with only the initial stages of mandibular dysfunction, which is consistent with research and meta-analyses regarding the frequency of TMD symptoms and indicators (42, 43).

Table 1. *The prevalence of occlusal disharmonies in young adults of the Republic of Serbia*

Occlusal variables	The number of examinees	Percent
Reduced number of natural teeth	98	19.6
Reduced number of teeth which contact the antagonists	142	28.4
Outstanding RCP-ICP slide	117	23.4
Protrusive, laterotrusive and mediotrusive interferences	143	28.6

Evaluation of the Helkimo's occlusal index (Oi) confirmed the presence of occlusal discrepancies in 45.5% of young adults, most frequently in the central part of Serbia— 62.1%, mostly caused by presumably poorer hygiene habits, a consequently higher percentage of missing teeth due to caries, periodontal diseases or irresponsible and early tooth extractions. The relevant literature contains no reliable evidence for an occlusal contribution to TMD (8-10, 13, 16, 17), but an absence of any relationship should not be inferred because this would imply an absence of any relationship between form and function (39-41).

The comparative analysis of the values of the Helkimo's Di and Oi in this study points to a certain association between occlusal factors and TMD signs/symptoms; however, this association should not be overstated because the signs/symptoms of TMD were confirmed in 70.85% of subjects with occlusal discrepancies, as well as 58.45% of subjects without such discrepancies. According to the X-Test analysis, differences between these findings, significant at the level of $p < 0.01$, point to a certain occlusal association because the prevalence of TMD signs/symptoms was considerably higher in the group with occlusal discrepancies. This association can only be declared because of the lack of a clear etiologic background of confirmed TMD signs/symptoms in the examined groups and because of unsatisfactory differentiation between patients and normal controls.

Within the limits of this study, the following conclusions can be drawn:

1. The results point to the considerable prevalence of TMDs in the population of 500 young adults in the Republic of Serbia. It was confirmed that 327/500 (65.4%) of the examined subjects had at least one sign/symptom of TMD. The prevalence was somewhat higher in samples from Vojvodina—73/101 (72.3%) and the central part of Serbia—137/193 (71.5%), and somewhat lower in the sample from Belgrade 116/206 (56.3%).

2. The most prevalent signs and symptoms of TMDs in the examined population group were:
- Disturbed mandibular kinematics (deviations, deflections on mouth opening, restricted mouth opening) confirmed in 245/327 (74.9%) subjects in the complete sample.
- TMJ sounds on mandibular opening/closing confirmed in 126/327 (38.5%) subjects.
- Masticatory muscle tender to palpation, confirmed in 34/327 (10.4%) subjects.
- TMJ tender to palpation, confirmed in 18/327 (5.8%) subjects.
- Pain during mandibular movements confirmed in 17/327 (5.2%) subjects.

3. According to the Helkimo's dysfunction index scale, the majority of signs/symptoms of TMDs observed in the examined population group were of mild intensity and corresponded mainly with the Di I and Di II values of the dysfunction index. The severe symptoms, like pain on mandibular movements (Di III), were observed in only 17/327 (5.2%) of the subjects. Although high in prevalence, the signs/symptoms of TMDs in this population group seem to present the mild or initial stages of TMDs.
4. According to the values of the Helkimo's occlusal index (Oi), occlusal disturbances were found in 54.2% of young adults in the total sample. The prevalence of occlusal disturbances was somewhat higher in the sample from the central part of Serbia, where 62.1% of subjects presented some kind of occlusal disharmony. The majority of subjects had mild or severe protrusive, laterotrusive or mediotrusive interferences (23.4%–28.6%) and a reduced number of natural teeth contacting the antagonists (17.8%–28.4%).

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Author Contributions

Conceptualization, methodology, investigation, formal analysis, and writing—original draft preparation, S.D.; Data curation, formal analysis, and validation, V.S.; Investigation, data curation, visualization, writing—review, editing, and supervision, S.D.P.

Statement of Ethics

This study was part of a project of the Ministry of Science and Technology of the Republic of Serbia No. 1549. Participation was voluntary and uncompensated. All participants provided written informed consent prior to completing the questionnaire.

Statement of Competing Interest

The authors declare no conflict of interest.

Statement of Data Availability

The data supporting the findings of this study are included in the article.

Statement of Generative AI Use

No generative AI was used.

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AFMN BIOMEDICINE

ORIGINAL ARTICLE

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BODY COMPOSITION IN PHYSICALLY ACTIVE MALE STUDENTS ASSESSED USING MULTI-FREQUENCY BIOELECTRICAL IMPEDANCE ANALYSIS

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Body composition is strongly influenced by physical activity and dietary habits. Male students enrolled in Physical Education and Sport programs represent a distinct population because their curricula include systematic physical activity that directly affects physiological characteristics. This study aimed to establish a representative body composition profile of physically active male Physical Education and Sport students using a multi-frequency bioelectrical impedance analyzer (BIA) and to evaluate the reliability and consistency of measurements across two assessments. A total of 108 male students aged 19–25 years participated in the study. Body composition was assessed using ten indicators: body fat percentage, visceral fat index, body mass index (BMI), muscle mass, skeletal muscle mass, sarcopenic index, total body water, body weight, height, and phase angle. Descriptive statistics, paired-samples t-tests, effect size analysis (Cohen's d), and Bland–Altman analyses were used to evaluate agreement, reliability, and measurement consistency between pre- and post-test assessments. No statistically significant differences were observed between assessments for any parameter ($p > 0.05$). Effect sizes were trivial across all indicators, indicating negligible changes between measurements. Bland–Altman analysis revealed bias values close to zero and relatively narrow 95% limits of agreement, confirming good measurement reliability and reproducibility. Physically active male students exhibited a stable and reproducible body composition profile. The obtained values may serve as reference data for body composition assessment in similar populations. Although the BIA method demonstrated high reliability, it appears more sensitive to moderate-to-large changes than to very small individual physiological fluctuations.

Keywords: pattern, physiological profile, reliability assessment

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INTRODUCTION

Body composition is influenced primarily by physical activity level, diet, lifestyle, and other factors. In addition to commonly used indicators such as body mass index (BMI) and body fat percentage, contemporary research also considers parameters such as the sarcopenic index and total body water (TBW), which provide deeper insight into muscle quality and hydration status (1). The sarcopenic index (2) reflects the proportion of skeletal muscle relative to body size, and it is an indicator of functional muscle status, while TBW is closely related to fat-free mass and overall physiological balance. These parameters allow for a more comprehensive evaluation of body composition in relation to physical activity and nutritional habits. Nowadays, physical activity levels among students are often low (3,4), which affects body image and contributes to weight management issues such as overweight and obesity by increasing body fat percentage and creating a risk for chronic diseases associated with physical inactivity (5). However, some studies have found no significant differences in body composition between students involved in sports and those who are not (6). Not all physical activity programs lead to significant changes in students' body composition; the regularity of activity is a critical factor (7,8), with moderate-intensity continuous training showing greater benefits for improving body composition (9). Longitudinal studies have reported an increasing trend in students' BMI over four consecutive years, alongside a decline in physical fitness, particularly in upper limb strength (10). There is a clear association between BMI and physical fitness, as well as between physical activity levels, body composition parameters, and physical performance. Specifically, the proportion of adipose tissue is negatively correlated with physical condition (11). Moderate leisure physical activity has also been linked to lower body fat percentages among students (12).

Diet is another key factor influencing body composition. While a varied and healthy diet is desirable, many students adopt lifestyles incompatible with healthy eating (13). Unhealthy diets are associated with higher obesity prevalence, emphasizing the need for nutritional recommendations targeted at students (14). Lebanese male students demonstrated better dietary habits, greater nutrition knowledge, and maintained normal BMI values; a mild to moderate correlation was observed between dietary habits and body composition, especially body mass, fat mass, and skeletal muscle mass (15). Associations have also been found between sugar consumption and visceral fat, bone mass, and muscle mass, whereas fruit and vegetable intake correlates with lower fat percentages (16). The Mediterranean diet positively influences body composition, associating with lower levels of visceral and subcutaneous adipose tissue, BMI, and waist circumference (17). Physical activity combined with a varied diet is essential for achieving optimal body weight and preventing nutritional and health problems (18).

The living environment appears to have little impact on body composition, as rural and urban students exhibit similar average values across most body composition indicators (19). In one study, El-Hayek Fares et al. (20) found that 40% of students were overweight or obese; 52.5% of males required dietary changes, with many showing lower fitness scores. Using bioelectrical impedance analysis, university students were found to have normal BMI but elevated body fat mass. Despite most reporting as active via the International Physical Activity Questionnaire (IPAQ), nearly half were actually less active or sedentary (21). Students tend to gain weight and body fat during their academic studies, especially in the first year, which affects BMI (22). Significant differences in fat mass, lean mass, skeletal muscle percentage, and overall body mass were identified between students with excessive weight and those with normal weight using bioimpedance methods (23).

Regarding body fat, no significant differences were observed between active and inactive male students; however, other indicators like body water and muscle mass showed significant differences (24). Among male students specializing in physical education and sport, BMI and fat percentage were within normal ranges, and the proportion of overweight and obese students was lower than that of other Ecuadorian male youth (25). Similar results were found for Lebanese students, where sports students had normal body weight and healthier body composition profiles compared to students from other fields (26). Students enrolled in sports programs also demonstrated better body composition indicators and blood pressure levels compared to their peers in other courses, particularly for waist and hip circumferences, muscle mass, and bone mineral content (27). Nonetheless, approximately 20% of students are overweight despite positive attitudes toward physical activity; without regular exercise, these attitudes are insufficient. Offering a diverse range of sports and recreational activities could encourage youth participation and improve quality of life (28).

Obesity and overweight negatively correlate with self-esteem, which tends to be lower in these groups compared to physically active students who exhibit higher self-esteem

and better health status (29). Students with larger body silhouettes often experience dissatisfaction, body image concerns, and lower self-esteem (3). More than half of the physical education students in a study by Rocha et al. (30) exhibited distortions in self-body perception, which raises concerns for future professionals in the physical activity field. Male students averaged 38% skeletal muscle mass and 24% body fat, reflecting high levels of physical activity (31).

The level and regularity of physical activity and diet primarily influence body composition in male students. Male students in physical education and sport represent a distinct group due to the higher physical activity demands in their curricula compared to students in other fields. Previous studies that have reported differences in body composition among male students, typically attributed to variations in physical activity, diet, and lifestyle. Our research focuses on determining a body composition pattern for Romanian male students in physical education and sport. There is a lack of information on this population in journals indexed by Web of Science, and our study aims to fill this gap by providing reliable data.

This study aimed to establish a reference pattern of body composition in physically active young males enrolled in a Physical Education and Sport program using a multi-frequency bioelectrical impedance analyzer. Specifically, the objectives were to (a) assess ten key body composition indicators, (b) evaluate the reliability and agreement between two consecutive assessments using the Bland–Altman method, and (c) determine whether statistically significant differences exist between the two assessments.

It was hypothesized that physically active male students enrolled in the Physical Education and Sport program would exhibit a consistent and reliable body composition pattern, with values aligning with those reported for healthy, active youth populations and showing no significant differences between repeated assessments.

METHODS

The subjects involved in this research were 108 male students enrolled in the Physical Education and Sport program at Ștefan cel Mare University of Suceava. All participants provided written informed consent before participation. The study was conducted in accordance with the Declaration of Helsinki. It was approved by the Ethics Research Committee of Ștefan cel Mare University of Suceava (protocol code no. 64, approved on May 23, 2022).

Study design

In designing the study, we established the following inclusion criteria: male sex, age between 19 and 25 years, and active student status. Exclusion criteria included: female sex, age below 19 or above 25 years, and non-student status.

During the 8-week experimental period, participants engaged in four hours of structured physical activity per week as part of their academic curriculum, as well as an additional average of six hours of voluntary physical activity weekly.

To minimize measurement variability, assessments were conducted twice, at baseline (week 1) and post-intervention (week 8), at the same time of day and under similar conditions for all participants.

Instrumentation and data collection

Body composition was assessed using a multi-frequency bioelectrical impedance analyzer (Tanita MC-780 MA) in standard mode, following the manufacturer's guidelines. Participants were instructed to avoid food, caffeine, and vigorous physical activity for at least three hours before testing to ensure consistent measurement conditions.

The device provided data on ten body composition parameters: body fat, visceral fat, BMI, muscle mass, skeletal muscle mass, sarcopenic index, total body water, weight, height, and phase angle.

Statistics

Descriptive statistics (mean ± standard deviation) were calculated using IBM SPSS Statistics, version 26. To evaluate agreement and reliability between pre- and post-test measurements, the Bland–Altman method was applied to examine individual differences and assess the interchangeability of results by calculating the bias and the 95% limits of agreement for each parameter. A bias value close to zero indicates the absence of systematic error and no consistent tendency to overestimate or underestimate values in the second assessment. Furthermore, narrower limits of agreement reflect higher measurement reliability and consistency. One-way analysis of variance (ANOVA) was used to assess the homogeneity of the study sample. A paired samples t-test was then conducted to identify any statistically significant differences between the two assessments. The level of significance was set at $p < 0.05$. Cohen's d was used to quantify the actual magnitude of the improvement in strength and velocity, where a value of 0.8 or higher meant a large effect, and the difference was substantial.

In addition, the smallest worthwhile change (SWC) was calculated for each parameter as 0.2 × SD (baseline), representing the minimal meaningful change. The comparison between SWC and the Bland–Altman limits of agreement (LoA) was used to further interpret measurement sensitivity, where SWC values smaller than LoA indicate limited ability to detect small but meaningful changes.

RESULTS

The ANOVA method confirmed that participants were relatively uniform in terms of age, height, and weight, with no statistically significant differences observed ($p > 0.05$).

More specifically, one-way ANOVA revealed no statistically significant between-subject variability for baseline characteristics, with low within-group dispersion relative to total variance, indicating a high degree of sample homogeneity. The absence of significant F-ratios ($p > 0.05$) suggests that individual differences in age, height, and body mass did not contribute meaningfully to variability in the dataset. Additionally, the relatively small standard deviations observed for height (±7.02 cm) and BMI (±3.82 kg/m^2), alongside moderate dispersion in body mass (±14.33 kg), further support the conclusion that the sample was sufficiently homogeneous for subsequent parametric analyses.

Furthermore, one-way ANOVA results ($p > 0.05$ across all baseline characteristics) confirmed sample homogeneity, indicating that variability within the group was random and not influenced by subgroup stratification, thereby justifying the use of parametric analyses.

Importantly, the homogeneity of variance assumption was considered satisfied, as reflected by the consistent spread of values across participants, which reduces the likelihood of bias in inferential comparisons and strengthens the internal validity of the study design.

The descriptive statistics and comparative results for the pre-test and post-test assessments are presented in Table 1. For each indicator, the pre- and post-test mean values were highly comparable, and no statistically significant differences were found, as confirmed by the paired samples t-test ($p > 0.05$). The parameters closest to the significance threshold were skeletal muscle mass ($p = 0.099$) and total body water ($p = 0.109$), suggesting a tendency toward minor physiological variation rather than systematic change.

Effect size analysis revealed trivial Cohen's d values across all body composition indicators (approximately −0.16 to 0.11), indicating negligible differences between the two assessments. The combination of non-significant paired-samples t-test results and very small effect sizes confirms that the observed variations were minimal and unlikely to reflect meaningful physiological changes. Instead, these findings suggest a high degree of measurement consistency and temporal stability over the study period.

These findings support the reliability and stability of the bioelectrical impedance analyzer used in this study, as the group and individual values remained nearly constant over the two-month testing interval, with only minor natural fluctuations observed.

To further examine measurement consistency, Bland–Altman analysis was applied to all ten indicators. The analysis provided bias estimates, 95% limits of agreement (LoA), and smallest worthwhile change (SWC) values, allowing a comprehensive evaluation of measurement reliability and sensitivity (Table 2).

The calculated bias values for all variables were very close to zero (ranging from −0.17 to 0.23), indicating the absence of systematic measurement error and demonstrating that the device did not consistently overestimate or underestimate values during the second assessment. The narrow bias estimates across all indicators confirm excellent agreement between repeated measurements.

Table 1. *Statistical analysis for body composition*

Indicator	Pre-test (X ± SD)	Post-test (X±SD)	Bias	Lower LoA	Upper LoA	t	p	Cohen's d
Body fat (%)	17.30 ± 6.85	17.36 ± 6.80	0.06	-2.74	2.87	0.457	0.648	0.044
Visceral fat index	3.27 ± 2.72	3.30 ± 2.77	0.03	-0.92	0.97	0.598	0.551	0.058
Body mass index	23.71 ± 3.86	23.77 ± 3.76	0.07	-1.27	1.4	1.013	0.313	0.097
Muscle mass (%)	78.57 ± 6.49	78.52 ± 6.45	-0.04	-2.64	2.55	-0.334	0.739	-0.032
Skeletal muscle mass (%)	45.62 ± 3.97	45.49 ± 3.93	-0.13	-1.72	1.46	-1.665	0.099	-0.16
Sarcopenic index	8.47 ± 0.97	8.46 ± 0.94	-0.01	-0.45	0.43	-0.574	0.567	-0.055
Total body water (%)	58.11 ± 4.72	57.95 ± 4.70	-0.16	-2.24	1.91	-1.617	0.109	-0.156
Weight (kg)	76.18 ± 14.33	76.41 ± 14.17	0.23	-3.92	4.37	1.11	0.269	0.107
Height (cm)	179.02 ± 7.05	179.03 ± 7.09	0.01	-0.32	0.34	0.576	0.566	0.055
Phase angle (°)	6.85 ± 0.62	6.83 ± 0.71	-0.02	-0.79	0.76	-0.487	0.628	-0.047

Legend: X – average; SD - standard deviation;

Table 2. *Bland–Altman agreement statistics and smallest worthwhile change (SWC) for body composition indicators in males (n = 108)*

Body composition indicator	Bias	Lower LoA (95%)	Upper LoA (95%)	SWC
Body fat (%)	0.06	-2.75	2.87	1.37
Visceral fat index	0.03	-0.92	0.97	0.54
Body mass index (kg/m²)	0.07	-1.27	1.4	0.77
Muscle mass (%)	-0.04	-2.64	2.55	1.3
Skeletal muscle mass (%)	-0.13	-1.72	1.46	0.79
Sarcopenic index	-0.01	-0.45	0.43	0.19
Total body water (%)	-0.17	-2.24	1.91	0.94
Weight (kg)	0.23	-3.92	4.37	2.87
Height (cm)	0.01	-0.32	0.34	1.41
Phase angle (°)	-0.02	-0.79	0.76	0.12

The 95% limits of agreement were relatively narrow for most parameters, confirming good absolute reliability. Specifically, variables such as height (−0.32 to 0.34 cm), sarcopenic index (−0.45 to 0.43), phase angle (−0.79 to 0.76°), and visceral fat index (−0.92 to 0.97) demonstrated particularly tight agreement, indicating high measurement precision. Conversely, wider limits were observed for body weight (−3.92 to 4.37 kg), body fat percentage (−2.75 to 2.87%), muscle mass percentage (−2.64 to 2.55%), and total body water (−2.24 to 1.91%), reflecting greater biological and measurement variability at the individual level.

Importantly, comparison of the LoA with SWC values revealed that for all variables, the LoA exceeded the corresponding SWC, indicating that the magnitude of measurement error was greater than the smallest practically meaningful change. Consequently, the device appears to be more suitable for detecting moderate-to-large physiological changes than for identifying very small individual changes over time.

Overall, the Bland–Altman analysis confirmed a high level of concordance between the two testing sessions and provided strong evidence for the reproducibility and reliability of the multi-frequency bioelectrical impedance analyzer in assessing body composition among physically active young adult males.

The graphical representation (Figure 1) illustrates the Bland–Altman plots for all body composition indicators, showing the relationship between the mean of paired measurements and their differences. The bias line was located close to zero in all panels, while the majority of observations were distributed within the 95% limits of agreement, indicating satisfactory agreement between repeated measurements.

No evidence of proportional bias was observed, as the differences remained randomly distributed across the measurement range for all variables. Although slightly greater dispersion was evident for body weight, body fat percentage, muscle mass percentage, and total body water, the overall pattern remained consistent with acceptable measurement agreement.

The combined interpretation of Bland–Altman analysis, SWC comparison, ANOVA homogeneity testing, and effect size magnitude indicates that the instrument demonstrates high reliability for group-level assessments. However, because the LoA were consistently wider than the SWC thresholds, caution is warranted when interpreting very small individual-level changes, which may fall within the expected measurement error of the device.

DISCUSSION

This study aimed to establish a representative body composition pattern among male students of Physical Education and Sport using a multi-frequency BIA across key indicators. This objective was addressed through a comprehensive analytical approach that integrated multiple body composition parameters in order to provide a detailed and reliable profile of physically active young males.

Although BIA is widely recognized as a valid and practical method for assessing body composition, it is less precise compared to dual-energy X-ray absorptiometry (DXA) or magnetic resonance imaging (MRI). Its reliability can be influenced by several factors, including device calibration, operator proficiency, participant characteristics, and environmental conditions (32). Despite these limitations, BIA remains an advantageous method due to its simplicity, safety, mobility, affordability, and non-invasive nature (33). In contrast to DXA or MRI, BIA allows quick field assessments, making it particularly useful in educational and athletic environments. Moreover, BIA devices provide adjustable modes (standard or athletic) and incorporate gender, age, and height, while differences among devices are typically related to calibration, frequency spectrum, and electrode configuration (34).

By integrating results from multiple statistical analyses, this study provides a clearer understanding of the body composition profile of male students engaged in structured physical activity. The novelty lies in defining this pattern across ten distinct indicators: body fat, visceral fat, BMI, muscle mass, skeletal muscle mass, sarcopenic index, total body water, body weight, height, and phase angle. Although the sample was relatively homogeneous in terms

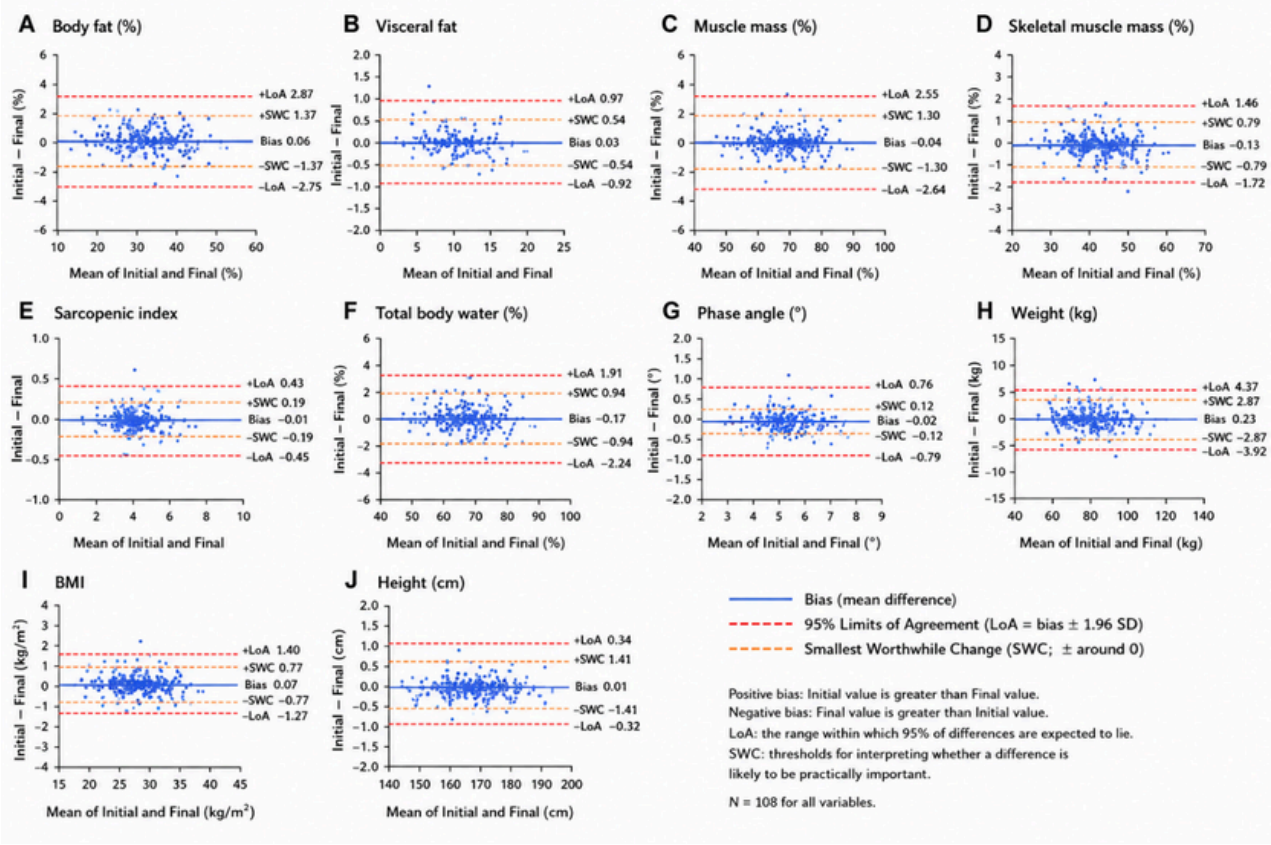


Figure 1. *Data distribution for each body composition indicator (A-J) at Bland-Altman analysis*

of age, height, and weight, inter-individual differences in body type, energy demands, and metabolic characteristics highlight the necessity of defining specific body composition reference patterns according to gender and activity level. Such data are particularly relevant in the context of modern lifestyle and technological changes that affect overall health, physical fitness, and well-being.

To establish structural framing for these parameters, the BIA device used in this study classifies results into three colour-coded categories—normal (green), below normal (orange), and above normal (red)—based on gender, age, and physical activity level (35), which simplifies clinical evaluation. For instance, for body fat percentage, values between 8% and 20% were considered normal. Approximately 60% of participants were within this range, while smaller proportions fell below 8% or above 20%. These results correspond closely to findings by Alarcón et al. (25), who reported similar body fat distributions among male university students. The mean body fat percentage in the present study (17.3%) was slightly higher than the 12% reported elsewhere (15), potentially due to differences in training volume or dietary habits.

Most participants exhibited visceral fat index values below the normal range (4–12.5), indicating favorable fat distribution. In terms of BMI, the majority of students fell within normal limits, with only a small proportion being underweight and roughly one-third classified as overweight. These trends align with previous studies conducted among male university students (20,25,28,36). The average BMI in our study corresponded closely to data reported in other populations of physically active young men.

Regarding muscle-related parameters, the majority of participants displayed normal or above-normal muscle mass and skeletal muscle mass, confirming the positive influence of regular physical activity. The sarcopenic index values were predominantly within normal limits, with only a few cases suggesting lower bone density and a potential risk for osteopenia. Total body water values were also within normal physiological limits, while phase angle results indicated a generally healthy cellular condition, although a few individuals presented values that may warrant further health monitoring.

Our findings correspond well with earlier studies conducted on similar populations. Petřeková et al. (18) reported body composition results for weight, skeletal muscle mass, body fat, total body water, and BMI comparable to those obtained in the present study. Cárdenas Quintana et al. (31), however, observed lower values for weight, height, and skeletal muscle mass but higher BMI, body fat, and visceral fat percentages. Similarly, Valenzuela Contreras et al. (11) reported comparable height and weight but higher fat percentages.

In addition, the Bland–Altman analysis performed in the present study demonstrated bias values close to zero and relatively narrow limits of agreement across all body composition indicators, supporting the stability and repeatability of the measurements over the eight-week interval. The absence of statistically significant differences between assessments, together with trivial effect sizes, further supports the temporal stability of the measured variables. Although the limits of agreement exceeded the corresponding SWC values for all variables, indicating limited sensitivity for detecting very small individual changes, the overall agreement between repeated measurements remained high, confirming the suitability of the device for monitoring body composition at the group level.

Interestingly, a previous study conducted at the same university by Leuciuc et al. (37), involving a smaller sample size and a more limited set of body composition variables, reported greater variability in participant characteristics. The larger sample and broader range of indicators included in the present investigation provide a more comprehensive characterization of body composition among male students of Physical Education and Sport.

In interpreting this collective dataset, it should also be noted that our sample included students participating in wrestling, combat sports, and rugby, disciplines that demand distinct anthropometric profiles, which may partly explain the observed variations across individual indicators and highlight the baseline diversity inherent to complex sporting cohorts.

Strengths, limitations, and implications

The main strength of this study lies in its inclusion of over 70% of all male students enrolled in Physical Education and Sport at Ștefan cel Mare University of Suceava, as well as in its comprehensive assessment of ten major body composition indicators. However, several limitations must be acknowledged: the study's single-institution context, a relatively small sample size, the narrow age range (19–25 years), and the exclusive focus on male participants. Moreover, differences in BIA device settings, calibration, and proprietary software algorithms may have influenced results.

In addition, the study did not include systematic monitoring of participants' voluntary physical activity outside structured academic requirements, which may have introduced uncontrolled variability in energy expenditure and body composition outcomes. Likewise, dietary intake during the intervention period was not controlled or monitored, limiting the ability to account for nutritional influences on body composition parameters. Furthermore, the potential influence of prior sporting background and accumulated sport experience was not explicitly controlled, which may have contributed to inter-individual differences, particularly given the heterogeneous engagement in sports such as wrestling, combat sports, and rugby among participants.

Future research should include larger, multi-institutional, and gender-diverse samples to enhance generalizability. Establishing normative reference values across different populations, levels of physical activity, and body weight categories could contribute significantly to public health and sports science. Future studies should also incorporate objective monitoring of physical activity levels (e.g., accelerometry or training logs), standardized dietary assessment protocols, and stratification according to prior sport experience in order to better isolate training effects from background-related adaptations.

Practical and public health relevance

Considering that over 80% of young adults globally do not meet recommended physical activity levels—which negatively impacts their body composition, health, and quality of life (38)—the results of this study emphasize the importance of continuous promotion of physical activity and healthy lifestyle habits among university students. The established reference values can serve as a practical tool for educators, coaches, and health professionals in monitoring and improving the physical status of young adults engaged in sports and physical education.

This study aimed to establish a representative pattern of body composition among physically active male university students enrolled in the Physical Education and Sport program, using a multi-frequency BIA. The results confirmed a high degree of consistency and reliability across ten key body composition indicators—body fat percentage, visceral fat index, BMI, muscle mass, skeletal muscle mass, sarcopenic index, total body water, weight, height, and phase angle.

The absence of statistically significant differences between pre- and post-test assessments, together with the bias values close to zero and the relatively narrow 95% limits of agreement identified through Bland–Altman analysis, supports the stability and reproducibility of the applied measurement method. These findings validate the main hypothesis that active male students maintain a consistent body composition profile, comparable to previously established healthy ranges reported in the literature.

The identified body composition values and agreement characteristics may serve as useful reference data for evaluating body composition in young, physically active males, particularly in sports and academic environments. Individuals whose body composition indicators fall within the observed ranges may be considered representative of this population, whereas substantial deviations may warrant additional medical, nutritional, or training-related evaluation. Furthermore, although the Bland–Altman analysis demonstrated high measurement reliability, the comparison between the limits of agreement and the smallest worthwhile change indicated that the device is better suited for detecting moderate-to-large changes than very small individual physiological fluctuations.

To build substantively upon these baseline patterns, future research should extend these findings by including larger and more heterogeneous samples, incorporating both sexes, different age categories, and various physical activity levels, to establish more comprehensive normative data for youth and adult populations in physical education and sport sciences. Longitudinal studies employing multiple assessment methods, including criterion techniques such as DXA, would further contribute to the validation and refinement of body composition reference values.

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Author Contributions

Conceptualization, V.L.F., E.V.L., D.P.F., V.T.G., T.D., D.P., and S.B.; Data curation, D.P. and S.B.; Formal Analysis, D.P. and S.B.; Funding acquisition, V.L.F., E.V.L., D.P.F., V.T.G., and T.D.; Investigation, V.L.F., E.V.L., D.P.F., V.T.G., and T.D.; Methodology, V.L.F., E.V.L., D.P.F., V.T.G., and T.D.; Project administration, V.L.F., E.V.L., D.P.F., V.T.G., and T.D.; Resources, V.L.F., E.V.L., D.P.F., V.T.G., and T.D.; Software, D.P., and S.B.; Supervision, V.L.F., E.V.L., D.P.F., V.T.G., T.D., D.P., and S.B.; Validation, V.L.F., E.V.L., D.P.F., V.T.G., T.D., D.P., and S.B.; Visualization, V.L.F., E.V.L., D.P.F., V.T.G., T.D., D.P., and S.B.; Writing – original draft, V.L.F., E.V.L., D.P.F., V.T.G., T.D., D.P., and S.B.; Writing – review & editing, V.L.F., E.V.L., D.P.F., V.T.G., T.D., D.P., and S.B.

Statement of Ethics

The study was approved by the Ethics Research Committee of Ștefan cel Mare University of Suceava (protocol code no. 64, issued on May 23, 2022). and conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent before participation.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availability

The data analyzed during this study are included in the published article. 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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ENDOTHELIUM-MEDIATED VASORELAXANT EFFECTS OF CHOKEBERRY (*ARONIA MELANOCARPA*) EXTRACT, JUICE, AND WASTE: AN EXPERIMENTAL STUDY IN ISOLATED RAT AORTA

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Aronia melanocarpa (chokeberry, aronia) is rich in polyphenols and associated with vascular protective effects; however, comparative data on different aronia-derived products and their mechanisms remain limited. This study aimed to compare the vasorelaxant effects of aronia berry extract (AE), aronia waste extract (AWE), and aronia juice (AJ), and to investigate their underlying mechanisms. Preparations from organically grown berries (western Serbia) were chemically characterized using spectrophotometric methods and HPLC. Vasorelaxant activity was assessed in isolated thoracic aorta rings from Wistar rats precontracted with potassium chloride (KCl) or phenylephrine (PE). All preparations induced concentration-dependent relaxation. In KCl-precontracted rings, AE showed the greatest potency (EC_{50} = 0.97 ± 0.02 mg/mL), followed by AJ (1.74 ± 0.08 mg/mL) and AWE (1.95 ± 0.12 mg/mL). In contrast, under PE-induced contraction, AWE exhibited the highest activity (EC_{50} = 0.58 ± 0.07 mg/mL), followed by AJ (0.96 ± 0.06 mg/mL) and AE (1.27 ± 0.11 mg/mL). Pre-incubation with the nitric oxide synthase inhibitor L-NAME significantly attenuated relaxation responses, increasing EC_{50} values for AWE (0.59 ± 0.03 to 2.37 ± 0.01 mg/mL), AE (0.92 ± 0.01 to 4.38 ± 0.05 mg/mL), and AJ (0.95 ± 0.02 to 2.78 ± 0.03 mg/mL) ($p < 0.01$), indicating a key role of endothelium-derived nitric oxide. These findings demonstrate that aronia-derived products, including processing waste, exert potent NO-mediated vasorelaxant effects and may represent promising functional ingredients for improving vascular function. Further clinical studies are warranted to confirm these effects in humans.

Keywords: *Aronia melanocarpa,* waste extract, vasorelaxation, nitric-oxide pathway, rat aorta

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INTRODUCTION

Cardiovascular diseases (CVD) remain a leading cause of morbidity and mortality worldwide, with endothelial dysfunction and impaired vasorelaxation representing critical early events in their pathogenesis (1,2). Endothelium-derived nitric oxide (NO) is a key regulator of vascular tone, and reduced NO bioavailability is closely associated with hypertension, atherosclerosis, and other vascular disorders. Recent reviews have highlighted that impaired endothelium-dependent vasodilation and chronic inflammation are central to the initiation and progression of CVD, and that interventions targeting endothelial function are a promising strategy for prevention and management (2). Increasing evidence suggests that dietary strategies rich in bioactive phytochemicals may improve endothelial function and promote vasorelaxation. A recent narrative review concluded that polyphenol-rich foods, including berries, consistently improve flow-mediated dilation, a clinical measure of endothelial function, and are associated with enhanced NO bioavailability and reduced cardiovascular risk (4).

Berries are recognized as an important dietary source of polyphenolic compounds, including anthocyanins, flavonols, and phenolic acids, which have been consistently associated with cardiovascular protection (5,6). Anthocyanin-rich products and other polyphenol-rich foods have been shown to enhance the expression and phosphorylation of endothelial nitric oxide synthase (eNOS), increase NO production, and improve vasodilatory responses, thereby mitigating endothelial dysfunction and oxidative stress (4,7). Experimental and clinical studies indicate that regular consumption of berry fruits and berry-derived products improves endothelial function, enhances NO-mediated vasodilation, and reduces oxidative stress and vascular inflammation (4,8). These vascular effects are attributed to both direct modulation of endothelial signaling pathways, including eNOS activation, and indirect antioxidant mechanisms (9).

Among berry species, *Aronia melanocarpa* [Michx.] Elliott (aronia, chokeberry) has attracted considerable scientific interest due to its exceptionally high content of polyphenols, particularly anthocyanins and proanthocyanidins (10,11). Aronia preparations, including juices and standardized extracts, have demonstrated strong antioxidant, anti-inflammatory, and vasoprotective properties in various experimental models (12,13). In isolated vascular tissues, aronia juice induces potent endothelium-dependent vasorelaxation, primarily mediated by enhanced NO release following redox-sensitive activation of endothelial nitric oxide synthase (eNOS) through Src/PI3K/Akt signaling pathways (14). More recent evidence further confirms that polyphenol-rich *Aronia melanocarpa* juice sustains eNOS activation via phosphorylation and increased expression in endothelial cells, supporting its potential role in vascular protection (15).

In vitro studies further support these observations, showing that polyphenol-rich aronia extracts significantly increase NO production and eNOS phosphorylation in cultured endothelial cells in a concentration-dependent manner (16). Moreover, human intervention studies have reported improvements in endothelial function, assessed by flow-mediated dilation, as well as reductions in blood pressure following consumption of aronia-based products or anthocyanin-rich berry extracts (17,18). Collectively, these findings highlight the potential of aronia polyphenols to modulate vascular tone and endothelial homeostasis.

In recent years, attention has increasingly focused on valorizing berry processing by-products, particularly chokeberry waste materials, as sustainable sources of bioactive compounds with potential vascular benefits. Pomace and other residues remaining after juice production retain substantial amounts of polyphenols, including anthocyanins, flavan-3-ols, and phenolic acids, often at concentrations comparable to or even exceeding those found in the corresponding juices (11,19,20). Recent studies further emphasize that chokeberry pomace represents a rich and underutilized source of antioxidant phytochemicals suitable for functional food and nutraceutical applications (21,22). Utilizing chokeberry waste extracts not only supports circular economy and sustainable food production but also represents a promising approach for the development of functional ingredients and nutraceuticals.

Despite growing evidence supporting the vasorelaxant properties of berry-derived polyphenols, the relative contribution of different aronia preparations—including juices, whole-fruit extracts, and waste-derived extracts—and the precise molecular mechanisms underlying their vascular effects remain incompletely understood. Further investigation into these mechanisms may provide valuable insights for the development of novel dietary strategies and functional products that aim to improve vascular function and cardiovascular health.

The novelty of the present study lies in the direct comparative evaluation of vasorelaxant effects of three different aronia-derived preparations (berry extract, juice, and processing waste extract) under identical experimental conditions in isolated rat aorta. In particular, this study

provides new insight into the vascular activity of chokeberry processing by-products (pomace-derived extract), which remain insufficiently investigated despite their high polyphenolic content. In addition, the study contributes to understanding the relative contribution of NO-mediated mechanisms in these preparations under both receptor-mediated (phenylephrine) and depolarization-induced (KCl) contraction models.

METHODS

Plant material, extracts, and juice preparation and chemical characterization

Aronia (chokeberry) juice and extracts were prepared from organically cultivated berries harvested in western Serbia. Juice was first obtained from frozen–thawed fruits using a Hydropress 90 L (Speidel, Germany), while the remaining pressed pomace was collected as by-product material for waste extract production. Both whole berries and pomace were dried at 40°C for 48 h (Instrumentaria ST 01/02, Croatia), milled, and sieved (0.75 mm).

Dried samples were macerated with 50% ethanol (1:20, w/v) under previously optimized conditions for polyphenol recovery (23–25). Ethanol was removed under reduced pressure at 60°C (Buchi rotavapor R-114). The juice, whole berry extract, and waste extract were then freeze-dried after pre-freezing at −80°C, using a lyophilizer (Beta 1-8 Freeze Dryer, Martin Christ, GmbH, Osterode am Harz, Germany) at −60°C and low pressure.

The final lyophilized preparations, aronia berry extract (AE), aronia waste extract (AWE), and aronia juice (AJ), were stored in sealed glass containers at −4°C until further analysis.

The chemical composition of the preparations, including total polyphenols, flavonoids, anthocyanins, proanthocyanidins, and individual compounds, was determined using spectrophotometric methods and high-performance liquid chromatography (HPLC), following the protocols reported by Milutinović et al., 2024 (25).

Vasorelaxant activity of the AE, AWE, and AJ

The vasorelaxant activity of chokeberry fruit extracts and juice was evaluated using isolated thoracic aorta rings from Wistar rats. The effects of the chokeberry extract (AE), the extract obtained from the fruit residue after juice pressing (AWE), and chokeberry juice (AJ) were assessed on induced contractions of isolated aortic segments. The influence of these preparations on vascular contractility was examined in the presence of KCl, the nitric oxide synthase inhibitor L-NAME, and phenylephrine (PE). Verapamil, a calcium channel blocker, was used as a positive control. All experiments were performed using thoracic aortic rings isolated from six independent male Wistar rats (n = 6). Throughout the manuscript, n refers to the number of biological replicates (individual animals).

Experimental animals

All animal experimental procedures were approved by the Ethics Committee and conducted in accordance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes (approval issued by the Ministry of Agriculture, Forestry and Water Management – Veterinary Directorate, decision No. 323-07-04034/2018-05/2).

Male Wistar albino rats (180–220 g) were obtained from the animal facility of the Scientific Research Center for Biomedicine, University of Niš Faculty of Medicine. Animals were housed under standard laboratory conditions (20–24°C, 12 h light/dark cycle) with free access to food and water. Food was withdrawn 24 h prior to the experiment. The study involved a total of six male Wistar rats.

Isolation and preparation of thoracic aorta rings

The isolation of thoracic aorta rings was performed as previously described (26). Briefly, rats were sacrificed, the thoracic cavity was opened, and the thoracic aorta was carefully excised. The tissue was immediately placed in ice-cold, oxygenated Krebs solution, cleaned of surrounding connective tissue, and cut into rings 3–5 mm in length.

Aortic rings were mounted in a 20 mL organ bath containing Krebs solution under a resting tension of 1 g and allowed to equilibrate for at least 30 min at 37°C before the experiments. The Krebs solution consisted of 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2 \cdot 2H_2O$, 2.6 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 5.5 mM glucose (pH 7.4), continuously aerated with 95% O_2 and 5% CO_2.

Changes in isometric tension were recorded using a force transducer (TSZ-04-E, Experimetria Ltd., Budapest, Hungary) and analyzed with the SPEL Advanced ISOSIS data acquisition system (Experimetria Ltd., Budapest, Hungary).

Assessment of vascular contractility and vasorelaxation

To evaluate the effects of chokeberry preparations on vascular reactivity, aortic rings were precontracted with potassium chloride (K^+ 80 mM) or phenylephrine (PE, 1 µM). Vasorelaxant responses to AE, AWE, and AJ were examined in endothelium-intact rings.

To investigate the involvement of nitric oxide (NO), aortic

rings were preincubated with L-NAME (0.1 mM) for 20 min before contraction with phenylephrine (PE, 1 µM). Vasorelaxant responses to the tested preparations were subsequently evaluated under the same experimental conditions.

To verify endothelial integrity, aortic rings were precontracted with phenylephrine (1 µM), followed by the addition of acetylcholine. Only rings exhibiting more than 70% relaxation in response to acetylcholine were considered endothelium-intact and included in subsequent experiments, whereas rings not meeting this criterion were excluded.

Chokeberry extracts and juice were applied cumulatively at concentrations ranging from 0.001 to 1 mg/mL. Each concentration was allowed to act until the vascular response reached a stable plateau before adding the subsequent concentration. The next concentration was applied only after no further change in isometric tension was observed, ensuring that cumulative concentration–response curves were generated under steady-state conditions.

Vasorelaxation was expressed as the percentage reduction of the initial contraction induced by the respective vasoconstrictor, which was taken as 100%. Accordingly, in KCl-precontracted rings, relaxation was calculated relative to the KCl-induced contraction, whereas in phenylephrine (PE)-precontracted rings, relaxation was calculated relative to the PE-induced contraction. Verapamil was used as a positive vasorelaxant control and was applied cumulatively at concentrations ranging from 0.01 to 1 mg/mL.

The inhibitory effects of the tested preparations were calculated from the area under the contraction curve relative to baseline and expressed as percentage inhibition (27,28).

The concentration range (0.001–1 mg/mL) was selected based on preliminary experiments as it was considered suitable for assessing concentration-dependent vasorelaxant responses while minimizing potential nonspecific physicochemical effects associated with higher concentrations of crude herbal preparations, including reduced solubility, precipitation of constituents, increased viscosity, and alterations of the organ bath medium.

Statistical analysis

Statistical evaluation was performed using SPSS software (version 20.0; SPSS Inc., Chicago, IL, USA). Data are presented in tables and figures as mean ± standard deviation. The normality of data distribution was assessed with the Shapiro–Wilk test. Differences between two groups were analyzed using the independent Student's t-test for normally distributed data or the Mann–Whitney test for non-normal data. Comparisons between multiple groups were conducted using either one-way ANOVA or the Kruskal–Wallis test, depending on the data suitability. When ANOVA was applied, post-hoc tests were conducted for pairwise group comparisons. Statistical significance was set at $p < 0.05$.

RESULTS

Chemical characterization of the AE, AWE, and AJ

The phytochemical composition of AE, AWE, and AJ was comprehensively characterized in our previous study using spectrophotometric assays and HPLC analysis (25). A concise summary of the major bioactive constituents is presented in Table 1. All preparations contained high levels of polyphenolic compounds, including phenolic acids, flavonoids, anthocyanins, and proanthocyanidins. Among the investigated preparations, AWE exhibited the highest content of most quantified polyphenolic constituents, whereas differences in the relative abundance of individual compounds were observed among AE, AWE, and AJ. These compositional differences may contribute to the differences in their vasorelaxant activities observed in the present study.

Vasorelaxant activity of the AE, AWE, and AJ

The vasorelaxant effects of aronia extracts and juice on isolated rat thoracic aorta were evaluated in the presence of elevated K^+ concentration (80 mM) in the first experimental series (Figure 1). Contraction of aortic smooth muscle was induced by adding KCl to the organ bath, and the response progressively decreased following the cumulative application of aronia preparations at concentrations of 0.001 to 1 mg/mL.

Among the tested samples, AE demonstrated the most pronounced activity, with an EC_{50} value of 0.97 ± 0.02 mg/mL, producing the highest and statistically significant relaxation of aortic smooth muscle ($p < 0.01$). Conversely, AWE exhibited the weakest vasorelaxant potential against KCl-induced contractions (EC_{50} = 1.95 ± 0.12 mg/mL). Aronia juice (AJ) showed slightly greater efficacy compared to AWE, with an EC_{50} value of 1.74 ± 0.08 mg/mL.

Verapamil, used as a positive vasorelaxant control, produced a concentration-dependent relaxation response, with an EC_{50} of 0.00077 ± 0.00001 mg/mL ($p < 0.01$). Differences among EC_{50} values obtained for AE, AWE, AJ, and verapamil were assessed using one-way ANOVA, which

Table 1. *Total phenolics, anthocyanins, flavonoids, proanthocyanidins, and HPLC quantification of individual compounds in AE, AWE, and AJ (adapted from Milutinović et al.,2024 (25)).*

	AE	AWE	AJ	*P*
Compounds	Amounts			
Total phenolics content*	261.71 ± 35.26[a]	605.67 ± 64.58[b]	495.91 ± 25.85[c]	0
Total anthocyanins**	95.24 ± 15.33[a]	423.81 ± 23.17[b]	111.32 ± 10.72[a]	0
Total flavonoids***	39.25 ± 6.57[a]	29.36 ± 1.83[a]	29.21 ± 1.89[a]	0.038
Total proanthocyanidins****	97.1 ± 5.56[a]	129.28 ± 21.9[a]	105.32 ± 5.51[a]	>0.05
Chlorogenic acid#	2.29 ± 0.35[a]	3.72 ± 0.47[b]	2.77 ± 0.36[a]	0.012
Cyanidin-3-*O*-galactoside#	0.88 ± 0.07[a]	5.21 ± 0.4[b]	1.32 ± 0.07[a]	0
Cyanidin-3-*O*-glucoside#	0.12 ± 0.02[a]	0.77 ± 0.1[b]	0.15 ± 0.01[a]	0
Cyanidin-3-*O*-arabinoside#	0.37 ± 0.06[a]	2.31 ± 0.14[b]	0.41 ± 0.05[a]	0
Quercetin-3-*O*-rutinoside#	0.21 ± 0.03[a]	0.04 ± 0.00[b]	0.12 ± 0.02[c]	0
Quercetin-3-*O*-galactoside#	0.13 ± 0.01[a]	Traces	0.01 ± 0.00[b]	0
Quercetin-3-*O*-glucoside#	0.14 ± 0.01[a]	Traces	0.02 ± 0.00[b]	0

Each value represents the mean of n = 3 ± standard deviation.
* mg GAE/g of AE, AWE or AJ.
** mg cyanidin-3-O-glucoside equivalents/g of AE, AWE or AJ.
*** mg catechin/g of AE, AWE or AJ.
**** mg catechins equivalent/g of AE, AWE or AJ.
# mg/g of AE, AWE or AJ.
[a,b,c] Different letters in the rows indicate a statistically significant difference in the content of active compounds between AE, AWE or AJ, based on the ANOVA test (Tukey post-hoc, $p < 0.05$).

Table 2. *EC_{50} values for KCl-induced and PE-induced contractions obtained with AE, AWE, AJ, and verapamil (positive control)*

	EC_{50} values for KCl-induced contractions (mg/ml)	EC_{50} values for FE-induced contractions (mg/ml)
AE	0,97 ± 0,02 [a]	1,27 ± 0,11 [a]
AWE	1,95 ± 0,12 [b]	0,58 ± 0,07 [b]
AJ	1,74 ± 0,08 [b]	0,96 ± 0,06 [c]
Verapamil	0,00077 ± 0,00001[c]	

Statistical significance was assessed by one-way ANOVA followed by Tukey's post-hoc test, and data are expressed as mean ± SEM. Different superscript letters within the same column denote statistically significant differences in EC_{50} values (Tukey's post-hoc test; $p < 0.01$). Specifically, groups sharing different letters differ significantly, while groups sharing the same letter do not differ significantly.
Abbreviations: AE, aronia fruit extract; AWE, aronia waste extract; AJ, aronia juice; PE, phenylephrine

confirmed statistically significant variation between treatments (Table 2).

In the second experimental series, aortic rings were precontracted by the addition of phenylephrine (PE; 1 µM) to the organ bath. Verapamil, used as a positive vasorelaxant control, demonstrated a concentration-dependent relaxation response.

The strongest vasorelaxant effect against PE-induced contractions of rat aorta was observed for AWE, which exhibited the lowest EC_{50} value (0.58 ± 0.07 mg/mL, $p < 0.01$). The chokeberry extract (AE) produced a weaker relaxation response (EC_{50} = 1.27 ± 0.11 mg/mL), whereas the juice demonstrated greater efficacy (EC_{50} = 0.96 ± 0.06 mg/mL) (Figure 2).

A comparison of EC_{50} values for the extracts, juice, and verapamil in the PE-induced contraction model is shown in Table 2.

The vasorelaxant activity of AE, AWE, and AJ was further evaluated in the presence of the nitric oxide synthase inhibitor L-NAME (0.1 mM), applied after phenylephrine-induced precontraction, to clarify the involvement of NO-mediated mechanisms. Since endogenous NO contributes to vascular relaxation, inhibition of its synthesis markedly attenuated the effects of all tested preparations.

These results suggest that the vasorelaxant responses of AE, AWE, and AJ are predominantly NO-dependent (Figure 3). Under PE stimulation, AWE showed the highest potency (EC_{50} = 0.59 ± 0.03 mg/mL), whereas L-NAME significantly shifted its EC_{50} to 2.37 ± 0.01 mg/mL ($p < 0.01$).

Similarly, blockade of NO production increased EC_{50} values for AE and AJ to 4.38 ± 0.05 mg/mL and 2.78 ± 0.03 mg/mL, respectively, compared with significantly lower values obtained under control conditions (0.92 ± 0.01 mg/mL and 0.95 ± 0.02 mg/mL; $p < 0.01$).

DISCUSSION

Elevated blood pressure is a major risk factor for cardiovascular diseases and stroke. Even a modest reduction in systolic blood pressure by 3 mmHg has been estimated to lower stroke mortality by 8% and coronary heart disease mortality by 5% (29). A direct relationship between blood pressure and cardiovascular mortality has been observed in adults aged 40–89 years (30). Maintaining lower blood pressure throughout life is therefore crucial, highlighting the importance of identifying natural compounds and preparations that can help reduce blood pressure. Early dietary interventions in mild hypertension may also delay the need for pharmacological treatment (31).

Consequently, research into the effects of bioactive natural products on vascular health is of considerable interest (32). Several studies have shown that fruits and fruit juices, particularly berries such as blackcurrants and cranberries, can contribute to blood pressure reduction (33,34). Blackcurrants have also been reported to exhibit strong vasorelaxant properties (33).

Chokeberry juice and extracts are highly rich in polyphenolic compounds and exhibit strong antioxidant activity, as previously reported (25), indicating their potential utility in conditions linked to oxidative stress. Since oxidative stress plays a key role in the development of hypertension, suppression of reactive oxygen species may contribute to lowering blood pressure (35,36). Epidemiological and clinical evidence suggests that regular consumption of anthocyanin-rich foods is associated with a lower risk of cardiovascular diseases, including coronary heart disease (5,37,38). A recent meta-analysis of prospective cohort studies reported that higher dietary anthocyanin intake correlates with reduced incidence of coronary heart disease and total cardiovascular events (39).

Clinical trials further support these findings, showing that anthocyanin-rich interventions, such as blackcurrant extracts, improve endothelial function and modestly reduce blood pressure and inflammatory markers (40,41). These data indicate that dietary anthocyanins may contribute to cardiovascular protection and could be considered as part of preventive or adjunctive strategies. Nonetheless, long-term, large-scale studies are needed to confirm their effects on specific cardiovascular outcomes such as myocardial infarction.

Chokeberry preparations have been shown to reduce oxidative stress and vascular inflammation, major contributors to cardiovascular dysfunction, suggesting significant cardioprotective potential through their antioxidant and anti-inflammatory properties. Both in vitro and *in vivo* studies indicate that polyphenol-rich chokeberry extracts and juices improve endothelial function, inhibit pro-inflammatory pathways, and decrease oxidative damage in vascular tissues (42–44). These findings support the potential use of chokeberry products as functional dietary interventions for cardiovascular health.

Emerging evidence suggests that chokeberry preparations exhibit significant vasorelaxant activity, predominantly through endothelium-dependent mechanisms involving increased NO bioavailability, activation of endothelial nitric oxide synthase (eNOS), and attenuation of oxidative stress in vascular tissues (15,45). These effects are primarily attributed to enhanced endothelial nitric oxide (NO) release (46,47), although K^+ channel activation has also been implicated in mediating polyphenol-induced relaxation (33,48,49). Given the central role of K^+ channels in vascular smooth muscle contraction (50), it is likely that part of the vascular effects of chokeberry preparations is mediated via K^+ channel activation. Polyphenols can modulate vascular tone by inhibiting Ca^{2+} channels and/or activating K^+ channels in vascular cells (51).

Rhythmic contractions of vascular smooth muscle can occur spontaneously or in response to agonists (52,53). These contractions are preceded by membrane hyperpolarization through Ca^{2+}-activated K^+ channels (54,55). For example, phenylephrine (PE) induces rhythmic contractions via membrane depolarization, leading to Ca^{2+} influx through voltage-dependent channels (53,56). PE stimulation of α-adrenergic receptors causes smooth muscle depolarization and vasoconstriction; conversely, activation of endothelial NO synthase (eNOS) by intracellular Ca^{2+} triggers NO release, which promotes K^+ efflux via protein kinase G and subsequently repolarizes vascular smooth muscle through K^+ channel opening (57).

Our results demonstrate that chokeberry preparations exhibit significant vasorelaxant activity in the isolated rat aorta model. The observed relaxation was predominantly mediated by nitric oxide (NO), as evidenced by the marked attenuation of vasorelaxation following administration of L-NAME, a nitric oxide synthase inhibitor. This finding indicates that NO release from the endothelium plays a central role in the mechanisms underlying the vascular effects of chokeberry extracts and juice. Furthermore, relaxation responses were more pronounced in phenylephrine-induced contractions compared to those induced by KCl, although AE, AWE, and AJ effectively relaxed aortic smooth muscle in both conditions.

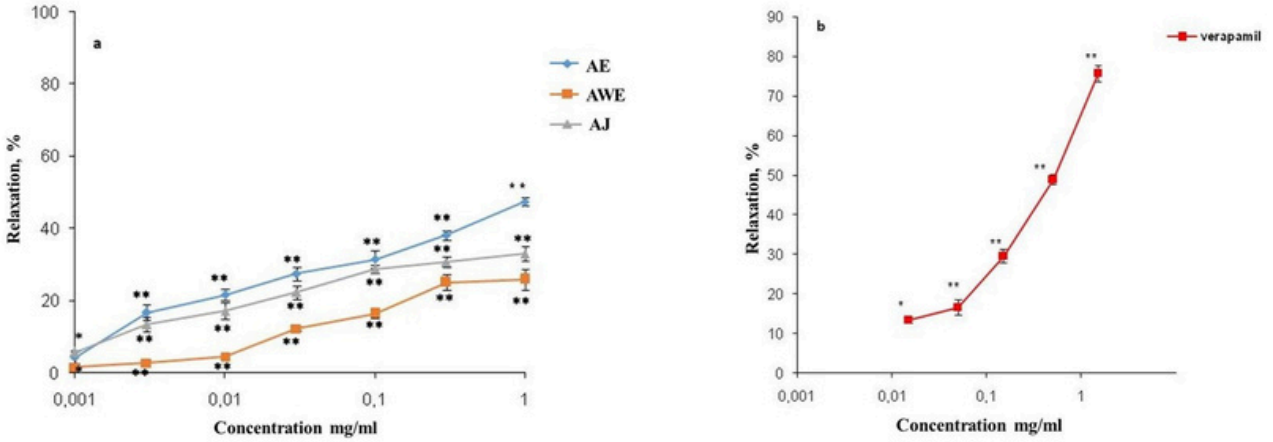


Figure 1. *Relaxant effects of AE, AWE, AJ (a) on KCl-induced contractions of isolated rat aorta. The concentration–response curve of verapamil, included as the positive control, is presented separately (b) to facilitate direct comparison among the three chokeberry preparations. Each point represents the mean ± SD of responses obtained from thoracic aortic rings isolated from six independent rats (n = 6) (Student's t-test, *p < 0.05, **p < 0.01).*

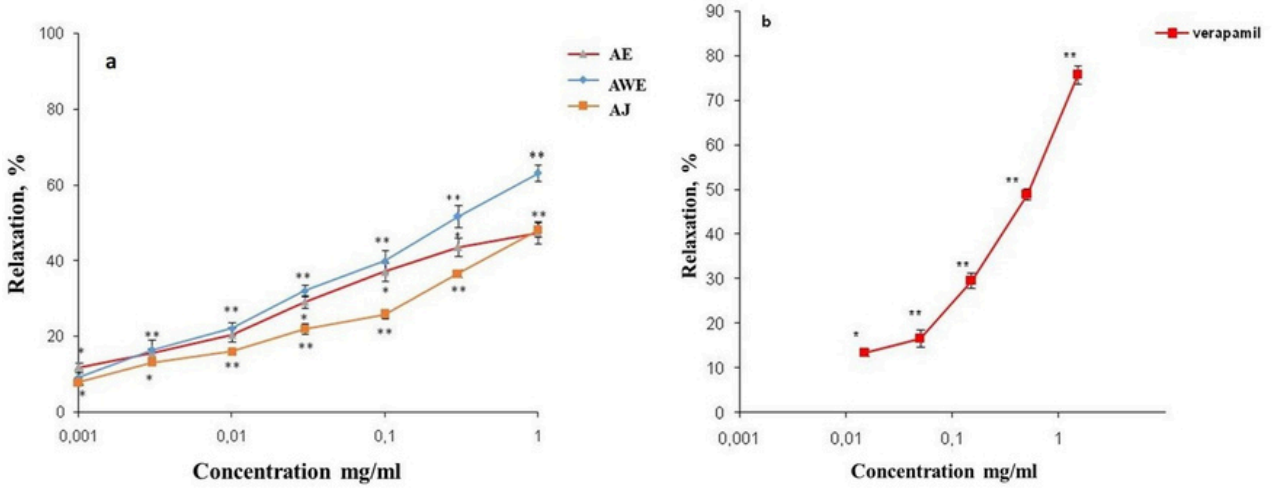


Figure 2. *Relaxant effects of AE, AWE, and AJ (a) on PE-induced contractions of isolated rat aorta. The concentration–response curve of verapamil, included as the positive control, is presented separately (b) to facilitate direct comparison among the three chokeberry preparations. Each point represents the mean ± SD of responses obtained from thoracic aortic rings isolated from six independent rats (n = 6) (Student's t-test, *p < 0.05, **p < 0.01).*

These results are consistent with previous studies demonstrating that polyphenol-rich berry extracts induce endothelium-dependent vasorelaxation through enhanced NO bioavailability. For example, flavonoid-rich extracts from berries such as blackcurrant and blueberry have been shown to increase endothelial nitric oxide synthase (eNOS) activity and improve nitric oxide-mediated relaxation in isolated vascular tissues (47,58,59). Similar mechanisms have been proposed for anthocyanins, which can stimulate NO release and improve endothelial function in both experimental and clinical settings (7,39).

The greater vasorelaxation observed in phenylephrine-induced contractions compared to KCl-induced contractions is also supported by the literature. Phenylephrine triggers contraction via α-adrenergic receptor activation and subsequent increases in intracellular Ca^{2+}, whereas KCl depolarizes the membrane more directly through the opening of voltage-gated calcium channels (53,56). Endothelium-dependent mechanisms, particularly NO release, tend to have a stronger modulatory effect in agonist-induced contractions than in depolarization models, which aligns with our findings.

Moreover, polyphenols have been shown to exert vascular effects not only through NO-dependent pathways but also via modulation of ion channels involved in smooth muscle tone. Specifically, activation of potassium (K^+) channels and inhibition of calcium (Ca^{2+}) influx have been implicated in the vasorelaxant actions of berry polyphenols (48,49). These

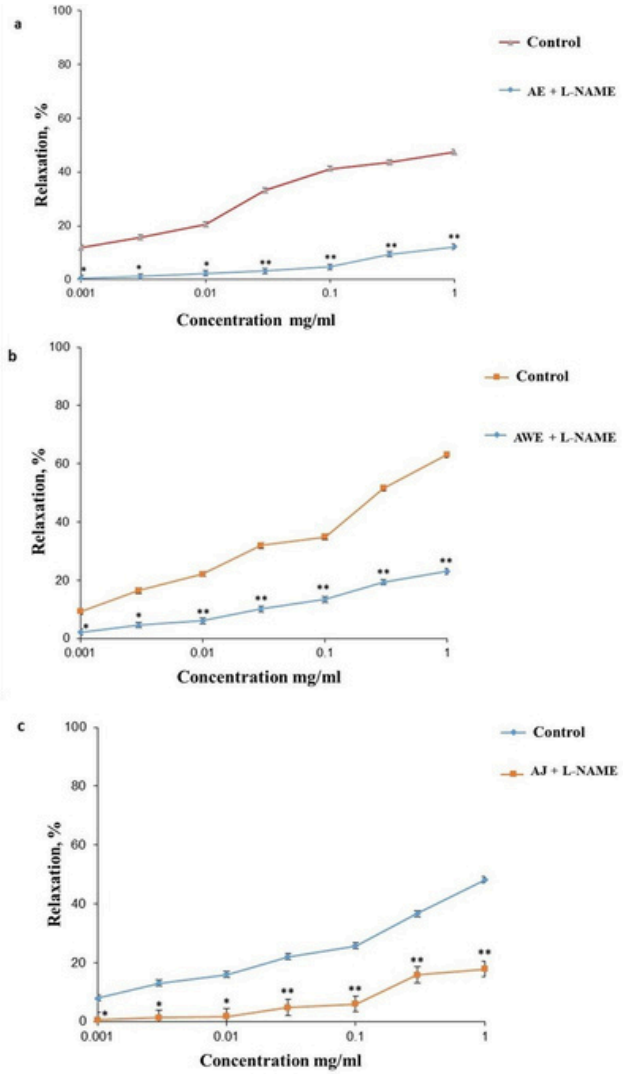


Figure 3. *Relaxant effects of AE (a), AWE (b), and AJ (c) on contractions of isolated rat aorta in the presence of L-NAME. Each point represents the mean ± SD of responses obtained from thoracic aortic rings isolated from six independent rats (n = 6) (Student's t-test, *p < 0.05, **p < 0.01).*

mechanisms likely contribute to the robust relaxation responses seen in our phenylephrine model and further support the cardiovascular relevance of chokeberry preparations.

Taken together, our findings reinforce the concept that chokeberry extracts and juice can positively influence vascular function through multiple converging pathways, primarily related to NO bioavailability and endothelial modulation. This aligns with growing evidence that polyphenol-rich diets, especially those containing anthocyanins, are beneficial for vascular health and may help mitigate risk factors for hypertension and other cardiovascular diseases.

Although concentration-dependent vasorelaxation was observed for all tested preparations, the concentration–response curves did not reach a clear plateau within the investigated concentration range, indicating that maximal vasorelaxation may not have been achieved. Previous studies have evaluated higher concentrations of crude plant extracts, including 0.01–10 mg/mL for Alchemilla vulgaris extracts (60) and up to 16.91 mg/mL for a polyphenol-rich extract of Steganotaenia araliacea (61), demonstrating that broader concentration ranges can be employed in studies of vascular reactivity. However, because the present study investigated complex crude aronia-derived preparations, concentrations above 1 mg/mL were not evaluated in order to minimize nonspecific physicochemical effects, such as increased viscosity, reduced solubility, precipitation of constituents, and alterations of the organ bath medium, which could interfere with the interpretation of vascular responses. Therefore, the absence of a clear plateau should be considered a limitation of the present study. Future investigations should evaluate a broader concentration range and/or purified fractions to better define the maximal vasorelaxant efficacy of these preparations.

Despite the valuable mechanistic insights provided in this study, several additional limitations should be acknowledged. Although the involvement of nitric oxide was investigated using L-NAME, other potential mechanisms, including endothelium removal, prostaglandin-mediated pathways, and potassium or calcium channel modulation, were not evaluated. Therefore, while our findings strongly suggest a predominant role of NO-mediated signaling, the contribution of other endothelial- and smooth muscle-dependent mechanisms cannot be excluded. Further studies investigating these pathways are warranted to provide a more comprehensive understanding of the vasorelaxant mechanisms of aronia-derived preparations.

The results demonstrated significant vasorelaxant activity of chokeberry preparations in the isolated rat aorta model. Relaxation was largely mediated by nitric oxide (NO), as evidenced by the reduction in aortic relaxation following L-NAME, an NO synthase inhibitor. Relaxation responses were more pronounced in phenylephrine-induced contractions compared to KCl-induced contractions, yet extract, waste extract, and juice effectively relaxed the aortic smooth muscle in both models. These findings suggest that aronia berry extract, waste by-product, and juice may be beneficial for blood pressure regulation or as an adjunct to antihypertensive therapy, although confirmation in clinical studies is required.

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Author Contributions

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Statement of Ethics

All experimental procedures were approved by the Ethics Committee and conducted in accordance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes (approval issued by the Ministry of Agriculture, Forestry and Water Management – Veterinary Directorate, decision No. 323-07-04034/2018-05/2).

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 included in the article.

Statement of Generative AI Use

No generative AI was used.

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ORIGINAL ARTICLE

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ATTITUDES AND PRACTICES TOWARDS COVID-19 AND SEASONAL FLU VACCINES AND VACCINATION IN THE GENERAL POPULATION AND AMONG MEDICAL PROFESSIONALS

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The COVID-19 pandemic has led to the development of several types of vaccines, among which messenger RNA (mRNA)-based vaccines have represented a significant breakthrough. This study aimed to evaluate attitudes towards COVID-19 and seasonal influenza vaccination among the general population and individuals with a medical background. A questionnaire was developed to collect sociodemographic data, personal health information, vaccination status, and information on participants' attitudes towards and knowledge of vaccines. The profile of the typical participant was an employed woman with tertiary education, living with another person in an urban area and reporting generally good health. The typical participant had received at least two doses of the Sputnik V vaccine. Participants generally expressed favourable attitudes towards regular COVID-19 vaccination. The results showed no apparent differences between participants with and without a medical background regarding vaccine choice, the decision to receive a third dose, or seasonal influenza vaccination. Similarly, no apparent differences were observed in attitudes towards the AstraZeneca vaccine or mandatory COVID-19 vaccination between respondents with and without a medical background.

Keywords: COVID-19, seasonal flu, vaccine, attitudes toward vaccination

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INTRODUCTION

The COVID-19 pandemic, caused by the SARS-CoV-2 virus, has profoundly impacted global health, leading to widespread illness, disruption of daily life, and significant socio-economic challenges. Just before the outbreak, in 2019, the WHO recognized vaccine hesitancy as a threat to global health. According to the WHO, vaccine hesitancy "threatens to reverse progress made in tackling vaccine-preventable diseases." The pandemic may have influenced these trends. Before the COVID-19 pandemic, the prevalence of vaccine hesitancy, as assessed using the conventional Parental Attitudes toward Childhood Vaccines (PACV) Scale, ranged from 5% to 34.7% across different countries. In Turkey, vaccine hesitancy and refusal have increased over the past decade; cases of vaccination refusal were previously rare but have risen rapidly since 2015. A survey conducted in Turkey between September and December 2021 found that 9.38% of parents were hesitant about vaccination, while 70.14% reported needing additional information regarding childhood immunization (1). According to Higgins et al., vaccination hesitancy and parental attitudes toward mandatory (routine) immunization in Colorado, USA, remained similar before and during the COVID-19 pandemic, at approximately 20.4%, a finding consistent with European data (2). In Europe, influenza vaccination coverage among healthcare workers (HCWs) varies by season; across the 2015/2016, 2016/2017, and 2017/2018 seasons, coverage ranged from 16% to 63% (median 30%). Belgium, England, and Wales have traditionally maintained good coverage (close to the WHO target level), while Italy, Norway, Hungary, Slovenia, and Eastern European countries generally report the lowest rates (3). In the 2021 season, England recorded the highest coverage among HCWs, at 76.4%. Over the period 2018–2023, vaccination coverage among HCWs in England ranged from 69% in 2018 to 49.6% in 2023, reaching a peak of 76.79% in 2021. Ireland likewise reported one of the highest HCW seasonal influenza vaccination rates in Europe, ranging from 42% to 71.28% over the same period, with a rate of 48.3% in 2023 (4). The greatest global success in seasonal influenza immunization among HCWs was achieved during the 2021 season. According to an Italian study, approximately 30.5% of HCWs reported changing their minds about influenza vaccination during the COVID-19 pandemic and subsequently chose to be vaccinated (5). It is a paradox that, although a high percentage of HCWs acknowledge the importance of influenza vaccination, coverage still falls short of the target level.

Public health strategies such as testing, contact tracing, quarantine, and social distancing play a crucial role in controlling the spread of the virus. Additionally, medical management includes supportive care for mild cases, hospitalization for severe cases, and the use of antiviral medications or monoclonal antibodies in certain situations (6). Nonetheless, widespread vaccination campaigns represent a key component in preventing severe illness and reducing the overall impact of the pandemic on public health.

To date, the European Medicines Agency (EMA) has authorized six COVID-19 vaccines for use and marketing, while the World Health Organization (WHO) is reviewing applications from 14 manufacturers for emergency use listing and/or prequalification. Depending on the production technology, vaccines differ in formulation and storage requirements. Vaccines based on innovative technology include messenger RNA (mRNA) vaccines. Comirnaty® (developed by BioNTech and Pfizer) and Spikevax (formerly the COVID-19 Moderna vaccine), both mRNA-based, were available for administration in Serbia. Viral vector vaccines available in the Republic of Serbia included Vaxzevria® (formerly the Oxford–AstraZeneca COVID-19 vaccine) and Gam-KOVID-Vac (Sputnik V), whereas Jcovden® (formerly the Janssen COVID-19 vaccine) was not marketed in the country. The inactivated SARS-CoV-2 vaccine (Vero cell), manufactured by Sinopharm (BIBP), was also available to citizens of the Republic of Serbia. In addition, vaccines based on viral protein subunit and/or recombinant protein subunit platforms are currently available, while ongoing research may lead to the development of novel vaccine platforms or the modification of existing vaccines (7).

The COVID-19 vaccination campaign in Serbia began as a voluntary, emergency-recommended immunization program in response to the global health crisis caused by SARS-CoV-2. The campaign officially began between December 28 and December 30, 2020, starting with the vaccination of residents of social care institutions, who were identified as the highest-risk group. The first dose of SARS-CoV-2 vaccine administered in Serbia in December 2020 was the Pfizer-BioNTech vaccine (Comirnaty, BNT162b2), the first vaccine to receive emergency use authorization both globally and in Serbia.

The Chinese vaccine (Sinopharm BBIBP-CorV) was introduced in late January 2021, followed by the Russian vaccine (Gam-KOVID-Vac, Sputnik V) in February. Additionally, the Covishield and Vaxzevria (ChAdOx1 nCoV-19/AZD1222) vaccines began to be administered in March 2021. Together, these vaccines formed the core of Serbia's

national vaccination program against SARS-CoV-2, aiming to achieve widespread immunity and control the pandemic.

Vaccination was not mandatory for any segment of the population, including healthcare workers; instead, it was strongly recommended based on medical risk assessment and public health priorities. The campaign followed a risk-based prioritization model, with subsequent groups including healthcare professionals (starting January 5, 2021), the elderly, individuals with chronic illnesses, and other vulnerable or high-exposure populations.

Vaccination was organized through an online platform that allowed individuals to register their interest and receive information about vaccine availability. The program expanded progressively as vaccine supplies increased, with booster doses becoming available to eligible individuals beginning in August 2021. This approach ensured that vaccination efforts were tailored to the risk level of each group, balancing public health priorities with respect for individual choice.

In the Republic of Serbia, the COVID-19 vaccination campaign was accompanied by information disseminated by the authorities through various traditional and digital media (8). Legitimate vaccine information was provided primarily by National Regulatory Authorities and healthcare providers (9), as well as traditional media outlets such as national television, national newspapers, and local newspapers. During an immunization campaign, vaccine choice may depend on several factors, most of which are deeply personal but can also be shaped by external influences. A study conducted among the Serbian population identified five main reasons for hesitancy toward COVID-19 vaccination: concern over side effects, doubts about effectiveness, insufficient testing, mistrust of authorities, and conspiracy theories (8). Vaccine hesitancy is commonly defined as a spectrum of behaviors ranging from outright refusal to delayed acceptance of vaccination. However, some scholars conceptualize vaccine hesitancy as an attitude closely associated with, but not identical to, vaccination behavior in general (10). Considering the above, the present study aimed to evaluate the attitudes of medical professionals and the general public toward COVID-19 and seasonal influenza vaccination in Serbia. General data on respondents' well-being, vaccination profiles, and sources of vaccination information were also collected and analyzed to provide a clearer picture of vaccine hesitancy.

METHODS

Participants

For this study, a questionnaire based on relevant KAP (Knowledge, Attitudes, and Practices) research guidelines was used. Prior to data collection, approval was obtained from the Ethics Committee of the Faculty of Pharmacy, University of Belgrade (approval number 1434/2, dated 14 July 2021).

The questionnaire was developed at the Department of Social Pharmacy and Pharmaceutical Legislation for the thesis "Willingness to Vaccinate and Information Provided to the General Public about COVID-19 Vaccines" (candidate: Andjela S. Marinković; mentor: Prof. Dr. Dušanka Krajnović). Additional questions concerning willingness to receive a COVID-19 booster dose (third dose) were incorporated later for this research; however, this extension did not alter the ethical framework or violate any principles of ethical conduct and therefore did not require further approval. Additionally, the author and mentor of the same thesis granted permission for the use of the questionnaire in this research and contributed to the data analysis and the preparation of this paper.

All participants were verbally informed about the objectives, procedures, and purpose of the study prior to participation. They were informed that participation was entirely voluntary and that they could withdraw at any time without any negative consequences. Participants provided verbal consent to participate, and the anonymous nature of the study further minimized any potential risk to their privacy. The questionnaire did not contain any identifying information, such as names, surnames, or contact details, or any other data that could be used to identify participants, ensuring complete anonymity of all responses. Collected data were used exclusively for statistical analysis and presented in aggregate form. All electronic data were securely stored on a password-protected device accessible only to the research team. Given the nature of the study, which did not involve the collection of personal identifiers, the risk to participants was assessed as minimal. The study was conducted in accordance with fundamental principles of ethical research and applicable law.

This study included a total of 150 participants who completed the questionnaire between October 1 and October 31, 2021, evenly divided into two groups: 74 members of the general population and 76 healthcare workers. This distribution was designed to enable a direct comparison of knowledge, attitudes, and practices (KAP) regarding COVID-19 and seasonal influenza between the

general public and medical professionals.

Due to technical constraints and the pandemic environment, a convenience sampling method was used, including adult residents of Serbia who met the inclusion criteria: being 18 years of age or older, having the capacity to provide informed consent, and being able to complete the questionnaire independently.

Participants with medical education—including physicians, pharmacists, nurses, and other medical staff—were included to gather specific insights from individuals with professional exposure to infectious diseases and frequent patient interaction.

The sample size of 150 respondents was determined based on methodological guidelines for small- to medium-scale KAP studies, as well as practical considerations such as available time, resources, and access to respondents, given the constraints posed by the COVID-19 pandemic, including limited access to participants. The selected sample size allowed for timely data collection while maintaining a reasonable degree of precision, in line with similar rapid-response surveys conducted under emergency public health conditions. This number was deemed sufficient to perform basic descriptive and comparative statistical analyses with an acceptable level of reliability and precision. The combination of random and purposive sampling provided a balanced and contextually relevant overview of the two key population groups, reconciling timeliness and logistical feasibility with acceptable statistical rigor.

Questionnaire structure

The first part of the questionnaire addressed sociodemographic data, including age, family status, education level, and financial status. The second part addressed personal health, examining respondents' attitudes and health conditions. The level of interest in one's health was assessed using a five-point Likert scale (not interested, slightly interested, moderately interested, very interested, and strongly interested), while overall health status was assessed using a four-point Likert scale (very poor, poor, average, and excellent). Respondents were also asked to evaluate their well-being over the preceding two weeks using the following response options: all the time, most of the time, more than half the time, less than half the time, sometimes, never. During the survey, respondents demonstrated a high level of interest in their personal health, with 44.7% reporting that they were interested "a lot" and 36.0% indicating that they were "very interested." Regarding their self-rated health status, 42.0% of respondents rated their health as good, 38.8% as average, 13.3% as excellent, and 4.7% as poor. The third part addressed influenza and COVID-19 vaccination. Seasonal influenza vaccination behavior was assessed using a single-item question: "Have you been vaccinated?", with a binary response option (Yes/No). The results showed that 26.7% of respondents reported receiving the seasonal influenza vaccine annually, while 73.3% reported that they did not. No statistically significant differences were observed in seasonal influenza vaccination rates between respondents with a medical background and those from the general population. Among respondents with a medical background, 25.0% reported receiving the vaccine, compared with 28.4% of respondents from the general population. The corresponding proportions of unvaccinated respondents were 75.0% and 71.6%, respectively. These findings are consistent with results from an Italian study conducted before the COVID-19 pandemic, which reported seasonal influenza vaccination rates ranging from 5% to 34% among HCWs (5). Data from the Italian study conducted during the 2021 influenza season showed that influenza vaccination coverage among HCWs reached 53.6% ($p < 0.001$), whereas no comparable coverage was observed in our study (5). The fourth section addressed the type of COVID-19 vaccine received, as well as respondents' attitudes towards and knowledge of COVID-19 vaccination.

Statistical analysis

Categorical variables were summarized using frequencies and percentages and presented in tabular form to assess vaccination readiness and public awareness of COVID-19 and seasonal influenza vaccines. Normality of data distribution was tested using the Kolmogorov–Smirnov test. The χ^2 test was used to compare categorical variables in univariate analysis. Statistical significance was defined as $p < 0.05$. Statistical analysis was performed using SPSS v.18 (Chicago, IL, USA).

RESULTS

Sample structure

A total of 150 respondents participated in the study, of whom 104 (69.3%) were female, and 46 (30.7%) were male. Of the total sample, 76 (50.7%) respondents had medical-science education, while 74 (49.3%) had no medical education, allowing for comparisons of their responses (Table 1). Regarding age distribution, 4.67% of respondents were aged 18–30 years, 40.00% were aged 31–45 years, and 55.33% were over 45 years of age. The target group

was selected based on sociodemographic and health-related characteristics, including age, sex, employment status, income, and health status, as well as the age categories for which the COVID-19 vaccine was approved. The majority of respondents had a monthly income ranging from 40,000 to 150,000 RSD and lived with other household members (Table 1).

Health and daily activities of the study population

At the time of the interview, respondents reported above-average interest in their health and generally rated their health status as average or good. It is also worth noting that approximately 40% of respondents had a chronic illness (Table 2). In this section, respondents were asked about their emotional and affective state during the two weeks preceding the survey. According to their responses, respondents felt cheerful, relaxed, calm, and engaged with matters of interest, and generally felt fulfilled most of the time during the two weeks before completing the questionnaire (Table 2).

Vaccination profile of the study population and attitudes towards vaccination

Respondents rarely reported receiving the seasonal influenza vaccine, and most had not received it in the same year they received the COVID-19 vaccine (Table 3). Approximately 90% of respondents had received two doses of the COVID-19 vaccine (Table 3). In response to the question "What is the main reason for vaccination against COVID-19?", most respondents cited two reasons: concern for their own health and a sense of responsibility toward their family. Conversely, unvaccinated respondents reported that they had not been vaccinated either due to a prior Covid-19 infection or due to a general attitude against

Table 1. *Sociodemographic characteristics of the respondents from the total sample*

Question number	Question	Answer	Total (%)
1	Place of residence	<10,000 people	1.3
		10,000-100,000 people	12.7
		>100,000 people	86
2	Gender	Male	30.7
		Female	69.3
3	Job-related category	Student	3.3
		Employed	86
		Retired	10.7
4	Education level	Primary	2
		Secondary	42
		Highest education (Master or PhD)	56
5	Do you have medical education	Yes	50.7
		No	49.3
6	Total monthly income	< 20,000 RSD	2
		20,000-39,999 RSD	9.4
		40,000-79,999 RSD	34.2
		80,000-150,000 RSD	34.9
		>150,000 RSD	19.5
7	Living with	Alone	18.7
		With someone	81.3

Table 2. *Health and daily activities-related questions and answers from the study population*

Question number	Question	Answer	Total (%)
1	How much interest you have in your health	Slightly	1.3
		Moderately	18
		Above average	44.7
		Intensive	36
2	Subjective health status	Very bad	0.7
		Bad	4.7
		Average	38.7
		Good	42.7
		Excellent	13.3
3	Do you have any chronic medical condition	Yes	42.7
		No	57.3
4	I felt cheerful and in good spirits*	Never	0
		Almost never	20.8
		Less than half of the time	21.5
		More than half of the time	34.2
		Most of the time	21.5
		All the time	2
5	I felt calm and at ease*	Never	3.3
		Almost never	16.7
		Less than half of the time	28
		More than half of the time	32
		Most of the time	18
		All the time	2
6	I felt refreshed and relaxed*	Never	8
		Almost never	24.7
		Less than half of the time	32
		More than half of the time	24
		Most of the time	8
		All the time	2.7
7	My daily life was filled with things that interested me*	Never	0.7
		Almost never	273
		Less than half of the time	22
		More than half of the time	28
		Most of the time	21.3
		All the time	0.7

*The period of time the participants were referred to recall while answering included the previous two weeks

Table 3. *Vaccination profile and attitudes towards vaccination of the study population*

Question number	Question	Answer	Total (%)
1	Do you receive seasonal flu vaccine regularly?	Yes	26.7
		No	73.3
2	Have you received the seasonal flu vaccine this year?	Yes	32.7
		No	67.3
3	Have you received a vaccine against COVID-19?	Yes – one dose	2
		Yes – two doses	90.7
		No	7.3
4	What is the main reason for vaccination against COVID-19?*	Traveling – COVID passport	6.7
		Care for health	48.9
		Following recommendations	3.5
		Faster pandemic resolution	18
		Responsibility and care for family	44.8
		Employees request	2.8
3	What is the reason for not taking vaccines against COVID-19?	Had COVID-19	41.7
		I am not in a risk group	16.7
		I do not take vaccines generally	33.3
		I do not want to	8.3

*More than one answer was available

Table 4. *Vaccination profile of the study population*

Question number	Question	Answer	Total (%)
1	If you are vaccinated for COVID-19, what vaccine was it?	Pfizer/BioNTech COVID19	22.7
		Sputnik V(Gam-KOVID-Vak)	40.4
		AstraZeneca	7.8
		Sinopharm (BBIB–CoV)	28.4
2	If you received two doses of vaccine, do you want to receive a third?	Yes	94
		No	5.7
3	Would a third dose of vaccine be the same as the first two?	Yes	55
		No	45
4	Should vaccination be mandatory?	Yes	70
		No	30
5	Do you fall into a group of respondents with high risk?	Yes	32.7
		No	67.3

vaccination (Table 3). Among the available vaccines, respondents most often chose Sputnik V, followed by Sinopharm, then Pfizer/BioNTech, and finally Vaxzevria® (Oxford-AstraZeneca), chosen by approximately 8% (Table 4). Approximately 94% of respondents received two vaccine doses, and 55% believed that a third dose should be of the same vaccine as the first two (Table 4). Additionally, 70% of respondents believed that vaccination should be mandatory in Serbia. When asked whether they belonged to a risk group, approximately 32% responded affirmatively (Table 4). Vaccine refusal was generally low (8); however, some respondents indicated that they would refuse certain vaccines, predominantly Oxford-AstraZeneca (Table 5). Approximately 65% of respondents stated that the Vaxzevria® (Oxford-AstraZeneca) vaccine would not be an option for them, as its safety profile and effects were subject to additional monitoring in some countries. Data presented in Table 5 show that 87.2% of respondents believe it is necessary to continue adhering to epidemiological measures even after vaccination, while 12.8% believe it is not. Additionally, 9.4% of respondents believe that the COVID-19 vaccine can cause infertility, while 90% believe it does not. When respondents suspected they had a COVID-19 infection, they were most likely to confirm this with a medical practitioner, with a significantly smaller proportion relying on other sources of information (Table 5). Respondents reported obtaining information about COVID-19 vaccination primarily from medical doctors and official websites, but also, to a considerable extent, from various social media platforms (Table 5).

Interviewed respondents reported that 60% had not searched the internet for information about COVID-19 vaccines (Figure 1). They reported either requiring assistance in distinguishing false from accurate information or experiencing little difficulty in doing so (Figure 2). Approximately 90% of respondents believed they should follow epidemiological recommendations after vaccination (Figure 3). Television was the primary source of information related to COVID-19 and vaccines, ahead of medical practitioners, with pharmacists and social media cited least frequently (Figure 4). The findings indicate that respondents placed particular importance on comprehensive coverage of topics (59.3%), up-to-date information (51.3%), and information from official sources (52.0%).Furthermore, 64.7% of respondents considered the presentation of differing opinions important (Figure 5). Most respondents rarely reported experiencing difficulties in understanding instructions related to therapy or medication or in following similar procedures, and generally felt confident completing medical questionnaires during examinations.

Table 5. *Knowledge and attitudes about COVID-19 vaccination*

Question number	Question	Answer	Total (%)
1	Is there a COVID-19 vaccine that you would refuse to take?	Pfizer/ COVID19	1.4
		Sputnik V(Gam-KOVID-Vak)	4.2
		AstraZeneca	13.2
		Sinopharm (BBIB–CoV)	4.9
		Moderna (Moderna mRNA 1237)	4.9
		I would not refuse any vaccinee	69.4
		I would refuse every vaccinee	1.4
2	Would you receive a vaccine whose effects are being closely monitored in other countries (e.g., AstraZeneca)?	Yes	34
		No	66
3	Should you follow epidemiological recommendations after you receive two doses of vaccine?	Yes	87.2
		No	12.8
4	Do you think that the COVID-19 vaccine can cause infertility?	Yes	9.4
		No	90.6
5	What was your main source of information in case you suspect COVID-19 infection?	Social media	11
		Television	16.4
		Medical practitioner	60.6
		Pharmacist	5.1
		Health-related web pages	16.7
		Official web pages*	7.4
		Other	0.7
6	What was your main source of information about COVID-19 vaccines?**	Social media	11.4
		Television	25.4
		Medical practitioner	48.7
		Pharmacist	6.1
		Health-related web pages	14.3
		Official web pages*	34.2
		Medical journals for professionals	10.7

*Official web pages given by the government of RS and the Ministry of Health (Public Health Institute Batut); **some respondents gave more than one answer.

Comparison between the attitude of medical and non-medical personnel

Table 6 indicates that there were no statistically significant differences in vaccine choice based on respondents' health education background. There was likewise no statistically significant difference in preference for a third dose between respondents with medical education and the general public. No statistically significant differences were found in responses regarding seasonal influenza vaccination between the two groups. Similarly, no statistically significant differences were observed in attitudes toward mandatory vaccination between the two subgroups, suggesting that medical education was not a significant factor in these responses (Table 6).

DISCUSSION

This study was conducted among the general population in October 2021. The most common respondent profile was that of an employed woman with the highest level of education (a Master's or PhD degree), living with someone in a place of residence with a population exceeding 100,000 and reporting a monthly income between 80,000 and 150,000 RSD. Nearly equal proportions of respondents fell into the 40,000–79,999 RSD and 80,000–150,000 RSD income categories (34.2% and 34.9%, respectively). These characteristics are broadly consistent with the socio-economic profile of Serbian society; however, other segments of the population, particularly those without employment or higher education, were underrepresented (11). In some countries, attitudes toward vaccination have been influenced by financial circumstances when vaccination was not provided free of charge (12). In Serbia, however, vaccination was provided free of charge; therefore, financial background should be considered separately from factors that may directly influence the vaccination process.

Examining attitudes toward COVID-19 and seasonal influenza vaccination helps us understand public opinion and improve vaccination awareness. This is particularly relevant given the resurgence of diseases that had previously been significantly reduced, such as pertussis, rubella, and measles (13). In Serbia, approximately 70% of respondents do not receive the influenza vaccine annually (Table 3), consistent with findings from a study conducted in April/May 2021, "Readiness of the General Population for Vaccination against COVID-19," which reported that only 8.2% of respondents received the seasonal influenza vaccine regularly. Even during the 2020/2021 season, the

Table 6. *Comparison between the vaccine profile in respondents with and without medical education*

Question	Answer	Respondents (number, %)		Statistical parameters
		With medical education	Without medical education	
Vaccine type	Pfizer/BioNTech COVID19	19 (25.3%)	13 (19.7%)	χ^2 (Df) = 4.683 (4); p = 0.321
	Sputnik V(Gam-KOVID-Vak)	34 (45.3%)	23 (34.8%)	
	AstraZeneca	5 (6.7%)	6 (9.1%)	
	Sinopharm (BBIB–CoV)	17 (22.7%)	23 (34.8%)	
	None	0 (0%)	1 (0.7%)	
Third dose	Yes	71 (94,7%)	62 (93.9%)	χ^2 (Df) = 0.350 (1); p = 0.852
	No	4 (5,3%)	4 (6.1%)	
Flue vaccination	Yes	19 (25%)	21 (28.4%)	χ^2 (Df) = 0.219 (1); p = 0.640
	No	57 (75%)	53 (71.6%)	
Would you receive AstraZeneca?	Yes	24 (31.6%)	27 (36.5%)	χ^2 (Df) = 0.402 (1); p = 0.562
	No	52 (68.4%)	47 (63.5%)	
Obligatory vaccination?	Yes	56 (73.7%)	49 (66.2%)	χ^2 (Df) = 0.402 (1); p = 0.562
	No	20 (26.3%)	25 (33.8%)	

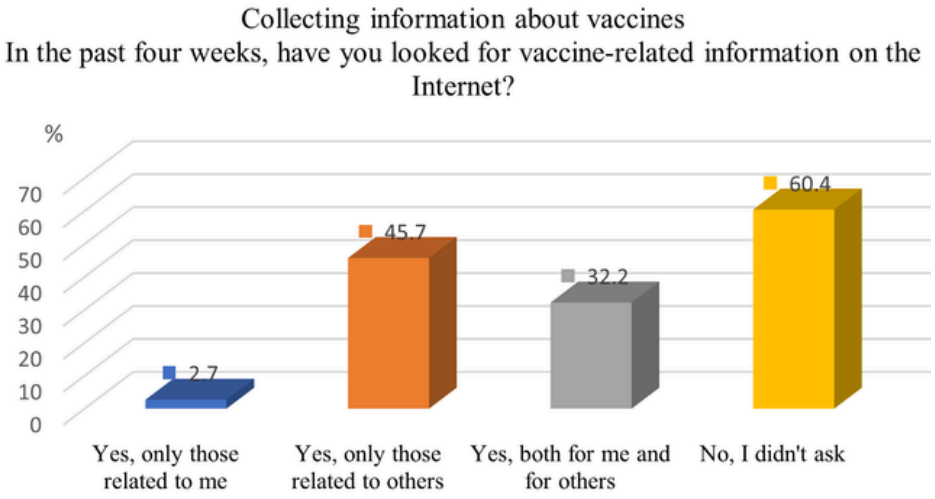


Figure 1. *In the past four weeks, have you looked for vaccine-related information on the Internet?*

same study reported a rate of 9.1% (14). These results align with epidemiological data on influenza vaccination rates among individuals aged 65 and older in Serbia, at 15.2% for the 2022 season and 14.03% for the 2023 season, according to WHO immunization data for Serbia (15). Although influenza is not widely perceived by the general population as a major health threat, a large-scale study has estimated that influenza is responsible for between 15,000 and 70,000 deaths worldwide annually (16). Seasonal influenza therefore continues to place a burden on the healthcare system. Given that 33% of respondents in our study do not receive vaccines in general, these findings could help inform future promotional campaigns aimed at overcoming identified barriers to immunization.

In the study population, approximately 91% of respondents had received two doses of the COVID-19 vaccine (Table 3), consistent with findings from other European population studies, such as those conducted in the UK and Denmark (17). In general, a second dose produces a more reliable immune response and greater effectiveness than a single dose (7). People receive COVID-19 vaccines to protect themselves and their families from the virus. By getting vaccinated, individuals reduce their risk of infection and of subsequently transmitting the virus to family members. Vaccination not only helps prevent illness and severe complications but also contributes to community immunity, which in turn helps protect vulnerable individuals at higher risk of severe illness, such as older adults, immunocompromised individuals, and those with underlying health conditions (chronic disease). Receiving the COVID-19 vaccine therefore benefits not only the individual

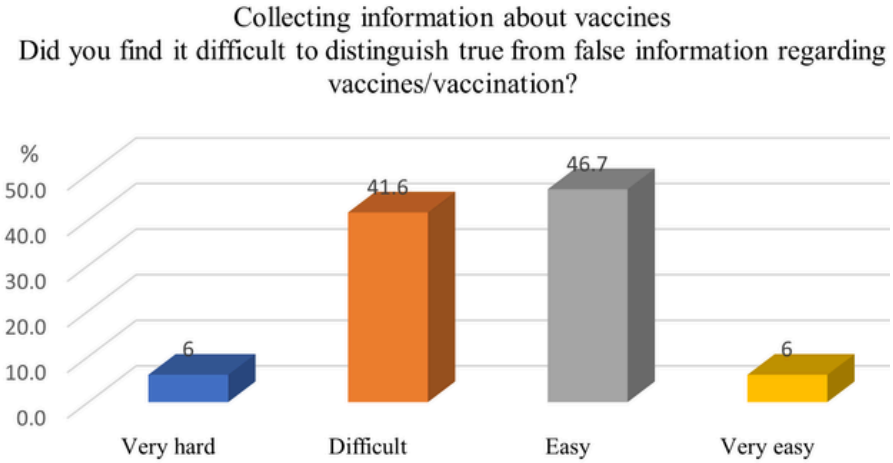


Figure 2. *Did you find it difficult to distinguish true from false information regarding vaccines/vaccination?*

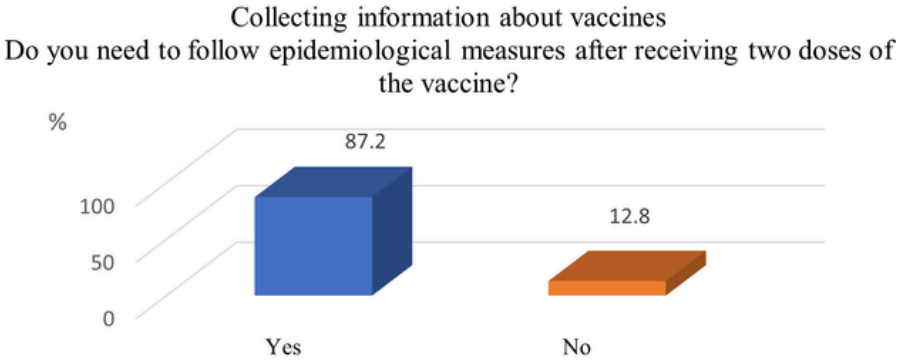


Figure 3. *Do you need to follow epidemiological measures after receiving two doses of the vaccine?*

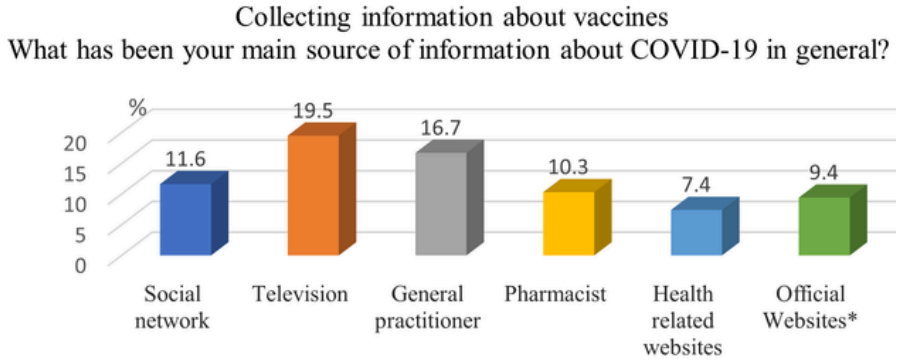


Figure 4. *What has been your main source of information about COVID-19 in general?*

but also plays a crucial role in safeguarding the health and well-being of their families and communities (18). Consistent with this, respondents in the study population reported that they were vaccinated to protect their own health and prevent transmission of infection within their families. This is further reflected in the attitude of 70% of respondents who believe that COVID-19 vaccination should be mandatory. A study conducted in the Balkan region similarly indicates a relationship between trust in political authorities and vaccination response (19).

Vaccine hesitancy has been identified as a significant global health threat, driven in part by misguided safety concerns that have contributed to the spread of diseases such as measles, diphtheria, and pertussis (20). Religious beliefs and the influence of political leaders may also play a significant role in vaccine refusal. Personal or philosophical beliefs are likewise relevant, with some individuals believing that immunity acquired through natural infection is superior to vaccination, that the side effects of vaccines outweigh their benefits, or that a healthy lifestyle alone is sufficient to prevent disease (21). In the present study population, the most commonly reported reason for not receiving the COVID-19 vaccine was the perception that vaccination was unnecessary following a previous COVID-19 infection (Table 3).

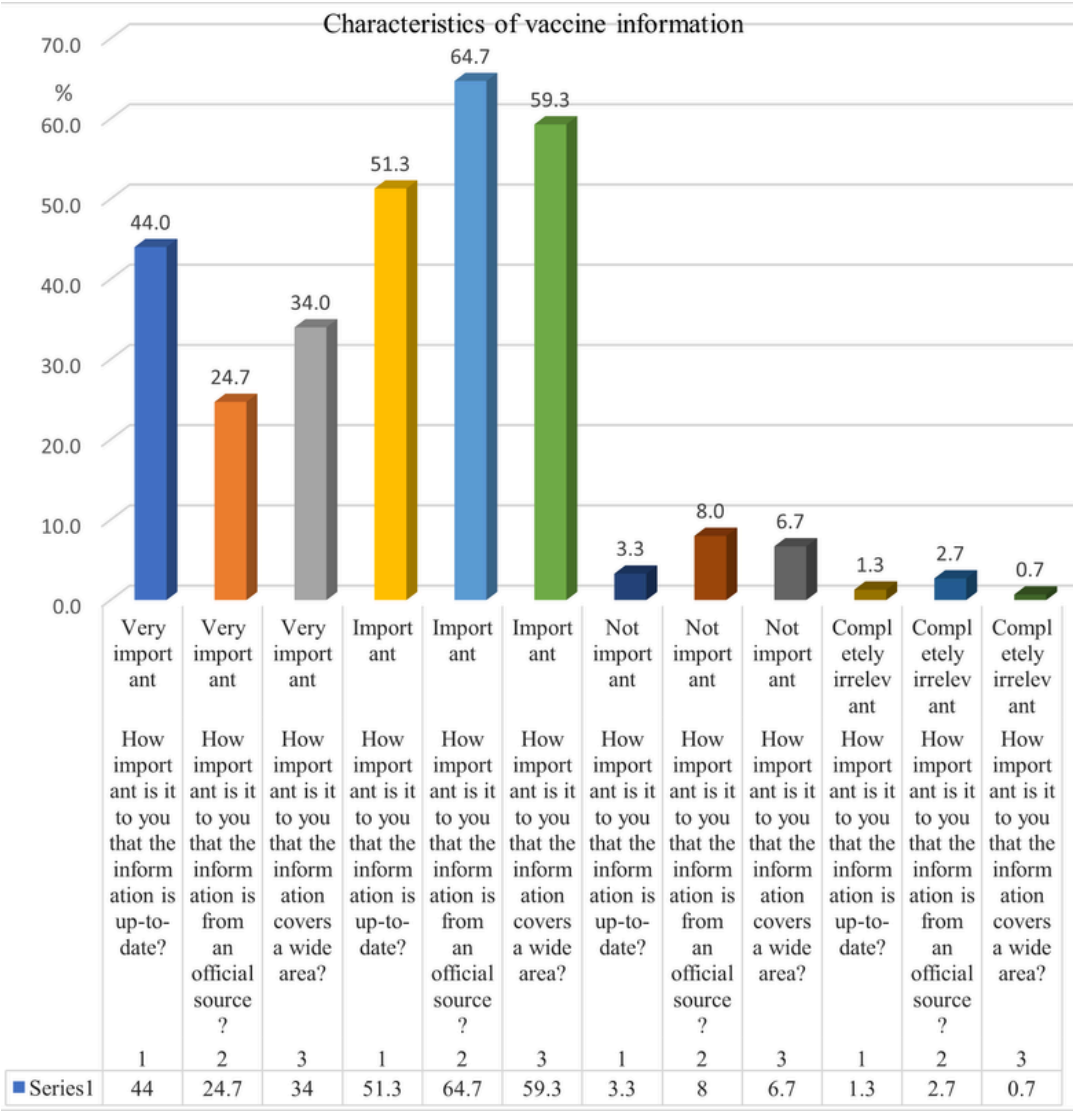


Figure 5. *Characteristics of vaccine information*

A meta-analysis showed that mRNA vaccine technology demonstrated the highest overall efficacy and effectiveness among the available vaccine types (8). During the period of this study, the only mRNA-based vaccine available in Serbia was Comirnaty® (Pfizer), chosen by approximately 21% of respondents—almost 50% fewer than Gam-KOVID-Vac (Sputnik V), the most frequently selected vaccine (Table IV). Compared with other data, this is a somewhat unusual finding; however, considering that COVID-19 immunization in the Republic of Serbia began in January 2021, when Sputnik V and Sinopharm were the most widely available vaccines and mRNA vaccine supplies were limited, this result is not unexpected. This finding only partially corresponds with the April/May 2021 study "Readiness of the General Population for Vaccination against COVID-19," in which the majority of respondents expressed interest in the Sinopharm vaccine (23.8%) and Comirnaty® (Pfizer-BioNTech) (19.8%) (14). COVID-19 vaccines developed using viral vector platforms to deliver genetic material and stimulate an immune response, such as Vaxzevria® (Oxford-AstraZeneca), have been associated with public concerns regarding various adverse events reported following both the first and second doses (22,23). Consistent with this, most respondents in the present study indicated that they would refuse this particular vaccine (Table 5), as its safety profile and effects were still under additional review in some countries at the time of the survey. During the pandemic, and even in the post-pandemic period, it is well

established that individuals with chronic conditions (e.g., chronic obstructive pulmonary disease, diabetes, obesity) are more prone to severe or complex clinical presentations of COVID-19 (24). Approximately 32% of respondents reported belonging to a higher-risk group, while approximately 42% reported having a chronic medical condition. Respondents reported an above-average interest in their health and generally considered their health status to be average or good. Patients with chronic illnesses should be vaccinated to help prevent disease progression (25). The majority of respondents reported taking greater-than-average care of their health and feeling in relatively good health overall. The presence of chronic diseases and heightened health concerns may represent factors that motivate respondents to seek vaccination and follow official recommendations.

In this section of the questionnaire, respondents were asked about their emotional and affective state during the two weeks preceding the survey. According to their responses, respondents felt cheerful, relaxed, calm, and engaged with matters of interest, and generally felt fulfilled most of the time during this period. One could argue that respondents experienced heightened anxiety during the pandemic, which may be reflected in their reported willingness to continue following Covid-19 recommendations even after vaccination. It has been reported that individuals with pre-existing mental health conditions, such as anxiety disorders or depression, are more prone to increased anxiety during a pandemic (26). Given the timing of the study, these findings suggest that respondents had largely returned to their everyday routines following the pandemic lockdown period.

Information relevant to vaccination decision-making, whether in favor of or against vaccination, can originate from numerous sources. During the pandemic, individuals faced considerable pressure, as it was often difficult to avoid such information. Since the onset of the pandemic, there has been a marked rise in conspiracy theories, which have been associated with reduced compliance with protective measures and vaccination (27). Approximately 40% of respondents had searched the internet for information about vaccines. Finding reliable information about vaccines and vaccination was a challenging task for most respondents. Claims regarding the potential for vaccines to cause infertility (28) have been associated with vaccine refusal in other populations, although such beliefs were not reported by respondents in the present study. Traditional information sources, such as television, were among the sources most frequently associated with vaccine hesitancy (29), while medical doctors appeared to have comparatively less influence on respondents' willingness to be vaccinated. Respondents in the present study indicated that they primarily received information about Covid-19 vaccination from their doctors or from official websites where government recommendations were published. This suggests that the population in Serbia received adequate information either from medically trained personnel or from official sources.

Adequate medical training equips healthcare professionals with a comprehensive understanding of vaccine safety profiles, efficacy, and administration protocols, enabling them to address patient concerns and provide accurate information. Moreover, a solid foundation in immunology and infectious disease allows healthcare providers to support public health efforts by promoting vaccine uptake, countering misinformation, and fostering community trust in vaccines and vaccination (30). Several studies have identified doctors and other medical professionals as having greater knowledge of, and more favorable attitudes toward vaccination (31,32). In the present study, however, no such discrepancy in knowledge or attitudes toward Covid-19 vaccination was found between medically trained and non-medically trained respondents in the Serbian population.

Study limitation

One limitation of this study relates to participant selection. Although the questionnaire was made publicly available (shared across several companies), it was completed predominantly by highly educated individuals, which does not proportionally represent the general population, including less-educated groups. Additionally, half of the respondents had a medical education, representing both a strength and a limitation of the study. While this enabled comparisons of attitudes and understanding of vaccines between medically and non-medically educated individuals, the composition of the sample may have influenced responses to questions concerning health preservation and self-management.

The results of this study show that the examined population, living predominantly in urban areas, was largely vaccinated with Sputnik V and showed limited interest in seasonal influenza vaccination. Respondents generally expressed positive attitudes toward vaccination overall, with many supporting mandatory Covid-19 vaccination. It is also worth noting that respondents expressed a high degree of distrust toward the Oxford-AstraZeneca vaccine, which was widely perceived as

experimental. Finally, and most notably, respondents — regardless of their medical education background — held very similar attitudes toward vaccine type and booster (third) dose choice, as well as toward seasonal influenza vaccination, sharing both an unfavorable view of the Oxford-AstraZeneca vaccine and a favorable view of mandatory Covid-19 vaccination. The Covid-19 pandemic serves as a reminder to both the general public and healthcare professionals of the pandemic potential of influenza and other infectious diseases.

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Author Contributions

Conceptualization, D.K. and V.M.; Investigation, K.P., A.M., and B.F.; Data curation, K.P., A.M., and B.F.; Formal analysis, A.M.G.; Writing – original draft preparation, K.P.; Writing – review and editing, D.K., V.M., B.F., and A.M.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 Faculty of Pharmacy, University of Belgrade, approval number 1434/2 issued on 14.07.2021. Prior to completing the survey, all participants were fully informed about the study's objective, nature, and the voluntary basis of their participation. The questionnaire was completely anonymous, non-invasive, and contained no sensitive personal data; therefore, formal written informed consent was not required. Participation and submission of the completed survey constituted implied informed consent.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availability

The datasets generated and/or analyzed during this study are included within this article and are available from the corresponding author upon reasonable request.

Statement of Generative AI Technologies Use

No generative AI was used.

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ORIGINAL ARTICLE

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ASSOCIATION OF CASPASE-1 GENE POLYMORPHISMS WITH RHEUMATOID ARTHRITIS SUSCEPTIBILITY AND TEMPOROMANDIBULAR JOINT INVOLVEMENT: AN IRAQI COHORT STUDY

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Caspase-1 (*CASP1*) is an integral part of the inflammasome, and rheumatoid arthritis (RA) is a complex, progressive, chronic inflammatory autoimmune disease. The present study aimed to investigate two aspects of RA: i) the association of *CASP1* gene polymorphisms with susceptibility to RA; and ii) the prevalence of temporomandibular joint (TMJ) involvement in patients with RA. This cross-sectional study included 90 participants: 60 patients with RA (20 newly diagnosed and untreated, 40 receiving combination therapy with methotrexate and etanercept) and 30 healthy controls. Three a priori-selected SNPs were screened: rs580253 (C>T), rs501192 (G>A), and rs56244680 (11-bp deletion TTCCATATTTA). Control variant allele frequencies were rs580253 T = 0.10, rs501192 A = 0.10, and rs56244680 del = 0.017. No significant differences in CASP1 genotype frequencies were observed among the treated, newly diagnosed, and control groups. Both components of the Helkimo index revealed significantly greater TMJ involvement in patients than in controls (both $p < 0.001$). Subjective TMJ symptoms (Ai >0) were reported by 28/40 (70.0%) treated and 10/20 (50.0%) newly diagnosed patients, compared with 5/30 (16.7%) controls. Objective TMJ dysfunction (Di >0) was observed in 30/40 (75.0%) of treated and 14/20 (70.0%) of newly diagnosed patients, compared with only 4/30 (13.3%) of controls, all of whom presented with mild dysfunction. Notably, treated patients reported more severe subjective symptoms than newly diagnosed patients ($p = 0.048$), whereas objective dysfunction did not differ significantly between these groups ($p = 0.303$). These findings highlight the importance of clinical TMJ assessment in all patients with RA, regardless of genetic background or treatment status.

Keywords: rheumatoid arthritis, caspase-1, gene polymorphism, temporomandibular joint, inflammasome, single nucleotide polymorphism

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INTRODUCTION

Rheumatoid arthritis (RA) is an inflammatory condition that predominantly impacts the joints of the extremities. One of its most common manifestations is persistent, recurrent joint inflammation (1–3). The disease typically affects multiple joints, characteristically presenting first in the periphery (4,5). While initial involvement of the temporomandibular joint (TMJ) as the first manifestation of RA is rare (6), TMJ involvement has been reported in 5%–86% of patients with RA, with bilateral involvement being the most common presentation (6).

When TMJ involvement is assessed using patient-reported symptoms, clinical examination, or radiographic imaging, the wide range of reported prevalence reflects differences in assessment methods among studies. Imaging studies, in particular, tend to detect higher rates of TMJ involvement than clinical assessment alone (7,8).

Early research has suggested that genetic variables are responsible for 50% to 65% of RA cases (9). Single nucleotide polymorphisms (SNPs) serve as valuable genetic markers for elucidating evolutionary history and identifying common genetic factors that account for heritable risk of prevalent diseases and influence progression of certain diseases (10). Previous studies have found a link between SNPs in multiple genes and the resistance or favorable response to treatment in patients with RA (11–13).

One of the primary features of RA is continuous inflammation (14). Identifying individual molecules and their roles in inflammation is crucial for understanding the RA etiology and creating novel therapeutics. A class of proteolytic enzymes known as caspases transduce signals that ultimately result in cell death (15). Caspase-1 (*CASP1*), in particular, plays a central role in inflammation as the primary activator of pro-inflammatory cytokines IL-1β and IL-18 through its function within the inflammasome complex (16).

The three *CASP1* polymorphisms (rs580253, rs501192, and rs56244680) were selected a priori based on existing evidence linking them to altered inflammatory responses in diverse non-RA cohorts, including those with restenosis and severe infectious diseases (17,18). Biologically, *CASP1* is a strong candidate for RA pathogenesis because it drives synovial inflammation and joint destruction by activating IL-1β and IL-18 within the inflammasome complex (16,19–21).

While previous studies have investigated other *CASP1* variants in rheumatoid arthritis (RA), including rs2409062 in Chinese populations (22) and rs572687 in a Brazilian cohort (23), the specific intronic variants rs580253 (C>T) and rs501192 (G>A), together with the 11-bp deletion variant rs56244680, have not been investigated in RA. Based on a comprehensive literature review and the potential functional significance of the 11-bp deletion, these three *CASP1* variants were selected as novel, hypothesis-driven candidates for assessing genetic susceptibility to RA in an Iraqi population.

The present study aimed to investigate two aspects of RA: i) the association of *CASP1* gene polymorphisms with susceptibility to RA; and ii) the prevalence of temporomandibular joint (TMJ) involvement in patients with RA.

METHODS

This cross-sectional study included 90 participants recruited from the outpatient rheumatology unit of Baghdad Teaching Hospital (Baghdad, Iraq) between mid-March 2023 and mid-September 2023, with the approval of the Scientific and Ethical Unit of Baghdad Teaching Hospital in Baghdad, Iraq (Approval No. 859, issued on 19 February, 2023). In accordance with the Declaration of Helsinki, written informed consent was obtained from all participants prior to their enrollment in the study.

A total of 60 patients with RA and 30 healthy controls were enrolled. The patient group consisted of 20 newly diagnosed, untreated RA patients and 40 RA patients receiving combination therapy with methotrexate and etanercept. The control group comprised 30 healthy individuals, matched for age and sex, with no history of systemic diseases or medication use.

The inclusion criteria were as follows:

For the patients with RA: i) age 20–60 years; ii) diagnosis of RA according to the 2010 ACR/EULAR classification criteria (24).

For the newly diagnosed subgroup: confirmed RA diagnosis within the past 3 months and no prior use of disease-modifying antirheumatic drugs (DMARDs) or biological therapy.

For the treated subgroup: established diagnosis of RA with stable methotrexate and etanercept combination therapy for ≥3 months.

For the healthy controls: i) age 20-60 years; ii) no history or current diagnosis of any systemic disease (including RA, other autoimmune diseases, diabetes, cardiovascular disease, or malignancy); iii) no current medication use; iv) age- and sex-matched to the patient group.

The following exclusion criteria were applied:

For the patients with RA: i) age <20 or >60 years;

ii) presence of other concomitant autoimmune diseases (e.g., systemic lupus erythematosus, psoriatic arthritis, or Sjögren's syndrome); iii) major comorbidities influencing systemic inflammation or TMJ status, including diabetes mellitus, chronic kidney or liver disease, malignancy, active infections; iv) history of TMJ trauma/surgery or pre-existing temporomandibular joint disorder; v) bruxism or other parafunctional habits; vi) pregnancy or lactation.

For the newly diagnosed subgroup: any prior DMARD/or corticosteroid exposure.

For the treated subgroup: poor medication adherence or dosage modification within the past 3 months.

For the healthy controls: i) age <20 or >60 years; ii) any known systemic disease (determined by structured interview); iii) any current medication use; iv) clinical symptoms suggestive of an undiagnosed disease (such as joint pain, morning stiffness, or oral dryness); v) a history of TMJ trauma/surgery, or any current TMJ symptoms; vi) bruxism or other parafunctional habits; vii) pregnancy or lactation; viii) a first-degree relative with RA (to minimize genetic confounding); and ix) age- and sex-matched to the patient group at the group level.

The control group's healthy status and absence of systemic disease were confirmed through a structured medical history interview and a brief physical examination, including assessment of vital signs, an oral examination, and bilateral TMJ palpation.

Disease activity assessment

Rheumatologists evaluated disease activity in all patients with RA using the Clinical Disease Activity Index (CDAI). The CDAI is a composite measure calculated as the sum of four components: i) tender joint count based on assessments of 28 joints; ii) swollen joint count based on assessment of 28 joints; iii) patient global assessment of disease activity (on a 0–10 cm visual analog scale); and iv) physician global assessment of disease activity (on a 0–10 cm visual analog scale).

CDAI scores were interpreted according to the following validated thresholds (25,26): remission, ≤2.8; low disease activity, >2.8 to ≤10.0; moderate disease activity, >10.0 to ≤22.0; and high disease activity, >22.0.

TMJ assessment

TMJ involvement was evaluated using the Helkimo index, which comprises two components based on the standardized criteria established by Helkimo (27) and described previously (28,29). The Helkimo anamnestic index (Ai) assesses subjective symptoms: 0, no symptoms; I, mild symptoms; and II, severe symptoms. The Helkimo clinical dysfunction index (Di) evaluates objective clinical findings: 0, no dysfunction; I, mild dysfunction; II, moderate dysfunction; and III, severe dysfunction. The Di score is based on five clinical criteria: mandibular mobility, TMJ function, muscle pain, TMJ pain, and pain during mandibular movement.

CASP1 genotyping

For the genetic association analysis, a post hoc power calculation was performed using the WINPEPI software package (version 11.65; Brixton Health). Assuming a minor allele frequency of approximately 10% for rs580253 and rs501192 (based on preliminary data and public databases for Middle Eastern populations), the study sample provided 80% power to detect large genetic effects [an odds ratio (OR) ≥3.5], 60% power to detect an OR ≥3.0, and <30% power to detect an OR <2.5.

These findings are based on the assumption that the significance threshold was $\alpha = 0.05$. For the deletion variant rs56244680, with an observed frequency of approximately 3% to 10%, power levels are markedly lower, yielding 45% power to detect an OR of 4.0. These calculations indicate that although the study had sufficient power to identify large genetic effects, it may fail to identify moderate or mild effects (OR <3.0). Thus, the negative findings for *CASP1* polymorphisms should be interpreted with caution, as they do not preclude smaller effect sizes that require larger cohorts to detect. Of note, the data on TMJ involvement were highly significant ($p < 0.001$), suggesting that the sample size was sufficient to detect the primary clinical outcome.

DNA extraction

To isolate DNA for *CASP1* genotyping, 2 mL of venous blood was collected from each participant into EDTA tubes. DNA concentrations ranged from 30 to 150 ng/μL, and the DNA quality was adequate for all 90 samples, with A260/A280 ratios ranging from 1.8 to 2.0. Furthermore, there were no sample processing failures that necessitated repeat collection. Extraction of genomic DNA was performed according to the manufacturer's instructions using the EasyPure® gDNA EE101-01 kit (TransGen Biotech Co., Ltd.). DNA concentration and purity were determined spectrophotometrically using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, USA).

Primer design and optimization

Primers were designed to amplify a *CASP1* region encompassing the three target SNPs (rs580253, rs501192, and rs56244680) and optimized to yield specific results. The primer sequences were as follows: forward, 5'-ACCTCTGACAGCACGTTCCT-3'; and reverse, 5'-GGTGTGGAAGAGCAGAAAGC-3'. To determine the optimal annealing temperature, a gradient PCR was performed across a temperature range of 55–65 ˚C. The annealing temperature yielding the clearest single band with minimal nonspecific amplification was 60˚C, which was subsequently used for all reactions.

PCR amplification conditions

PCR was performed in a total reaction volume of 25 µl containing 100 ng of genomic DNA, 0.4 µM of each primer (forward and reverse), 12.5 µl of 2× GoTaq® Green Master Mix (Promega Corporation) containing Taq DNA polymerase, dNTPs, $MgCl_2$, and reaction buffer, and nuclease-free water to make up the final volume. All PCR reactions were performed using a Veriti® 96-Well Thermal Cycler (Model No. 4375786, Applied Biosystems, Inc.). The thermal cycling protocol consisted of an initial denaturation at 95˚C for 5 min, followed by 35 cycles of denaturation at 95˚C for 30 s, annealing at 60˚C for 30 s, and extension at 72˚C for 45 s. The final extension was performed at 72˚C for 7 min, followed by a hold at 4˚C.

Product verification

PCR products were visualized by electrophoresis on a 2% agarose gel stained with ethidium bromide, alongside a 100-bp DNA ladder (Cat. no. DM003-R500; GeneDireX, Inc.) to confirm the expected product size. Gels were subsequently imaged using a UV transilluminator (BioDoc-It™ Imaging System; UVP).

Sequencing and genotype determination

PCR products were purified using the ExoSAP-IT™ Express PCR Product Cleanup Reagent (cat. No. 78201, Applied Biosystems, Inc.) according to the manufacturer's instructions. The purified products underwent Sanger sequencing on an ABI3730XL automated DNA sequencer (Applied Biosystems, Inc.) using the same primers as for PCR amplification. Both forward and reverse strands were sequenced to ensure accuracy.

Following sequencing, chromatogram files (.ab1) were imported into Geneious Prime software (version 2023.0; Biomatters) for analysis. Using the MUSCLE alignment algorithm, sequences were assembled into contiguous sequences (contigs) and aligned with the CASP1 reference sequence from GenBank (Accession No. NG_029124.2). This alignment was performed to screen for the three *a priori*-selected polymorphisms in the Iraqi population: rs580253 (C>T), rs501192 (G>A), and rs56244680 (11-bp deletion TTCCATATTTA). A visual examination of the electropherograms at the polymorphic sites allowed for genotype determination for each SNP at the following locations:

For rs580253 and rs501192, homozygosity was indicated by a single peak (reference or variant allele), while heterozygosity was indicated by two overlapping peaks of approximately equal height at the same position. For rs56244680 (11-bp deletion TTCCATATTTA), genotype determination was based on chromatogram patterns, as follows: i) homozygous wild-type (ins/ins) – a perfect match with the reference sequence and a clear single peak throughout the deletion region; ii) heterozygous deletion (ins/del), indicated by the presence of overlapping chromatogram peaks beginning immediately downstream of the deletion site; these overlapping peaks demonstrated the presence of two alleles of different lengths, one containing the 11-bp sequence and the other lacking it; iii) homozygous deletion (del/del), characterized by a sequence shortened by 11 base pairs with single, unambiguous peaks throughout the region, confirming that both alleles carry the deletion.

All sequences were independently examined by two investigators who were blinded to participants' clinical status. Any discrepancies were resolved by joint review and, when necessary, repeat sequencing. During the final analysis, only sequences with Phred quality scores >30 (with a base call accuracy of at least 99.9%) were considered.

Statistical analysis

All data were analyzed using IBM SPSS Statistics (version 26; IBM Corp.). Additional analyses were performed using WINPEPI (version 11.65) for power calculations and Hardy-Weinberg equilibrium testing, and GraphPad Prism (version 9; GraphPad Software, San Diego, CA, USA) for figure generation. For descriptive statistics, continuous variables (e.g., age) are presented as means ± standard deviations (SDs). Categorical variables (sex, genotype frequencies, and the Helkimo index categories) are presented as frequencies and percentages.

Demographic matching confirmation

Age: one-way analysis of variance (ANOVA) was used to compare mean age across the three study groups (newly diagnosed RA, treated RA, and control). Sex: the chi-squared (χ^2) test was used to compare sex distribution among the three groups. A p-value >0.05 for both tests was considered to support the comparability of the groups with respect to age and sex.

Hardy-Weinberg equilibrium (HWE)

For each SNP (rs580253, rs501192, and rs56244680), HWE was tested in the control group using the chi-square goodness-of-fit test (WINPEPI software). A p-value >0.05 indicated that the genotype frequencies did not significantly deviate from HWE.

Genetic association analysis

Genotype frequencies were compared among the three groups (newly diagnosed RA, treated RA, and control) using the chi-squared test or Fisher's exact test (when expected cell counts were <5), while allele frequencies were compared across groups using the chi-squared test. ORs and 95% confidence intervals (CIs) were computed using binary logistic regression (SPSS).

To assess the risk associated with carrying a variant allele (T for rs580253; A for rs501192), a dominant genetic model was applied by comparing variant allele carriers (heterozygotes and homozygous variants) to non-carriers (homozygous reference), with the control group serving as the baseline reference category. The OR was interpreted as follows: an OR >1.0 indicates an increased risk, an OR <1.0 indicates a decreased risk, and an OR =1.0 indicates no association. Statistical significance was established based on whether the 95% CI excluded 1.0.

TMJ involvement analysis

For the Helkimo Ai, the chi-squared test was used to analyze the distribution of Ai categories (0, I, and II) across the three groups. Similarly, the chi-squared test was applied to evaluate the distribution of the Helkimo Di categories (0, I, II, and III) among the groups. For pairwise comparisons, post hoc testing between specific groups (control vs. newly diagnosed, control vs. treated, and newly diagnosed vs. treated) was performed using the chi-squared test with a Bonferroni correction applied for multiple comparisons.

Multiple testing considerations

The genetic association analysis included three different SNPs that were independent of one another. To account for multiple comparisons, statistical significance was set at $p < 0.05$, unless specified otherwise for post hoc corrections.

RESULTS

Demographic characteristics

The demographic characteristics of patients with newly diagnosed RA, patients treated for RA, and healthy controls are presented in Table 1.

Age matching: the mean age ± SD was 40.65±10.21 years in the newly diagnosed group, 44.45±10.30 years in the treated group, and 39.90±9.99 years in the control group. One-way ANOVA revealed no statistically significant differences in mean age among the three groups ($F = 2.65$, $p = 0.08$), indicating successful age matching.

Sex matching: the newly diagnosed group included 18 females (90%) and 2 males (10%); the treated group consisted of 28 females (70%) and 12 males (30%); and the control group comprised 24 females (80%) and 6 males (20%). The chi-squared test confirmed no significant differences in sex distribution among the groups ($\chi^2 = 3.23$, $p = 0.20$), confirming successful sex matching.

Disease activity: the mean CDAI score was 24.6 ± 6.8 in the newly diagnosed group (indicating high disease activity) and 9.8 ± 4.2 in the treated group (indicating low disease activity).

Involvement of TMJ

Helkimo Ai: the distribution of the Helkimo Ai categories across the three study groups is presented in Table 2, part A. Statistically significant differences were observed among the groups ($p < 0.001$). Overall, subjective TMJ symptoms (Ai >0) were reported in 28/40 (70.0%) treated patients and 10/20 (50.0%) newly diagnosed patients, compared with 5/30 (16.7%) controls. The mean Ai score ± SD was 1.18 ± 0.87 in the treated group, 0.65 ± 0.75 in the newly diagnosed group, and 0.17 ± 0.38 in the controls.

Helkimo Di: breakdown of the categories comprising the Helkimo Di is presented in Table 2, part B, for the three different study groups, showing that statistically significant differences were observed among the groups ($p < 0.001$). Overall, objective TMJ dysfunction (Di >0) was observed in 30/40 (75.0%) treated patients and 14/20 (70.0%) newly diagnosed patients, compared with 4/30 (13.3%) controls, all

Table 1. *Demographic characteristics of the study groups*

Variable	Treated (n = 40)	Newly diagnosed (n = 20)	Control (n = 30)	Test statistic	df	P-value
Age (years), mean ± SD	44.45 ± 10.30	40.65 ± 10.21	39.90 ± 9.99	F = 2.65	2,117	0.08 (NS)
Sex, n (%)				$\chi 2 = 3.23$	2	0.20 (NS)
Male	12 (30%)	2 (10%)	6 (20%)	–	–	–
Female	28 (70%)	18 (90%)	24 (80%)	–	–	–
Mean CDAI score	9.8 ± 4.2	24.6 ± 6.8				

Data are presented as the mean ± standard deviation (SD) for continuous variables and as frequencies (percentages) for categorical variables. Age was compared across groups using a one-way analysis of variance (F = 2.65; df = 2, 117; p = .080), and sex distribution was analyzed using the chi-squared test ($\chi^2 = 3.23$; df = 2; P = 0.20); NS, not significant.

Table 2. *Anamnestic and dysfunction components of the Helkimo index across study groups*

A, Helkimo Ai						
Ai category (Ai)	Treated (n = 40)	Newly diagnosed (n = 20)	Control (n = 30)	$\chi 2$	df	p-value
No symptoms (Ai 0), n (%)	12 (30%)	10 (50%)	25 (83.3%)	–	–	–
Mild symptoms (Ai I), n (%)	9 (22.5%)	7 (35%)	5 (16.7%)	–	–	–
Severe symptoms (Ai II), n (%)	19 (47.5%)	3 (15%)	0 (0%)	–	–	–
Mean score ± SD	1.18 ± 0.87	0.65 ± 0.75	0.17 ± 0.38			
Overall Ai comparison	–	–	–	45.6	4	**<0.001**
B, Helkimo Di						
Dysfunction index (Di)	Treated (n = 40)	Newly diagnosed (n = 20)	Control (n = 30)	$\chi 2$	df	p-value
No dysfunction (Di0), n (%)	10 (25%)	6 (30%)	26 (86.6%)	–	–	–
Mild dysfunction (DiI), n (%)	11 (27.5%)	9 (45%)	4 (13.3%)	–	–	–
Moderate dysfunction (DiII), n (%)	15 (37.5%)	3 (15%)	0 (0%)	–	–	–
Severe dysfunction (DiIII), n (%)	4 (10%)	2 (10%)	0 (0%)	–	–	–
Mean score ± SD	1.33 ± 0.97	1.05 ± 0.94	0.13 ± 0.35			
Overall Di comparison	–	–	–	72.3	6	**<0.001**

Chi-squared test was used to analyze Ai and Di across groups, yielding a) for anamnestic index (Ai): $\chi^2 = 45.6$, df = 4, p <0.001; b) for dysfunction index (Di): $\chi^2 = 72.3$, df = 6, p <0.001. Fisher's exact test was applied where expected cell counts were <5, confirming significance.

Table 3. *Pairwise post hoc comparisons of the Helkimo anamnestic component among the study groups*

Groups	$\chi 2$ (Ai)	df (Ai)	p-value (Ai)	$\chi 2$ (Di)	df (Di)	p-value (Di)
Control vs. treated	28.4	2	<0.001	41.7	3	<0.001
Control vs. newly diagnosed	12.1	2	0.002	29.5	3	<0.001
Newly diagnosed vs. treated	6	2	0.048	3.6	3	0.303 (NS)

Pairwise post hoc comparisons were performed using the chi-squared test. Statistical significance was established at p <0.05, and non-significant differences are denoted as NS (p >0.05). Fisher's exact test was applied to confirm the results when expected cell counts were <5.

4 of whom had mild dysfunction (DiI). The mean Di score ± SD was 1.33 ± 0.97 in the treated group, 1.05 ± 0.94 in the newly diagnosed group, and 0.13 ± 0.35 in the controls.

Pairwise comparisons revealed distinct patterns for subjective versus objective TMJ measures (Table 3). For the Ai (subjective symptoms), a statistically significant difference was observed between newly diagnosed and treated patients (p = 0.048), with treated patients reporting more severe symptoms (47.5%) compared to newly diagnosed patients (15%), as presented in Table 2. In contrast, for the Di (objective findings), no significant difference was observed between these groups (p = 0.303), as shown in Table 3. Detectable TMJ dysfunction was highly prevalent in both the treated and newly diagnosed groups, indicating that TMJ pathology is a common finding regardless of current treatment status.

CASP1 gene sequencing

CASP1 gene sequencing revealed the presence of three single nucleotide polymorphisms, rs580253, rs501192, and rs56244680 in both the control group and patients with RA, as shown in Figure 1. Among these variants, the polymorphism rs580253 resulted from a cytosine-to-thymine (C>T) substitution, while rs501192 resulted from a guanine-to-adenine (G>A) substitution. Lastly, rs56244680 displayed the TTCCATATTTA sequence deletion.

CASP1 C/T (*rs580253)*: no statistically significant differences were observed among the three groups for genotype distribution (χ^2 = 2.01, p = 0.742) or allele frequencies (χ^2 = 0.76, p = 0.683), as presented in Table 4.

CASP1 G/A (*rs501192*): the observed genotype frequencies (proportion) were as follows: the AA genotype was found in 2 (10.0%) newly diagnosed patients, 0 (0.0%) treated patients, and 1 (3.3%) control; the GA genotype was found in 4 (20.0%) newly diagnosed patients, 6 (15.0%) treated patients, and 1 (3.3%) of the controls; the GG genotype was found in 70.0% of the newly diagnosed patients, 85.0% of the treated patients, and 83.3% of the controls (Table 5).

The A allele frequency was 8 (20%) in the newly diagnosed group, 14 in the treated group, and 6 (10%) in the control group. Statistical analysis revealed no significant differences in genotype distribution (χ^2 = 5.78, p = 0.218) or allele frequencies (χ^2 = 3.71, p = 0.156) among the three groups.

HWE testing for rs501192 yielded the following results: i) control group – observed frequency (proportion): GG=25 (83.3%), GA=4 (13.3%), AA=1 (3.3%) which was in HWE (χ^2 = 0.18, df = 1, p = 0.671); ii) newly diagnosed group – observed frequency (proportion): GG=14 (70.0%), GA=4 (20%), AA=2 (10%) revealing no significant deviation (χ^2 = 0.02, df = 1, p = 0.888); iii) treated group – observed frequency (proportion): GG=34 (85%), GA=6 (15%), AA=0 (0%), which was consistent with HWE (χ^2 = 0.26, df = 1, p = 0.610). These results confirm that genotype distributions for rs501192 were in HWE across all groups (all p >0.05), as presented in Table 5.

CASP1 (*rs56244680*): the rs56244680 11-bp deletion (TTCCATATTTA) was observed in both heterozygous and homozygous states across the study groups: i) newly diagnosed group, (n = 20) – two patients, 0.1 (10.0%) carried the deletion, one heterozygous (ins/del) and one homozygous (del/del); ii) treated group, (n = 40) – one patient, 0.025 (2.5%) carried the deletion in heterozygous form (ins/del); and iii) control group, (n = 30) – one individual, 0.033 (3.3%) carried the deletion in the heterozygous form (ins/del).

The overall frequency of deletion carriers (combining heterozygous and homozygous) did not differ significantly among the three groups (χ^2 = 2.15, p = 0.341), as illustrated in Figure 2. Due to the low number of these carriers, statistical comparison of heterozygous versus homozygous genotypes was not feasible.

HWE testing for rs56244680 (11-bp deletion) was performed, yielding the following results: i) control group – observed frequencies of ins/ins = 29, ins/del = 1, del/del = 0; due to the low expected frequency of the del/del genotype, Fisher's exact test was used, which confirmed no significant deviation from HWE (p = 1.000); and ii) newly diagnosed group – observed frequencies of ins/ins =18, ins/del = 1, and del/del = 1.

Fisher's exact test revealed consistency with HWE (p = 0.289); and iii) treated group – observed frequencies of ins/ins = 39, ins/del = 1, and del/del = 0, with Fisher's exact test confirming HWE (p = 1.000). These results confirm that genotype distributions for rs56244680 were in HWE across all groups (all p >0.05).

DISCUSSION

In the present study, both components of the Helkimo index confirmed substantially greater TMJ involvement in patients with RA than in controls (Table 2). Objective dysfunction was detectable in approximately three-quarters of the patient cohort, while the controls were almost uniformly asymptomatic, with only mild findings in a small minority. Dysfunction was comparably frequent in newly diagnosed and treated patients, indicating that TMJ pathology is established early in the disease course and persists despite systemic combination therapy.

Importantly, a clear discrepancy was observed between patient-reported symptoms and objective clinical findings. This divergence occurred despite the treated group exhibiting lower peripheral disease activity (CDAI: 9.8 ± 4.2 in the treated group vs. 24.6 ± 6.8 in the newly diagnosed group). This clinical dissociation has significant implications for routine practice.

The discovery that treated patients report more severe subjective symptoms despite having equal objective dysfunction ratings to newly diagnosed patients is surprising. Potential explanations include a longer overall disease duration leading to cumulative joint deterioration, or altered symptom awareness in patients undergoing long-term therapy. However, in the absence of imaging and temporal data, these remain purely speculative hypotheses.

Table 4. *Genotype and allele distributions of the CASP1 C/T (rs580253) polymorphism and associated odds ratios across the study groups*

A, Genotype											
Genotype	Control	Newly diagnosed	χ2	df	p-value	OR (95% CI)a	Treated group	χ^2	df	p-value	OR (95% CI)a
CC	25 (83.3%)	13 (65%)	3.2	2	—	1.00 (Ref)	33 (82.5%)	0.1	2	—	1.00 (Ref)
CT	4 (13.3%)	5 (25%)	1.6	2	0.20 (NS)	2.4 (0.54-10.51)	7 (17.5%)	0.3	2	0.60 (NS)	1.3 (0.34- 5.03)
TT	1 (3.3%)	2 (10%)	1.6	2	0.20 (NS)	3.8 (0.31-46.49)	0	0.3	2	0.20 (NS)	0.4 (0.0099-6.49)
B, Allele											
Allele	Control	Newly diagnosed	χ2	df	p-value	OR (95% CI)	Treated group	χ^2	df	p-value	OR (95% CI)
C alleleb	54 (90%)	31 (80%)	—	1	—	1.00 (Ref)	73 (90%)	—	1	—	1.00 (Ref)
T alleleb	6 (10%)	9 (20%)	2.9	1	0.09 (NS)	2.6 (0.84-8.03)	7 (10%)	0.07	1	0.80 (NS)	0.9 (0.27–2.71)

Data are presented as number of participants with percentage in parentheses for genotypes, and as allele frequency (proportion) with raw counts (variant alleles/total alleles) in parentheses for alleles. CI: confidence interval; df: degrees of freedom; NS: not significant (p >0.05); OR: odds ratio.

a: Odds ratios represent the risk of RA associated with each genotype or allele compared with the reference category (CC genotype or C allele) in the control group. OR >1.0 indicates increased risk; OR <1.0 indicates decreased risk.

b: The frequencies of alleles are presented as proportions, with rates ranging from 0 to 1.0. Raw counts are the number of variant alleles divided by the total number of chromosomes.

Genotype frequencies (CC, CT, TT) and allele frequencies were compared using chi-squared tests. In cases where the expected cell counts were less than five, such as in the case of the TT genotype in the treated group, Fisher's exact test was utilized.

No statistically significant differences were detected among the three groups for genotype distribution (χ^2 = 2.01, P = 0.742) or allele frequencies (χ^2 = 0.76, P = 0.683).

Table 5. *Genotype and allele distributions, χ^2, df, and odds ratios for CASP1 G/A (rs501192) across the study groups*

A, Genotype											
Genotype	Control	Newly diagnosed RA	χ2	df	p-value	OR (95% CI)a	Treated (n = 40)	χ2	df	P-value	OR (95% CI)a
GG	25 (83.3%)	14 (70%)	—	—	—	1.00 (Ref)	34 (85%)	—	—	—	1.00 (Ref)
GA	4 (13.3%)	4 (20%)	—	—	0.40 (NS)	1.7 (0.38-8.26)	6 (15%)	—	—	0.80 (NS)	1.1 (0.28–4.32)
AA	1 (3.3%)	2 (10%)	—	—	0.30 (NS)	3.5 (0.29-2.99)	0	—	—	0.30 (NS)	0.2 (0.00– 6.29)
Overall genotype χ2	—	—	2.14	2	0.34 (NS)	—	—	1.03	2	0.59 (NS)	—
B, Allele											
Allele	Control	Newly diagnosed RA	χ2	df	P-value	OR (95% CI)	Treated group	χ2	df	P-value	OR (95% CI)
G alleleb	54	32	—	—	—	1.00 (Ref)	66	—	—	—	1.00 (Ref)
A alleleb	6	8	2.63	1	0.10NS	2.2 (0.71–7.07)	14	1.64	1	0.20NS	1.9 (0.68–5.30)

Genotype frequencies (GG, GA, AA) were compared using chi-squared tests (df = 2). Allele frequencies (G vs A) were compared using chi-squared tests (df = 1). Fisher's exact test was applied where expected cell counts were <5 (e.g., AA genotype in the treated group), confirming the reported P-values.

[a]Odds ratios represent the risk of RA associated with each genotype or allele compared with the reference category (GG genotype or G allele) in the control group. OR >1 indicates increased risk; OR <1 indicates decreased risk. [b]Allele frequencies are presented as proportions (range 0-1). Raw counts show the number of variant alleles/total chromosomes. CI, confidence interval; HWE, Hardy-Weinberg equilibrium; OR, odds ratio; NS, not significant.

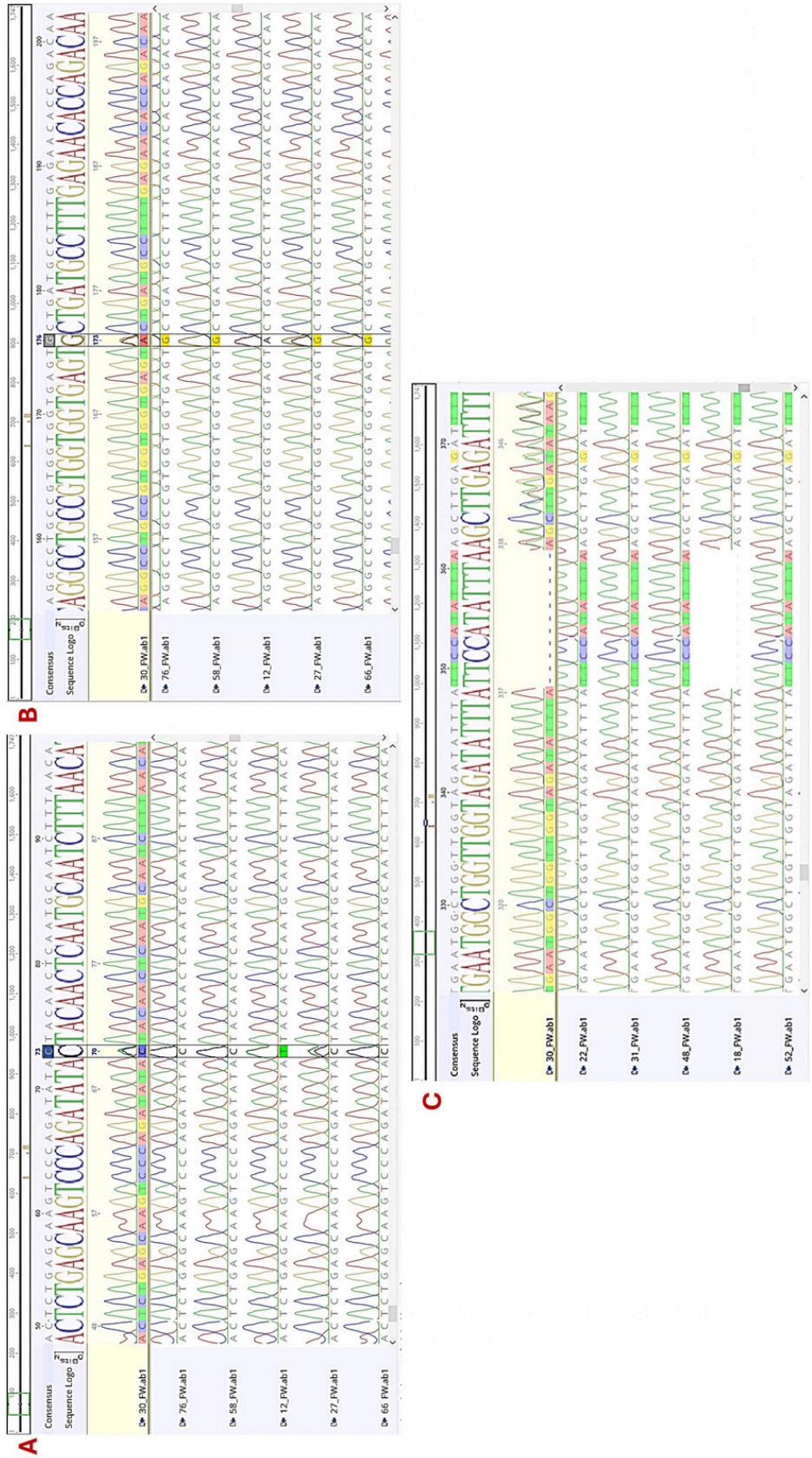


Figure 1. *Representative Sanger sequencing chromatograms of three CASP1 SNP regions from patients with rheumatoid arthritis and controls. (A) rs580253 (C>T): Homozygous CC shows a single blue peak, heterozygous CT shows overlapping blue and red peaks, and homozygous TT shows a single red peak. (B) (B) rs501192 (G>A): Homozygous GG and AA genotypes exhibit distinct single-peak patterns, while heterozygosity (GA) is indicated by overlapping black and green peaks. (C) rs56244680 (11-bp deletion, TTCCATATTTA): The deletion allele (Del) is distinguished from the normal allele (Norm) by a shift in peak alignment and a truncated consensus sequence. These panels validate the precise nucleotide alterations for the genotypes presented in Tables 4 and 5.*

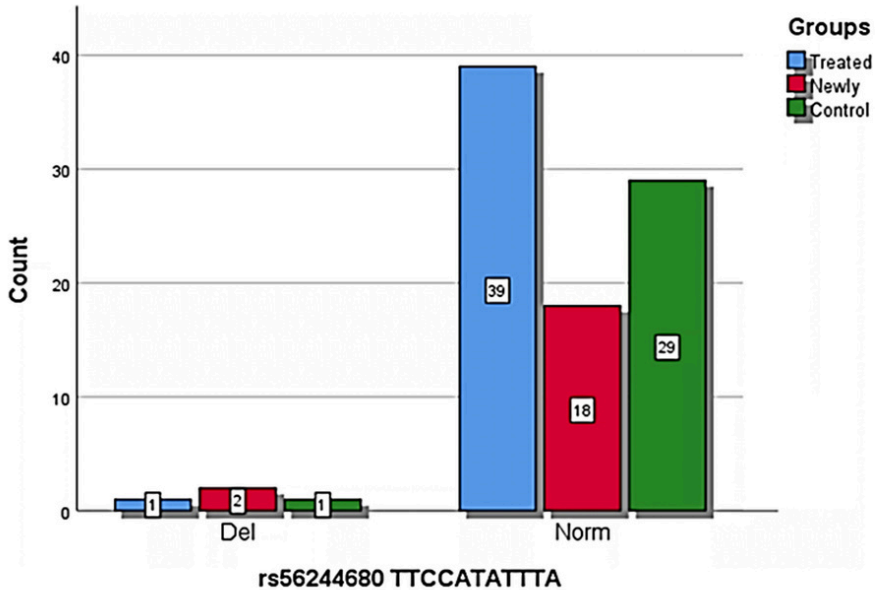


Figure 2. *Distribution of the CASP1 rs56244680 (TTCCATATTTA) variant across study groups. The count of individuals carrying the deletion (Del) and normal (Norm) variants is displayed for treated rheumatoid arthritis patients, newly diagnosed patients, and healthy controls. The Norm variant was predominant across all groups (39 treated, 18 newly diagnosed, 29 controls), while the Del variant was rare, occurring in 1 treated patient, 2 newly diagnosed patients, and 1 control. The figure shows the low frequency of the deletion variant and that its distribution is comparable and consistent across all study groups.*

The present study found no statistically significant associations between the investigated *CASP1* polymorphisms (rs580253, rs501192, and rs56244680) and RA susceptibility in the Iraqi cohort. Globally, genetic variation in *CASP1* has been explored across diverse populations, yielding inconsistent results. For instance, Addobbati et al. (23) reported no significant association for *CASP1* rs572687 and RA risk in a Brazilian cohort. This is in contrast to the findings of Liu et al. (22), who identified a significant correlation between *CASP1* rs2409062 and RA susceptibility in a Chinese population. These disparities between previous and the current study are likely attributable to population-specific genetic architecture.

The allele frequencies of the SNPs investigated in the Iraqi controls in this study were approximately 10.0% for both the rs580253 T allele and the rs501192 A allele, both of which were found in human subjects. When compared with patterns in Middle Eastern and Arab populations documented in public databases (for instance, the gnomAD Middle Eastern ancestry group) and limited regional research, these frequency rates appear to be compatible with such patterns (30).

In East Asian cohorts, however, specific *CASP1* polymorphisms show stronger effects on inflammasome-related features (22,31). These findings are in contrast to the current findings. There are a variety of potential mechanisms that may be responsible for differences that are specific to a population, including the following: i) when compared to East Asians, Iraqi and Middle Eastern populations may have lower or different minor allele frequencies. This may reduce the capacity to detect small effects or obscure the genetic background that determines risk. Additionally, allele frequencies may vary across different groups; ii) distinct linkage disequilibrium (LD) patterns: because the investigated SNPs are intronic, they have the potential to tag functional causal variants in certain populations but not in others. This occurs because LD blocks vary significantly among different ancestries. However, the LD structure in Arab populations may be different, potentially decoupling these variants from actually causal factors within *CASP1* or neighboring genes. For example, rs580253 and rs501192 exhibit moderate-to-high LD in Mexican cohorts, which have been analyzed in previous research on the inflammasome (17); iii) RA pathways exclusive to a particular ethnic group: the susceptibility and course of RA among Middle Eastern populations may entail distinct inflammatory or autoimmune pathways.

Genome-wide association studies in Arab populations have verified that European and Asian groups share contributions from HLA and documented non-HLA loci, while also suggesting the existence of population-specific modifiers or rare variations not captured here (32).

Activation of *CASP1* may play a less prominent role in the pathogenesis of RA in Iraqis compared with East Asians, or environmental and epigenetic factors may regulate the influence of this process. These factors highlight the importance of conducting genetic research on under-represented populations, such as Iraqis, to improve our understanding of RA variability. To determine whether these

CASP1 variations contribute modestly or whether other inflammasome-related genes increase risk in this demographic, larger replication studies involving many ethnic groups and including functional assays are required.

Beyond its function in apoptosis, *CASP1* serves as the principal inflammatory caspase mediating the generation of pro-inflammatory cytokines IL-1β and IL-18 through the activation of the inflammasome (20,33,34). Yang et al. (21) found that hyperactivated *CASP1* in neutrophils drives IL-18 and enhances RA activity, irrespective of NLRP3 inflammasome signaling, a pathway with direct relevance to RA etiology.

Preclinical evidence demonstrates that the *CASP1* inhibitor VX765 reduces the severity of collagen-induced arthritis in mouse models (35). Collectively, these findings position *CASP1* as a biologically plausible candidate for influencing RA susceptibility and warrant further investigation as a potential therapeutic target. This finding lends credence to the therapeutic possibility of targeting this axis.

Rheumatology practice is significantly impacted by the high prevalence of TMJ dysfunction among patients with RA: i) patients diagnosed with RA should undergo routine TMJ screening as part of the standard rheumatologic examination, utilizing established tools such as the Helkimo index. Early detection of TMJ involvement may allow for intervention before irreversible damage occurs; ii) treatment responses must be analyzed independently for TMJ symptoms, since improvements in peripheral joint counts or inflammatory markers may not correlate with TMJ outcomes; and iii) multidisciplinary management strategies are vital to provide comprehensive care.

These strategies should include rheumatologists, dentists, and maxillofacial experts. In addition to systemic immunomodulation, local therapies, such as intra-articular injections, physiotherapy, and occlusal appliances, must be considered; iv) it may be possible to facilitate earlier reporting and intervention by providing patients with information regarding TMJ symptoms, which include pain during mastication, joint clicking, and limited mouth opening; v) TMJ outcomes should be included as secondary endpoints in future therapeutic trials for RA to better understand how various medications influence this joint.

The present study has certain limitations, which should be mentioned. Its cross-sectional design allows for the identification of associations at a specific moment in time; however, it does not permit the demonstration of causality or monitoring of longitudinal changes in TMJ dysfunction among individual patients.

Due to the low minor allele frequencies of the investigated *CASP1* variants, the sample size was insufficient to perform valid sub-analyses correlating specific genotypes with TMJ severity phenotypes. We recommend this genotype-phenotype correlation as a specific objective for future multi-center studies with larger cohorts. Another limitation of our present study is the absence of baseline laboratory parameters, such as acute-phase reactants (CRP, ESR) and serological markers (RF, anti-CCP). While disease activity was rigorously captured using the acute-phase reactant-free CDAI, and all patients met standard classification criteria, the lack of baseline laboratory data limits the depth of immunological characterization of the cohort. Future studies should integrate these biomarkers to provide a more comprehensive clinical-serological profile.

Two primary findings emerged from this study. First, in our patient cohort, the investigated *CASP1* polymorphisms (rs580253, rs501192, and rs56244680) did not demonstrate a statistically significant association with rheumatoid arthritis in this Iraqi population. Consequently, while these specific variants did not appear to be major risk factors in this population, their potential role as minor modifiers requires further validation in larger cohorts. Second, the involvement of TMJ is relatively common in RA, affecting the vast majority of patients. Objective TMJ dysfunction did not differ significantly between treated and newly diagnosed patients, while subjective symptoms were more severe in the treated group, potentially reflecting a prolonged disease course and cumulative damage. This clinical dissociation underscores the necessity of routinely assessing the TMJ using both patient-reported outcomes and clinical examination, as well as the significance of adjuvant local therapies particularly targeting the TMJ. Taken together, these findings have two key implications for the management of RA: i) routine clinical assessment of TMJ function should be integrated into rheumatology practice; and ii) further large-scale multi-center studies are warranted to continue the search for genetic markers.

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Author Contributions

Conceptualization, W.L.A. and A.H.A.H; Methodology, B.H.A.G. and A.H.A.H.; Investigation, W.L.A. and A.H.A.H.; Formal Analysis, B.H.A.G. and A.H.A.H.; Writing – original draft, B.H.A.G. and A.H.A.H.; Writing – review & editing, W.L.A and A.H.A.H. All authors have read and approved the published version of the manuscript.

Statement of Ethics

The study protocol was approved by Baghdad Teaching Hospital Scientific and Ethical Unit (Approval No. 859, issued on February 19, 2023). All participants provided written informed consent prior to enrollment in accordance with the Declaration of Helsinki.

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 the published article. Additional data are available from the corresponding author upon reasonable request.

Statement of Generative AI Use

No generative AI was used.

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ORIGINAL ARTICLE

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CLINICAL SIGNIFICANCE OF *THYMUS SERPYLLUM* L. ESSENTIAL OIL IN PATIENTS WITH SYNCOPE

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Dysautonomia, reflecting autonomic nervous system dysfunction, is frequently observed in patients with syncope, particularly those with orthostatic hypotension. *Thymus serpyllum* L. (wild thyme) has demonstrated antioxidant, anti-inflammatory, and cardiometabolic properties in preclinical studies. This study aimed to evaluate the effects of *T. serpyllum* essential oil on autonomic function and selected biochemical markers in patients with syncope. This prospective, single-center study included 15 patients with a history of syncope who received a capsule formulation of *T. serpyllum* essential oil for three months. Autonomic function was assessed using beat-to-beat heart rate and blood pressure variability analysis, 24-hour Holter ECG monitoring, and ambulatory blood pressure monitoring. Biochemical parameters were evaluated at baseline and after treatment. After three months, the beat-to-beat analysis showed increased LFnu values and a higher LF/HF ratio, along with decreased HF values, indicating a shift toward sympathetic predominance. No significant changes were observed in 24-hour heart rate or blood pressure parameters. Biochemical analyses revealed significant reductions in AST and C-reactive protein levels after treatment. In this pilot study, three-month supplementation with *T. serpyllum* essential oil was associated with changes in autonomic balance and favorable reductions in selected inflammatory and biochemical markers in patients with syncope. Further and larger placebo-controlled studies are required to confirm these findings and clarify their clinical significance.

Keywords: syncope, autonomic nervous system, *Thymus serpyllum* essential oil, neurocardiology

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INTRODUCTION

Dysautonomia, defined as a dysfunction of the autonomic nervous system (ANS), encompasses a heterogeneous group of disorders characterized by variable clinical presentations. It may result from isolated ANS impairment or form part of a broader multisystem failure (1). This diversity arises from the ANS's critical role in the involuntary innervation of all non-motor end organs and its close interconnection with the central nervous system (CNS), where the boundaries between the two are not clearly delineated (2).

Among the most common clinical manifestations of dysautonomia are disorders of orthostatic intolerance, including orthostatic hypotension (OH), both initial and delayed forms, and postural orthostatic tachycardia syndrome (POTS). These conditions are frequently accompanied by symptoms such as dizziness, near-syncope, blurred vision upon standing, and transient loss of consciousness, i.e., syncope (3-5). Regarding syncope specifically, reflex syncope has been diagnosed in 56-73% of patients, whereas orthostatic hypotension accounts for 1-10% of cases (6).

In addition to ANS dysfunction in neurodegenerative diseases (such as Parkinson's disease and multiple system atrophy), dysautonomia has been extensively studied in patients with diabetes mellitus (DM). Notably, patients with DM and cardiac autonomic neuropathy (CAN) exhibit an associated five-year mortality rate of 25-50% (7). The development of autonomic neuropathy in DM is believed to result from inflammation induced by advanced glycation end products (AGEs) and the activation of the hexosamine, protein kinase C, and polyol pathways, coupled with increased oxidative stress (7). Ziegler et al. confirmed elevated oxidative stress by employing various biochemical markers in patients with diabetic CAN compared to a control group (8).

A wide range of pharmacological and natural agents with antioxidant properties have been identified that can counteract the adverse effects of these processes. Among them, *Thymus serpyllum* L. (wild thyme) has garnered significant attention due to its potent antioxidant effects demonstrated in both in vitro and in vivo studies (9). In addition to its essential oil constituents, *T. serpyllum* contains numerous bioactive compounds, including thymol, carvacrol, flavonoids, rosmarinic acid, phenolic acids, and terpenoids, which are considered largely responsible for its antioxidant, anti-inflammatory, and cardiometabolic activities (10,11). Experimental studies have shown that T. serpyllum extracts exhibit pronounced free-radical scavenging activity, inhibition of lipid peroxidation, metal-chelating properties, and protection against oxidative damage to biological molecules (12,13).

Wild thyme essential oil is widely used in the food industry as a flavoring agent and preservative, and its antimicrobial, anti-inflammatory, hepatoprotective, and cardiometabolic properties have been well-documented, particularly in rodent models (90. Recent reviews have further highlighted its immunomodulatory, antioxidant, and potential cardiovascular protective effects, which have been attributed to the synergistic action of multiple phytochemicals rather than a single active constituent (9,10). Notably, the hydrosolic (secondary) fraction of *T. serpyllum* essential oil demonstrates enhanced antibacterial and antifungal activity, and contains a markedly higher proportion of phenolic compounds than the primary, water-insoluble fraction (9).

Furthermore, using an aqueous extract of *T. serpyllum*, Mihailović-Stanojević et al. demonstrated a significant reduction in blood pressure and peripheral vascular resistance in hypertensive rats via a nitric oxide-independent mechanism, without affecting hemodynamic parameters in normotensive rats, alongside confirming its strong antioxidant capacity (14). Similarly, Miloradović et al. reported blood pressure normalization and reduction in oxidative stress in rats following a bolus injection of thyme extract (15). Additional experimental studies have shown that *T. serpyllum* preparations may reduce oxidative stress through flavonoid- and rosmarinic acid-mediated mechanisms while simultaneously exerting anti-inflammatory effects (10). The major constituents of the essential oil, including thymol and carvacrol, have also been associated with antioxidant, antimicrobial, and vasoregulatory properties (9,10). Although animal studies suggest that *T. serpyllum* may exert beneficial effects on the cardiovascular system in humans, further clinical research is required to validate these findings and clarify their clinical significance.

The primary aim of this study was to investigate the effects of a tablet formulation of *T. serpyllum* essential oil on autonomic nervous system function parameters and selected biochemical markers in patients presenting with syncope, with measurements obtained at baseline and three months after treatment.

METHODS

Study design and participants

This prospective study included 15 patients over the age of 18 who were evaluated at the Neurocardiology Laboratory of the Cardiology Clinic, Institute for Cardiovascular Diseases "Dedinje," between December 2024 and March 2025. The inclusion criterion was a documented history of syncope, defined as a transient loss of consciousness (TLOC) due to cerebral hypoperfusion, characterized by rapid onset, short duration, and spontaneous complete recovery (3). Exclusion criteria included: cardiogenic etiology of syncope; confirmed epilepsy as the cause of TLOC (diagnosed by a neurology specialist); orthostatic hypotension resulting from volume depletion or medication use; and the presence of primary autonomic neuropathy (e.g., multiple system atrophy, pure autonomic failure, Parkinson's disease).

Demographic data, including age and sex, were collected for all participants, alongside detailed medical histories covering comorbidities (hypertension, diabetes mellitus, thyroid disorders, systemic diseases, etc.) and the presence of OH, verified during a head-up tilt test and defined as a progressive and sustained fall in systolic blood pressure from baseline of >20 mmHg, a diastolic drop of >10 mmHg, or a decrease in systolic blood pressure to <90 mmHg (3). Each participant underwent: (1) beat-to-beat blood pressure monitoring in the supine position for 5 minutes; (2) 24-hour Holter ECG monitoring; (3) ambulatory blood pressure monitoring; and (4) venous blood sampling for laboratory analyses, including standard biochemical and hematological parameters (C-reactive protein, AST, ALT, GGT, urea, creatinine, uric acid, glucose, total cholesterol, LDL and HDL fractions, and triglycerides). All measurements were obtained at baseline and after three months of treatment with the tablet formulation of *T. serpyllum* essential oil.

All participants were informed of the study's purpose and the administration protocol for the investigational product. They received detailed instructions regarding dosage and timing. Written informed consent was obtained from each participant prior to enrollment. No additional medications such as statins, beta-blockers, calcium channel blockers, or any other agent known to affect the autonomic nervous system or the biochemical parameters under investigation were introduced during the study period.

The study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Institutional Ethics Committee of the Institute for Cardiovascular Diseases "Dedinje" (protocol code 6472, issued on 11 December 2024).

Study Protocol

Plant material of *Thymus serpyllum* L. was cultivated in Bavanište, Serbia (44°48′59.99″ N, 20°52′59.99″ E). Voucher specimens were deposited in the Herbarium of the Institute for Medicinal Plant Research "Dr Josif Pančić", Belgrade, Serbia (voucher numbers 302 and 311).

Essential oil from *T. serpyllum* was extracted using a semi-industrial distillation apparatus (SP-130) based on water and steam distillation principles. The isolated essential oil is a light yellow liquid with an aroma characteristic of the *Thymus genus*, and was subsequently subjected to inverse molecular distillation for further refinement.

The primary carrier for the highly purified essential oil was lyophilized honey. Acacia honey, rich in natural monosaccharides (approximately 70% combined glucose and fructose), was selected due to its favorable nutritional profile and stability during processing. Lyophilization was performed in three stages: freezing, primary drying, and secondary drying (desorption). Initially, the honey was frozen at temperatures ranging between -40 °C and -60 °C. During primary drying, sublimation occurred under reduced pressure and temperature, enabling the direct transition of ice into water vapor. Secondary drying, conducted at room or slightly elevated temperatures, removed residual moisture that did not crystallize during freezing.

Through this process, the lyophilized honey achieved stability, enabling long-term storage and preservation. After removing moisture, the honey was ground to eliminate lumps, creating a uniform, fine-grained powder that also served as a filler for capsules.

The final formulation consisted of 500 mg HPMC capsules containing 50 mg of *T. serpyllum* essential oil and 450 mg of lyophilized acacia honey as the carrier. Patients were instructed to take one to two capsules daily according to the treatment protocol.

The chemical composition of the essential oil was determined by gas chromatography–mass spectrometry (GC–MS) and ATR-FTIR analysis. GC-MS analysis was performed using an Agilent 7890A system (Agilent Technologies, Santa Clara, CA, USA) equipped with 5975C MSD and FID detectors. For chromatographic separation, a weakly polar HP-5MS (5% diphenyl- and 95% dimethyl-polysiloxane, 30 m × 0.25 mm, 0.25 µm film thickness) capillary column was used (Agilent Technologies, Santa Clara, CA, USA). EO samples were dissolved in dichloromethane at a concentration of 10 µL/mL and were injected in 1:10 split mode. Helium was used as the carrier gas at a constant pressure condition (16.255 psi). The oven temperature was programmed to increase from 60 °C to

300 °C at a rate of 3 °C per minute, followed by a final hold for 10 min. Mass spectra were obtained using the electron ionization (EI) mode across a scan range of 40–600 m/z, with the ion source maintained at 230 °C, the quadrupole set to 150 °C, and a solvent delay of 3 min.

Data processing was carried out using MSD ChemStation (Agilent Technologies, Santa Clara, CA, USA). Compound identification was performed by matching the experimental mass spectra with commercial libraries (NIST 17, Wiley 7, and Adams 4) using AMDIS 32 software (v2.73) along with the NIST search program (v2.3). Retention indices (RIs) were calculated using a homologous series of n-alkanes (C8–C32) and compared with the literature values. Relative abundances of the detected compounds were determined from GC-FID chromatograms.

ATR-FTIR analysis of *T. serpyllum* L. EO samples was recorded using an IRAffinity-1 FTIR spectrometer (Schimadzu, Kyoto, Japan). The spectra were recorded in the spectral range from 400 to 4000 cm−1 with a resolution of 4 cm−1 (256 scans). Afterwards, preprocessing including baseline correction, normalization, and smoothing was performed using the Spectragryph v1.2.15 software (Menges, 2018).

GC–MS analysis identified thymol (50.47%), p-cymene (23.56%), linalool (5.84%), 1,8-cineole (4.98%), carvacrol (3.18%), and caryophyllene oxide (2.28%) as the principal constituents of the essential oil. The complete chemical composition of the investigated *T. serpyllum* essential oil is presented in Supplementary Table S1.

Beat-to-beat heart rate and blood pressure variability were measured using the Task Force® Monitor system, which performs spectral analysis of heart rate and blood pressure via an autoregressive methodology, providing parameters such as power spectral density (PSD), frequency bands (VLF, LF, HF), baroreceptor reflex sensitivity (BRS), and baroreceptor effectiveness index (BEI) (16,17).

A 12-lead electrocardiograph was used for 24-hour Holter ECG monitoring, and recordings were reviewed by trained analysts. After manual artifact removal and correction of beat classifications, data were analyzed to determine mean heart rate and RR intervals. Heart rate variability (HRV) in the time domain was assessed using three standard parameters: SDNN, RMSSD, and PNN50.

Ambulatory blood pressure was recorded over 24 hours using an oscillometric device, with measurements obtained every 15 minutes during daytime hours. Analyses included systolic and diastolic blood pressure values for the entire 24-hour period and separately for daytime and nighttime intervals.

Statistical Analysis

All results are reported as counts and percentages, means with standard deviations, or medians with interquartile ranges (25th-75th percentiles), depending on the data type. The distribution of variables was assessed using the Shapiro-Wilk test and Q-Q plots. Paired measurements were compared using the paired-samples t-test for normally distributed data or the Wilcoxon signed-rank test for non-normally distributed data. Statistical analyses were performed using SPSS software (version 26.0), with a significance threshold set at $p < 0.05$.

RESULTS

Demographic data and comorbidities of the study population are presented in Table 1. The majority of patients were female and over 40 years of age. In addition to syncope, most patients also exhibited orthostatic hypotension. Hypertension, diabetes mellitus, and other comorbidities were present in fewer than 20% of patients.

The results of the beat-to-beat analysis before and three months after treatment are presented in Table 2.

LFnu was significantly higher following treatment (Figure 1), whereas HF expressed in ms^2 decreased. The LF/HF ratio (normalized units) was also significantly higher after treatment (Figure 2). Although baroreceptor parameters were lower after three months, the differences were not statistically significant.

Table 1. *Demographic data and comorbidities of the study population*

	N = 15
Demographic data	
Age (mean ± SD)	43.8 ± 17.80
Female (n,%)	13 (72.2%)
Comorbidities	
Syncope (n,%)	15 (100%)
OH (n,%)	13 (83.3%)
HTA (n,%)	3 (16.7%)
DM (n,%)	2 (11.1%)
Hashimoto thyroiditis (n,%)	1 (5.6%)
IBS (n,%)	0
Asthma (n,%)	0
COPD (n,%)	0
Hyperlipoproteinemia (n,%)	0

SD – Standard deviation; OH – Orthostatic hypotension; SOH – Syncope and OH; HTA – Hypertension; DM – Diabetes Mellitus;; IBS – Irritable Bowel Syndrome; COPD – Chronic Obstructive Pulmonary Disease

Table 2. *Beat-to-beat blood pressure and heart rate parameters of study population, before and 3 months after therapy*

	Before therapy N = 15 (n,%)	After 3 months N = 15	p value
Beat to beat Heart rate and Blood pressure (5 minutes recording)			
HR (bpm) (mean ± SD)	72.4 ± 10.9	73.6 ± 11.9	0.391[a]
SBP (mmHg) (mean ± SD)	106.7 ± 9.3	103.9 ± 8.3	0.348[a]
DBP (mmHg) (mean ± SD)	72.7 ± 6.4	71.6 ± 8.7	0.615[a]
MAP (mmHg) (mean ± SD)	82.7 ± 7	82.2 ± 8.1	0.813[a]
Short term HRV parameters			
LFnu (%) (mean ± SD)	60.8 ± 17.6	70.3 ± 11.4	0.019[a]
PSD (ms2) (Mdn (IQR))	685 (301 – 1211)	524 (335 – 1288)	0.9290[b]
VLF (ms2) (Mdn (IQR))	168 (49 – 361.5)	120 (44 – 362)	0.9170[b]
LF (ms2) (Mdn (IQR))	206 (146 – 426)	274 (139.5 – 355.5)	0.8070[b]
HF (ms2) (Mdn (IQR))	126.5 (61 – 462.5)	94 (68 – 229.3)	0.0500[b]
LF/HF (nu) (Mdn (IQR))	1.9 (.7 – 3.6)	2.3 (1.7 – 5)	0.0130[b]
LF/HF (ms2) (Mdn (IQR))	2 (.7 – 3.8)	2.2 (1.5 - 4)	0.1580[b]
Baroreceptors parameters			
BRS (ms/mmHg) (Mdn (IQR))	14 (7.5 – 20)	10 (6 – 15.8)	0.6740[b]
BEI (%)(Mdn (IQR))	113 (34.6 – 173.5)	61.3 (30.5 – 133)	0.2850[b]

HR – heart rate; bpm – beat per minute; SBP – systolic blood pressure; mmHg – millimeters of mercury; DBP – diastolic blood pressure; MAP – mean arterial pressure; LFnu – normalized low frequency of HRV ; PSD – power spectral density of HRV; VLF – VLF – very low frequency component of HRV; LF - low frequency component of HRV ; HF – high frequency component of HRV; LFnu/HFnu – normalized low frequency/ normalized high frequence ratio of HRV; LF/HF – low frequency/high frequency ratio of HRV; BRS – Baroreflex sensitivity; ms/mmHg – millisecond per millimeters of mercury; BEI - baroreflex sensitivity index; [a] – Paired Samples T test; [b] – Wilcoxon signed rank test

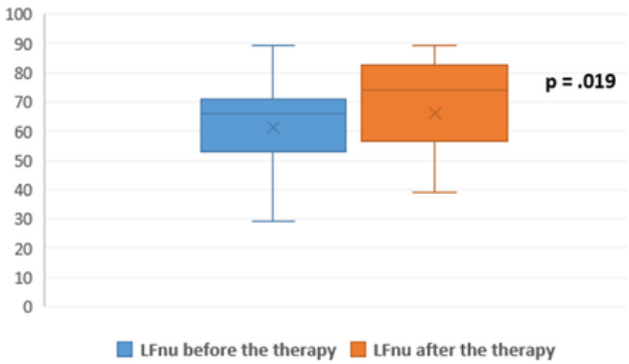


Figure 1. *Changes of LFnu after three months of therapy with Thymus serpyllum essential oil.*
For comparison, Paired samples T test was used.

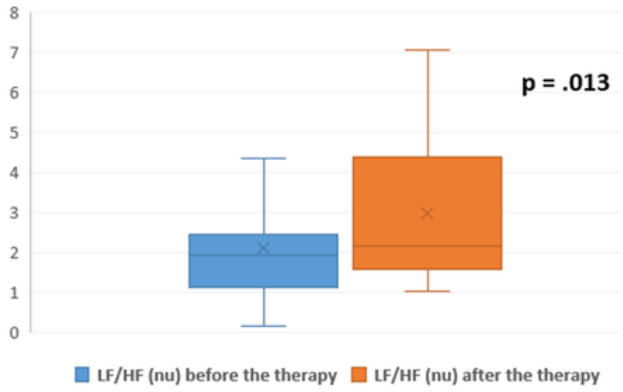


Figure 2. *Changes of LF/HF ratio expressed in normalized units after three months of therapy with Thymus serpyllum essential oil.*
For comparison, the Wilcoxon signed-rank test was used.

The 24-hour Holter ECG and ambulatory blood pressure parameters before and three months after treatment are presented in Table 3. Although time-domain HRV parameters and blood pressure values (systolic and diastolic) were higher at the end of treatment, the differences were not statistically significant.

The biochemical parameters before and three months after therapy are presented in Table 4. Levels of AST and C-reactive protein were significantly lower after the three-month treatment period (Figure 3 and Figure 4). No other statistically significant differences were observed.

Table 3. *24H Holter ECG and Ambulatory blood pressure parameters of the study population, before and 3 months after the therapy*

	Before therapy N = 15 (n,%)	After 3 months N = 15	p value
24h Holter ECG monitoring			
RRI (ms) (mean ± SD)	809.5 ± 125.4	838.2 ± 109	0.135[a]
HR (bpm) (mean ± SD)	79 ± 12.3	75.4 ± 7.5	0.348[a]
SDNN (ms) (mean ± SD)	135.8 ± 28.6	145.6 ± 31.8	0.170[a]
RMSSD (ms) (mean ± SD)	31.8 ± 14.3	33.4 ± 11.9	0.636[a]
PNN50 (%) (mean ± SD)	10.6 ± 9.9	12.8 ± 9.8	0.418[a]
AMBP (24h)			
SBP (mmHg) (mean ± SD)	111.4 ± 8.8	116.9 ± 8.7	0.091[a]
SBP day (mmHg) (mean ± SD)	114.4 ± 10.8	121.7 ± 8.9	0.128[a]
SBP night (mmHg) (mean ± SD)	102.6 ± 9.6	106.9 ± 8.4	0.244[a]
DBP (mmHg) (mean ± SD)	68.4 ± 8.3	71.7 ± 5.4	0.135[a]
DBP day (mmHg) (mean ± SD)	70.5 ± 9.5	74.6 ± 5.9	0.153[a]
DBP night (mmHg) (mean ± SD)	62 ± 7.8	63.2 ± 6.3	0.609[a]

RRI – RR interval; HR – Heart rate; ms – milliseconds; bpm – beats per minute; SDNN – Standard deviation of normal intervals; RMSSD –root mean square of successive differences; PNN50 – percentage of pairs of successive normal intervals that differ by more than 50 ms; SBP – Systolic blood pressure; DBP – Diastolic blood pressure; SD – standard deviation; [a] – paired samples t test

Table 4. *Biochemical Parameters of the study population, before and 3 months after the therapy*

	Before therapy N = 15 (n,%)	After 3 months N = 15	p value
Urea (mmol/L) (mean ± SD)	4.4 ± 1.1	4.5 ± 0.9	0.692[a]
Creatinine (μmol/L) (Mdn(IQR))	77.5 (70.5 – 84)	74 (72 – 85.5)	0.4800[b]
Glucose (mmol/L) (mean ± SD)	4.9 ± 0.5	4.9 ± 0.8	0.937[a]
AST (U/L) (Mdn(IQR))	25 (20 – 31)	22 (20 - 24)	0.0400[b]
ALT (U/L) (Mdn(IQR))	18 (16.5 – 39.5)	19 (14.5 – 26)	0.1720[b]
GGT (U/L) (Mdn(IQR))	13.5 (12 – 21.8)	14.5 (10.3 – 22.3)	0.4650[b]
Uric acid (μmol/L) (mean ± SD)	269.7 ± 74.7	253.9 ± 69.2	0.1430[b]
CRP (mg/L) (Mdn(IQR))	1.6 (0.8 – 3.4)	1 (0.8 – 1.4)	0.0080[b]
Triglycerides (mmol/L) (mean ± SD)	1.5 ± 0.6	1.3 ± 0.4	0.199[a]
Cholesterol (mmol/L) (mean ± SD)	5.9 ± 1	5.9 ± .9	0.952[a]
HDL (mmol/L) (mean ± SD)	1.4 ± 0.4	1.5 ± 0.4	0.392[a]
LDL (mmol/L) (mean ± SD)	3.9 ± 0.8	3.8 ± 0.9	0.522[a]

AST - Aspartate Aminotransferase; ALT - Alanine Aminotransferase; GGT - Gamma-Glutamyl Transferase; CRP – C reactive protein; HDL – High-Density Lipoprotein; LDL – Low-Density Lipoprotein; mmol/L - millimoles per liter; μmol/L - micromoles per liter; U/L - Units per Liter; mg/L – milligrams per liter; SD – Standard deviation; Mdn – Median; IQR – Interquartile range; [a] – Paired Samples T test; [b] – Wilcoxon signed rank test

DISCUSSION

The findings of this study indicate an enhancement of sympathetic tone with concurrent parasympathetic withdrawal, as recorded by beat-to-beat monitoring, without significant changes in blood pressure during either the 5-minute recordings or ambulatory blood pressure monitoring. In addition, decreased levels of AST and C-reactive protein were observed in patients with syncope treated with *T. serpyllum* essential oil.

As presented in Table 1, the majority of the participants in our study were female. This aligns with research, as it is, in fact, well established that the incidence of syncope is higher in women, who tend to experience syncopal episodes over longer periods of their lives and have more frequent episodes as compared to male patients (18). It is also notable that this study population was relatively healthy, with few significant comorbidities (Table 1).

One of the key observations, as shown in Table 2, is the increase in LFnu values and the LF/HF ratio of HRV, accompanied by a reduction in HF. Both LFnu and LF (ms^2), although the latter did not reach statistical significance, are

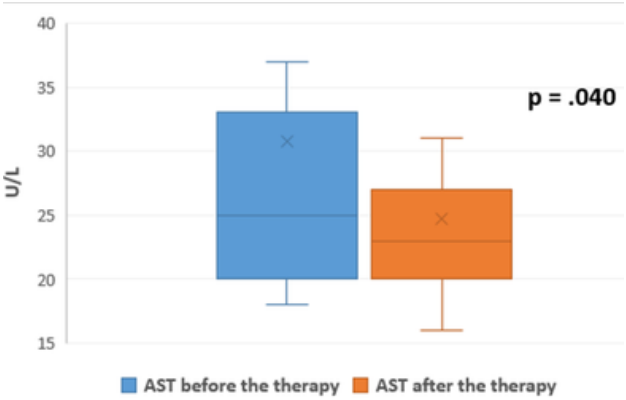


Figure 3. *Changes in AST (U/L) after three months of therapy with Thymus serpyllum essential oil.*
For comparison, the Wilcoxon signed-rank test was used.

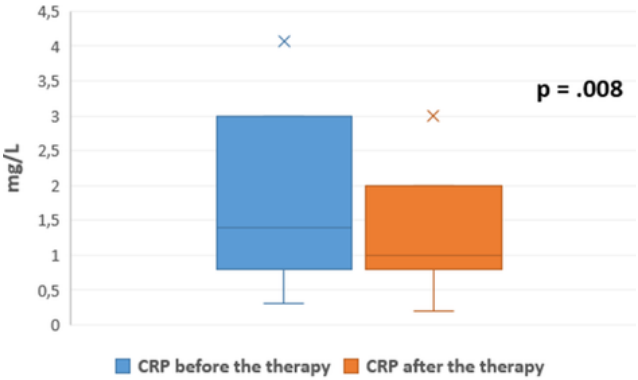


Figure 4. *Changes in CRP after three months of therapy with Thymus serpyllum essential oil.*
For comparison, the Wilcoxon signed-rank test was used.

considered markers of sympathetic activity (19,20). Furthermore, LFnu represents the relative contribution of LF activity, providing insight into autonomic balance with reduced sensitivity to total power variations; however, normalized units should be interpreted alongside absolute values (ms^2) to fully reflect the spectral distribution (21,22). Additionally, the LF/HF ratio, which showed a statistically significant increase after therapy in this study (Table 2), is commonly used to describe sympathovagal balance, with values above 2 typically indicating sympathetic predominance (19,20). HFnu (not shown here, as it is redundant—being derivable directly from 100% minus LFnu) and HF (ms^2) were both reduced following three months of therapy. HF, often referred to as the "respiratory band," is a key HRV marker of vagal (parasympathetic) activity (19) and is primarily modulated by respiration through respiratory sinus arrhythmia (19). It is also important to note that in short-term HRV recordings, especially during resting and supine conditions, the parasympathetic nervous system is the predominant contributor to HRV (19). Interestingly, these shifts in autonomic balance were not accompanied by significant changes in heart rate or blood pressure during the 5-minute recording period. Therefore, it may be inferred that, after three months of therapy, there was a shift towards sympathetic predominance with a concomitant reduction in parasympathetic tone in these patients. It is important to emphasize, as shown in Table 1, that nearly 85% of the study population had orthostatic hypotension as a key marker of sympathetic dysfunction prior to initiating therapy. Considering that these patients, who exhibited increased sympathetic tone after three months of treatment, are often managed conservatively (e.g., by avoiding provoking factors and using compression stockings) and, when necessary, with sympathomimetics such as midodrine, it would be reasonable to consider the potential use of *T. serpyllum* as an additional therapeutic option. However, further studies are required to clarify its role and confirm its efficacy in this specific patient population.

Regarding blood pressure, although values were higher after three months, no statistically significant changes were detected over the 24-hour period (Table 3). Animal studies have shown significant reductions in blood pressure and peripheral vascular resistance through a nitric oxide–independent mechanism, with rosmarinic acid identified as the main active compound using extracts of *T. serpyllum*. (14,15). Similar cardiovascular effects have also been reported in other species of the genus *Thymus*. In an experimental study, the essential oil of *Thymus* serrulatus produced dose-dependent reductions in arterial blood pressure, exhibiting vasodilatory and cardiac-depressant properties. These effects were attributed to α1-adrenergic antagonism and calcium channel blockade (23). These findings further support the potential cardiovascular activity of *Thymus* essential oils and suggest that members of this genus may influence vascular regulation through multiple mechanisms (23).

As shown in Table 4, notable changes in biochemical parameters were also observed after three months of therapy. While decreases were observed in the mean values of creatinine, uric acid, AST, and C-reactive protein, only the changes in the latter two reached statistical significance. Previous studies have demonstrated the potential hepatoprotective effects of *T. serpyllum* extracts in animal models, particularly in cases of alcohol-induced liver injury (24). The same study confirmed increased antioxidant enzyme activity and reduced oxidative stress (24).

Additionally, Jalil et al. highlighted the anti-inflammatory and antimicrobial properties of *T. serpyllum* extracts (9). Similar findings have been reported for carvacrol, one of the principal constituents of *Thymus* essential oils. A recent review summarized multiple experimental studies demonstrating that carvacrol consistently reduces oxidative stress, lipid peroxidation, and pro-inflammatory cytokine production while enhancing endogenous antioxidant defense systems, including superoxide dismutase, catalase, and glutathione-related pathways, thereby providing antioxidant and anti-inflammatory protection in various disease models (25). Furthermore, thymol, one of the key components of *Thymus* essential oils, has demonstrated cardioprotective effects in experimental models of adrenaline-induced myocardial injury (26). These effects were accompanied by reductions in AST, creatine kinase, and lactate dehydrogenase levels, restoration of antioxidant defenses through increased glutathione levels and reduced lipid peroxidation, as well as suppression of inflammatory mediators including NF-κB and IL-1β (26). In addition, thymol attenuated myocardial apoptosis through decreased caspase-3 expression and increased Bcl-2 expression, further supporting its antioxidant and anti-inflammatory properties (27).

In diabetic rabbits, although not observed in our study, a single high dose of 500 mg/kg of the aqueous extract of *T. serpyllum* reduced serum cholesterol, triglycerides, and LDL levels without affecting HDL levels (27). The absence of such pronounced effects in our study population may be attributed to (1) the fact that the study participants were patients with syncope who did not have significant liver disease or hyperlipoproteinemia, and only two participants had diabetes mellitus; (2) differences in dosage and mode of administration; and (3) potential interspecies metabolic differences between animal models and humans.

Study Limitations

This study has several significant limitations that should be acknowledged. First, the relatively small sample size restricts the generalizability of the findings. To draw more robust and reliable conclusions, future research should include a larger number of participants and incorporate a placebo-controlled arm to better delineate the specific effects of the investigated intervention.

Second, the lack of inclusion of patients with relevant comorbidities or specific diseases of interest may have limited the scope of biomarker evaluation. Although this study focused exclusively on patients with syncope, enrolling individuals with additional comorbid conditions would allow for a more comprehensive assessment of the potential effects of *T. serpyllum* on broader physiological and biochemical parameters.

Third, the follow-up duration was limited to three months. While this time frame was sufficient to detect short-term changes in autonomic parameters and selected biochemical markers, it may not have captured the sustainability of these effects or any potential delayed adverse outcomes. Longer-term studies are warranted to clarify the durability and safety of *T. serpyllum* supplementation.

Finally, the single-center design may limit the external validity of the findings, as the results obtained in a specialized neurocardiology laboratory may not be fully generalizable to other clinical settings or patient populations.

In conclusion, dysautonomia, reflecting dysfunction of the autonomic nervous system, is a key feature in patients with syncope, particularly those with orthostatic hypotension. This study demonstrated that a three-month course of *Thymus serpyllum* L. essential oil in patients with syncope was associated with increased sympathetic tone and parasympathetic withdrawal, as evidenced by beat-to-beat analysis, without significant changes in 24-hour blood pressure. Additionally, reductions in AST and C-reactive protein levels suggest potential hepatoprotective and anti-inflammatory effects of *T. serpyllum* in this patient population.

Based on these findings, *T. serpyllum* essential oil may be considered a potential adjunctive therapeutic option for patients with syncope, particularly those with orthostatic hypotension. Nevertheless, further well-designed studies with larger sample sizes and longer follow-up periods are warranted to confirm its efficacy and safety in this clinical context.

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Author Contributions

Conceptualization, B.M., S.P., N.P.; Methodology, B.M., S.P., and N.P.; Formal analysis, N.M., M.P.; Investigation, B.M. and N.M.; Data curation, N.M. and M.P.; Writing—original draft preparation, N.M., M.P.; Writing—review and editing, M.P. and M.B.; Supervision, B.M. and M.B.; Project administration, M.B. and M.P.; Funding acquisition, B.M. and M.B. All authors have read and agreed to the published version of the manuscript.

Statement of Ethics

This study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Institutional Ethics Committee of the Institute for Cardiovascular Diseases "Dedinje" (protocol code 6472, approval date: 11 December 2024).

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 the published article.

Statement of Generative AI Use

No generative AI was used.

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AFMN BIOMEDICINE

ORIGINAL ARTICLE

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A SEVEN-YEAR LONGITUDINAL STUDY OF TECHNICAL COMPLICATIONS IN REMOVABLE DENTURES

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This study aimed to analyze the frequency and type of technical complications in different categories of removable dentures. We examined clinical health records of 637 denture wearers who presented for repair and collected information on fracture type (tooth, base, clasp), arch location (maxilla vs. mandible), and denture type. Tooth fractures were the most common complication in both arches. In the upper arch, complete dentures showed the highest incidence of base fractures (20.1%), while in the lower arch, the most common complication was acrylic partial denture clasp fractures (56,7%), followed by base fractures (48.4%). Because the prevalence and distribution of denture fractures vary significantly by denture type and arch location, our findings emphasize the critical role of material selection and reinforcement techniques in extending the service life of removable dentures.

Keywords: denture fracture, removable dentures, resins, dental materials

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INTRODUCTION

According to a WHO report from 2022, elderly people make up one-sixth of the total population, and 4.44% of the whole population, which is 0.35 billion people, were affected by edentulism (1,2). Removable complete and partial dentures are the most common solution for oral rehabilitation, especially among elderly patients, when there is a lack of anatomical and financial support for a higher-quality solution. Aging is associated with a progressive reduction in both the quantity and quality of alveolar bone available for dental implant placement, potentially limiting the feasibility and predictability of implant-supported prosthetic rehabilitation. These dentures, despite the shortcomings, improve mastication, phonetics, facial esthetics, and general quality of life. In spite of major advances in fabrication techniques and the development of polymer-based dental materials, fractures of removable dentures continue to occur at similar or even increasing rates (3). The most common causes of denture fractures include the absence of intimate contact between the denture base and the underlying mucosa, occlusal discrepancies, material fatigue, and patient-related factors such as inadequate hygiene or accidental dropping of the denture. Bone remodeling and resorption after extractions occur rapidly (within a few weeks). This can lead to destabilization of dentures made by poly (methyl methacrilate) (PMMA), and potentially to fractures. Bone resorption occurs much more slowly over several years with long-term dentures. Immediate removable dentures inserted on the day of extractions require a specific protocol to prevent fractures, such as relining (4). Removable Cr-Co-Mo prostheses are considered permanent prostheses. They are most often made following the replacement of an existing prosthesis (immediate, temporary, or permanent). Based on their morphology and location, fractures can be categorized into artificial tooth fractures (adhesive, cohesive, or combined), denture base fractures, and clasp fractures. The frequency and type of fracture depend on multiple parameters, including the prosthesis design (complete denture, acrylic removable partial dentures (RPDs) with wire clasps, or cast metal framework denture with cast clasps), material composition, duration of use, and patient-specific habits. PMMA remains the most commonly used denture base resin due to its ease of manipulation, biocompatibility, and esthetics (5,6). However, PMMA exhibits brittle behavior and low impact resistance, which may increase its susceptibility to fracture under functional and parafunctional loading conditions (6). Current research efforts are increasingly focused on modifying PMMA with reinforcing agents such as fibers, nanoparticles, or forming interpenetrating polymer networks (IPNs) to improve its mechanical performance. The present study was conducted at the Clinic of Prosthodontics, School of Dental Medicine, University of Belgrade, with the aim of collecting and analyzing clinical data on the frequency, location, and type of denture fractures. Additionally, we discuss fracture patterns in relation to prosthesis type and present perspectives on modern repair strategies and potential material innovations designed to improve the longevity and resilience of removable dental prostheses. The aim of this study was to analyze the frequency and type of technical complications in different categories of removable dentures.

The study was reviewed and approved by the Ethics Committee of the School of Dental Medicine, University of Belgrade (No. 36/28).

METHODS

This retrospective study was performed at the Clinic of Prosthodontics, Faculty of Dental Medicine, University of Belgrade.

The data were collected from the 637 denture wearers who attended our Clinic for repair. The study was conducted over seven years, between April 30, 2017 and June 20, 2024.

The age of the study participants ranged from 45 to 85 years. Exclusion criteria were: neuromuscular diseases (Parkinsonism, ALS, multiple sclerosis), epilepsy, oncological patients, parafunctional movement of the mandible (bruxism), and excessive upper and/or lower jaw defects.

The data were analyzed using IBM SPSS Statistics v22 software (SPSS Inc., Chicago, IL, USA). Descriptive statistics parameters were used to evaluate the distribution of participants' gender, denture type (complete dentures, acrylic RPDs and CoCrMo RPDs), and period of fracture occurrence. Frequencies of categorical variables were analyzed using the Chi-square (χ^2) test or Fisher´s exact test, as appropriate. A significance level was set at 0.05.

RESULTS

Frequencies of gender, denture type and period of fracture occurrence

The frequency of the study participants by gender is shown in Figure 1.

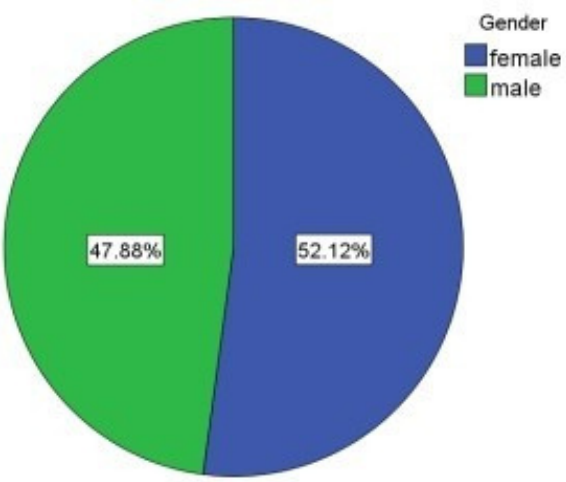


Figure 1. *Gender distribution of study participants*

Complete dentures were significantly more frequent in the upper jaw, while both acrylic and cast metal RPDs were more common in the mandible. The number of each denture type with some of the detected technical complications in the upper and lower jaw was 264 (60.3%) and 174 (39.7%), 129 (44%) and 164 (56%), and 111 (41%) and 160 (59%), respectively (Figure 2).

First-time fractures (63.7%) were more frequent than second or subsequent fractures. For acrylic dentures (complete and RPDs), nearly half of the registered fractures were detected in the first two years of denture delivery 285 (44.73%). For the CoCrMo RPDs, fractures occurred in only 9.2% of cases in the first two years.

Relation between fracture type and different dentures

Distribution of denture fractures according to denture type showed the highest incidence in complete dentures—174(27.3%), then acrylic removable partial dentures (RPDs)—164 (25.7%), and cobalt-chromium (CoCrMo) RPDs—160 (25.1%).

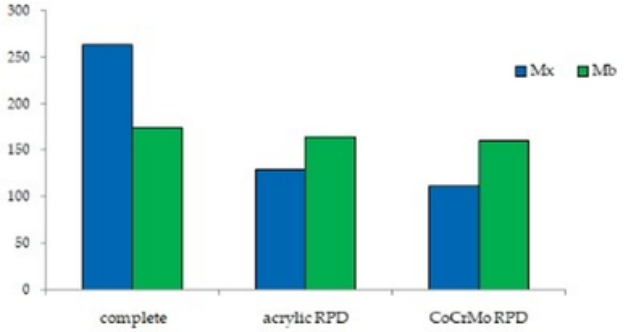


Figure 2. *Distribution of technical complications of each denture type for the upper (Mx) and lower (Mb) jaw*

Among maxillary (upper) dentures, artificial tooth fractures were the most prevalent—269 (42.2%) (Figure 3b), followed by denture base fractures—191 (30.0%) (Figure 3a), and clasp fractures—54 (8.5%). A similar behavior was observed in mandibular (lower) dentures, with tooth fractures accounting for 213 (33.4%) cases (Figure 4b), base fractures —154 (24.2%) (Figure 4a), and clasp fractures—67 (10.5%), respectively.

Analyzing the denture base fractures according to the denture type in the maxilla, the highest prevalence of fractures was noted in complete dentures —101 (20.1%), followed by acrylic RPDs—44 (8.7%), and CoCrMo RPDs— 9 (1.8%) cases, with a statistically significant association between base fracture and denture type ($p < 0.001$).

Oppositely, in the lower jaw, the highest incidence of base fractures was detected in acrylic RPDs—62 (48.4%). For complete dentures, that number decreased to 49 (38.3%), and for CoCrMo, RPDs it was 17 (13.3%). This association was also statistically significant ($p < 0.001$).

In the upper jaw, artificial tooth fractures were most commonly seen in complete dentures—113 (50.2%), whereas in CoCrMo RPDs, it was 58 (25.8%), and in acrylic RPDs 54 (24.0%). However, the association between artificial tooth fracture and denture type in the upper jaw was not statistically significant ($p = 0.192$).

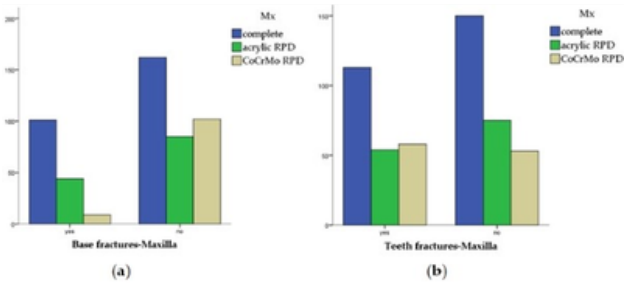


Figure 3. *Distribution of maxillary base and tooth fractures according to denture type*

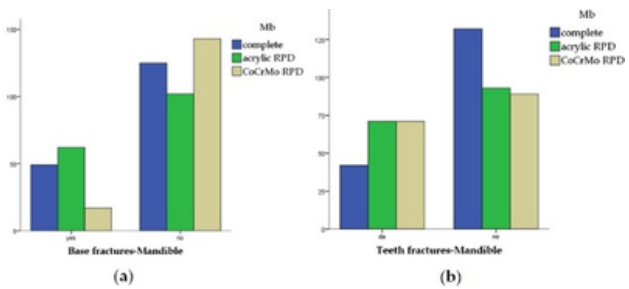


Figure 4. *Distribution of mandibular base and tooth fractures according to denture type*

In the lower jaw, artificial tooth fractures demonstrated comparable frequencies for acrylic and CoCrMo RPDs, 71 each (38.6%). Among complete denture wearers, half of them experienced tooth fracture complications, which was observed in 42 (22.8%) cases.

Among clasp fractures, the most frequent were in acrylic RPDs, involving 34 lower (56.67%) and 32 upper (65.3%) dentures. In Co-Cr-Mo RPDs, the incidence was 25 (42.4%) for lower and 17 (34.7%) for upper dentures.

Fracture type distribution by gender

Table 1 shows the frequency of different fracture types in mandibular dentures by gender. As seen, the type of mandibular denture fracture showed no statistically significant gender differences ($p > 0.05$).

Comparing similar results for maxillary dentures (Table 2), a statistically significant gender difference was observed only in tooth fractures, which were more prevalent in males (54.5%) than females (45.5%; $p = 0.019$), while differences in denture base and clasp fractures were not significant.

DISCUSSION

Fractures of removable dentures are among the most frequent secondary conditions, undermining both functional and esthetic outcomes. Despite improvements in prosthetic fabrication techniques, the present findings confirm that mechanical failure remains prevalent in both complete and partial dentures. This study is based on a clinical evaluation of fracture distribution and contributes to a better understanding of failure mechanisms and repair protocols, with a focus on material-based solutions. We processed the data only of complete, partial acrylic dentures and RPDs made after a long-term period of toothlessness, not immediately after tooth extraction. In the following text, we will describe other solutions for toothless patients.

Table 1. *Gender-based distribution of mandibular denture fracture types*

Gender	Female % (n)	Male % (n)	P value
Denture base	48.2% (92)	51.8% (99)	0.195
Tooth	53.2% (143)	46.8% (126)	0.688
Clasp	46.3% (25)	53.7% (29)	0.395

Table 2. *Gender-based distribution of maxillary denture fracture types*

Gender	Female % (n)	Male % (n)	P value
Denture base	53.9% (83)	46.1% (71)	0.644
Tooth	45.5% (97)	54.5% (116)	*0.019
Clasp	59.7% (40)	40.3% (27)	0.199

**Statistically significant difference.*

In order to better manage clinical complications, removable partial dentures with overlay represent an alternative to complete extraction (7), or if there are adequate anatomical structures, implant-supported dentures should be made (8).

Etiology and risks of denture fractures

The etiology of denture fractures is complex, including biomechanical, anatomical, material-related, and patient-specific factors. Accidental dropping of the denture remains the most frequently reported cause, particularly in complete dentures, aligning with previous reports that identify external trauma as the trigger in nearly one-third of cases (9). Additionally, poor adaptation of the denture base leading to uneven stress distribution was responsible for approximately 20% of fractures (10). Bone remodeling and resorption after extractions occur rapidly, which can destabilize dentures and, potentially, lead to fractures. Immediate removable dentures inserted on the day of extractions require a specific protocol to prevent fractures, relining to compensate for the lost tissue, stabilize the denture, and support healing after extractions (4), however, this is only a temporary solution. When bone resorption slows down, after a few months, a permanent removable denture should be made. Volumetric gaps between the denture base and the cast are considerably higher with conventional compression molding techniques compared to CAD-CAM milling, suggesting that poor adaptation significantly increases mechanical stress and a risk of fracture (11). Occlusal errors, deflective contacts, and unbalanced force vectors contribute to stress accumulation, particularly in non-stress-bearing zones such as the midline. From material perspective, defects introduced during processing (e.g., porosities, voids, residual stresses) significantly reduce fracture resistance. Patient behavior, including inadequate hygiene and cleaning practices, further contributes to the risk of damage (12). Evidence shows that among complete denture wearers, only approximately 40% achieved acceptable levels of prosthesis hygiene, with the majority demonstrating inadequate cleaning habits, behaviors known to contribute to denture deterioration and increased failure rates (13).

Accidental denture ingestion and aspiration are a recognized clinical emergency, particularly among the elderly, which may lead to serious gastrointestinal and pulmonary complications. It is often silent and may occur

due to poor prosthesis fit or fracture. Denture ingestion accounts for a small proportion of gastrointestinal foreign bodies but carries a higher risk of sigmoid and descending colon perforation, or duodenal obstruction due to sharp components. (14-17).

Fracture distribution and patterns

The current data from our study indicate that artificial tooth fractures were the most common, especially in the maxilla. Complete dentures predominantly exhibited midline base fractures, which were also reported in earlier studies that attribute with 50–70% of base failures to the biomechanically vulnerable midline zone (9,10). In contrast, base fractures in acrylic RPDs were more common in the mandibular arch, while clasp fractures were frequently associated with acrylic frameworks and wire clasps, which was already described by other researchers (18,19). These patterns highlight the impact of denture design, occlusal force distribution, and anatomical limitations on fracture distribution.

Treatment strategies and clinical protocols

Effective repair of fractured dentures implies proper surface preparation and appropriate material selection. The clinical workflow generally includes thorough cleaning, mechanical roughening (with carbide burs), and chemical conditioning using monomer or bonding agents to enhance bonding between two materials, both hot and cold polymerizing resins, which are the material of choice for chairside repairs due to their ease of use but limited mechanical properties. Improvements with nanofillers such us ZnO NPs have been shown to significantly increase flexural strength, bringing repaired dentures closer to their original performance levels (20). Additionally, nanoparticle incorporation (nano-ZrO_2, nano-SiO_2, TiO_2) has demonstrated improved stress distribution, fracture resistance, and interfacial adhesion in *in vitro* studies (8,12,21).

Advances in reinforced polymeric materials

Advanced polymeric systems provide potential for reducing fracture rates and improving repair outcomes. Interpenetrating polymer networks (IPNs), characterized by dual polymer matrices without covalent linkage, offer enhanced toughness and fatigue resistance due to superior energy dissipation properties. Nanoparticle-modified PMMA resins, incorporating materials such as silver (22), zirconia, or graphene (23), have demonstrated improvements in flexural strength, bond durability, and crack resistance (5). Nanoparticles implemented in PMMA matrix have also shown improved dispersion and mechanical integration (24,25), as well as antimicrobial efficacy (26). An increase in fracture toughness suggests enhanced resistance of PMMA against crack propagation and impact failure in reinforced PMMA. In clinical use, this may improve resistance to accidental impacts or high-load fractures. These modifications could extend the service life and improve the clinical durability of acrylic denture bases, despite a minor trade-off in flexural strength (27). Recent research also demonstrates self-healing polymers as an innovative direction (28). These systems rely on reversible covalent bonds or supramolecular interactions that enable autonomous structural repair, although their clinical application is still in early development. The use of these materials in everyday practice could drastically reduce the recurrence of fractures and improve long-term patient satisfaction. The results of this study reinforce the importance of aligning denture design and repair strategies with the evolving capabilities of polymer science. By incorporating fiber and nanoparticle reinforcements, and eventually adopting next-generation materials such as IPNs or self-healing systems, clinicians may significantly improve the longevity and biomechanical performance of removable dentures.

Additive manufacturing techniques in denture manufacturing offer advantages such as customization and rapid production but might exhibit weaknesses in mechanical strength and durability. Lee et al. evaluated and confirmed that incorporation of micro fillers demonstrated potential in enhancing the physicomechanical properties of the 3D printed denture base material (29). Studies have also shown that milled PMMA and dual-layer printing technique (compared to 3D-printed alternatives) improved the flexural and fracture resistance of denture base materials by eliminating interfacial weaknesses (30,31). A 3D-printed interim fixed dental prosthesis resin with different surface finishing procedures demonstrated favourable mechanical and biological properties, where glazing or mechanical polishing is recommended, but it should also be considered that the wear of the surface layer may be affected by the applied finishing procedure. Long-term wear resistance, staining, and intraoral aging should be evaluated in future research (32).

Based on the methodology applied in this study, we summarized the obtained results:

- Regarding the denture type, a statistically significant association was found between denture type and base fractures. Complete dentures were most commonly fractured in the upper jaw and acrylic RPDs in the lower jaw.
- Tooth fractures were more common in complete upper dentures.
- As far as gender-based distribution is concerned, mandibular denture fractures showed no significant differences between males and females. In the maxilla, tooth fractures were more common in males ($p = 0.019$), while base and clasp fractures were similar in both genders.
- Further investigations and implementation of nanoparticles or other fillers in the matrix of PMMA would improve stress distribution, fracture resistance, and lead to better quality of life for the denture wearers (19-21,33).

As the prevalence and distribution of denture fractures vary significantly by denture type and arch location, the findings of our results emphasize the critical role of material selection and reinforcement techniques in extending the service life of removable dentures.

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Author Contributions

Conceptualization, D.P.A. and M.M.L.; Investigation, D.P.A. and L.Z.; Data Curation, F.I. and M.L.; Formal analysis, S.D.; Writing- original draft preparation, D.P.A.; M.M.L., and I.Dj. Writing- review and editing, D.P.A, M.M.L, L.Z., M.L., and S.D. All authors have read and approved the published version of the manuscript.

Statement of Ethics

The study protocol was approved by the Ethics Committee of the School of Dental Medicine, University of Belgrade (Approval No. 36/28, issued on October 8, 2025). Written informed consent was obtained from the patient for the publication of this study and any accompanying datasets.

Statement of Competing Interest

The authors declare no relevant conflicts of interest.

Statement of Data Availability

The datasets generated and/or analyzed during this study are included within this article and are available from the corresponding author upon reasonable request.

Statement of Generative AI Use

No generative AI was used.

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AFMN BIOMEDICINE

ORIGINAL ARTICLE

INTRA-ABDOMINAL PRESSURE AS A MODULATOR OF BIOCHEMICAL INDICATORS OF HEPATIC FUNCTION AFTER LAPAROSCOPIC CHOLECYSTECTOMY

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Laparoscopic cholecystectomy is the standard treatment for gallbladder disease; the carbon dioxide pneumoperitoneum required for the procedure may transiently affect liver function. The aim is to examine the evaluation of the impact of different levels of intra-abdominal pressure (IAP) and ASA score on postoperative liver biochemical parameters. A prospective study included 90 patients divided into three groups according to IAP (13, 14, and 15 mmHg; 30 subjects each), with analysis of preoperative and postoperative parameters of liver function. The groups were comparable in terms of age, sex, and body weight, while differences were observed in the type of inflammation and total protein values. After surgery, changes in several biochemical parameters of liver function were recorded, including an increase in the values of direct bilirubin, AST, ALT, and ALP, as well as a decrease in LDH values. The values of total bilirubin, albumin, and total protein did not show significant postoperative changes. Comparison of the groups according to different values of intra-abdominal pressure did not show significant differences in most of the parameters examined, while a statistically significant difference was observed for LDH between the groups. ASA score was associated with postoperative changes in some biochemical parameters of liver function. Laparoscopic cholecystectomy leads to transient changes in liver biochemical parameters without clinically significant damage, whereby intra-abdominal pressure in the usual range has no significant effect, while the general condition of the patient has a greater role in the postoperative response.

Keywords: laparoscopic cholecystectomy, intra-abdominal pressure, liver function, pneumoperitoneum

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INTRODUCTION

Intra-abdominal pressure (IAP) is the static pressure within the abdominal cavity, which physiologically remains low but increases during pneumoperitoneum in laparoscopic surgery and in pathological conditions such as intra-abdominal hypertension and abdominal compartment syndrome. Elevated IAP may reduce splanchnic blood flow, including hepatic perfusion, leading to alterations in organ function. Intra-abdominal hypertension is defined as sustained IAP ≥12 mmHg, while pressures exceeding 20 mmHg are associated with impaired organ perfusion and dysfunction, including potential liver injury (1).

In the context of laparoscopic surgery, carbon dioxide pneumoperitoneum increases IAP to provide adequate visual and working space. Standard IAP values used in practice are usually between 12 and 15 mmHg, but there is evidence that even modest increases in IAP can have short-term effects on regional perfusion and physiological parameters, including hepatic blood flow and portal pressure. Studies have shown that increased IAP can lead to decreased hepatic blood flow and transient increases in liver enzymes (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)) (2).

A recent meta-analysis of included studies suggests that the use of lower IAP values (<10 mmHg) during laparoscopic procedures may be associated with fewer postoperative complications, lower pain scores, and shorter hospital stays, without increasing intraoperative risk. Although this analysis does not directly assess liver function, it supports the safety of using lower IAP values while highlighting the need for further investigation of the physiological effects of different IAP values on specific parameters of organ function (3).

Therefore, the aim of this study is to investigate the effect of different values of intra-abdominal pressure (13, 14, and 15 mmHg) on postoperative values of biochemical parameters of liver function and ASA score in patients undergoing laparoscopic cholecystectomy.

METHODS

This study was designed as a prospective, observational clinical study conducted to evaluate the effect of different values of intra-abdominal pressure (IAP) during laparoscopic cholecystectomy on postoperative liver function. The research was conducted at the Clinical Hospital Center Kosovska Mitrovica during a defined period of time of June 2025 to March 2026, with patients monitored from the time of admission to the early postoperative period and with the approval of the Ethics Committee of the institution (No. 3634, issued on June 2, 2025).

A total of 90 patients who underwent elective or urgent laparoscopic cholecystectomy due to acute or chronic cholecystitis were included in the study. Patients were divided into three groups (n = 30 in each group) according to the value of intra-abdominal pressure applied during surgery: 13 mmHg, 14 mmHg, and 15 mmHg. The distribution into groups was based on the pneumoperitoneum parameters set intraoperatively.

Criteria for inclusion in the study included adult patients of both sexes with a diagnosis of cholecystitis who were candidates for laparoscopic operative treatment. Exclusion criteria from the study included previously known chronic liver diseases, presence of malignant diseases, conversion to open surgery, and incomplete laboratory or clinical data.

Demographic data (age, gender), anthropometric data (body mass), as well as clinical parameters including type of inflammation (acute or chronic form of cholecystitis), presence of comorbidities (arterial hypertension, obesity, diabetes), and ASA score as an indicator of the general preoperative status of the patient were collected from all patients.

Laboratory analyses included the determination of biochemical parameters of liver function, which were measured at two time intervals: before surgery and in the early postoperative period. The analyzed parameters included direct and total bilirubin (μmol/l), aspartate aminotransferase (AST) (U/l), alanine aminotransferase (ALT) (U/l), alkaline phosphatase (ALP)v(U/l), albumin (g/l), total proteins (g/l), and lactate dehydrogenase (LDH) (U/l).

Biochemical parameters of liver function were determined from venous blood samples taken at two time intervals: preoperatively, as part of routine laboratory preparation within 24 hours before surgery, and postoperatively, on the first postoperative day. One limitation of this study is that the duration of the surgical procedure was not systematically recorded, which made it impossible to analyze its potential impact on postoperative biochemical parameters of liver function. Parameters such as duration of surgery, duration of pneumoperitoneum, and postoperative outcomes were not monitored because they were outside the primary research protocol of this study.

Statistical data processing was performed using the software package SPSS (Statistical Package for the Social Sciences), version 21. The normality of data distribution was tested with the Shapiro–Wilk test. Given the distribution of the data, continuous variables are presented as mean ± standard deviation or as median with interquartile range.

To compare continuous variables between multiple groups, a one-factor ANOVA test or Kruskal–Wallis test was used, depending on the normality of the distribution. A paired t-test or Wilcoxon test was used to compare dependent samples (preoperative and postoperative values). Categorical variables were analyzed using the Chi-square test. Statistical significance is defined as a value of $p < 0.05$.

RESULTS

In this research, a total of 90 patients undergoing laparoscopic cholecystectomy were analyzed; they were divided into three groups according to the value of intra-abdominal pressure during the operation (13, 14, and 15 mmHg). Demographic and clinical characteristics of patients, as well as changes in biochemical parameters of liver function before and after surgery, were examined. A special focus of the analysis was aimed at evaluating the impact of different levels of intra-abdominal pressure on the postoperative values of liver enzymes and other laboratory indicators, as well as the potential impact of the general health status of patients expressed through the ASA score.

Table 1 shows the preoperative demographic and clinical characteristics of patients distributed according to intra-abdominal pressure groups. The analysis of the basic characteristics did not reveal a statistically significant difference between the groups in terms of age, gender, and body weight, which indicates a good homogeneity of the studied groups. Also, most of the preoperative biochemical parameters of liver function did not differ significantly between the groups, which additionally confirms the comparability of the groups before the operation. However, a statistically significant difference was observed in the values of total proteins, as well as in the distribution of the type of inflammation, where certain groups had a higher prevalence of the acute or chronic form of cholecystitis. In terms of comorbidity and ASA score, no statistically significant difference was recorded between the groups. These findings indicate that the groups were largely uniform preoperatively, with some differences that should be considered when interpreting postoperative results.

By analyzing the preoperative and postoperative values of the biochemical parameters of the liver in the total sample, significant changes in certain parameters were observed

Table 1. *Preoperative demographic and clinical characteristics of patients according to intra-abdominal pressure (IAP) groups*

Variable	IAP 13 mmHg (n = 30)	IAP 14 mmHg (n = 30)	IAP 15 mmHg (n = 30)	p
Age, mean±SD	46.40 ± 16.64	51.07 ± 14.47	54.30 ± 12.83	0.096
Gender, n(%) Male Female	 14 (37.8%) 16 (30.2%)	 11 (29.7%) 19 (35.8%)	 12 (32.4%) 18 (34%)	0.725
Body mass, mean ± SD	85.53 ± 20.81	84.93 ± 12.45	83.57 ± 20.68	0.914
Direct bilirubin, med. (min-max)	3.3 (1.4-13.9)	3.35 (1.5-25.3)	2.8 (1.7-5.9)	0.407
Total bilirubin, med. (min-max)	11.6 (5.8-33.5)	11.8 (2.7-38.4)	15.45 (2.6-22.7)	0.157
AST, med. (min-max)	32.5 (3-137)	26.5 (2-91)	23 (8-61)	0.198
ALT, med. (min-max)	30 (2-167)	25 (2-61)	21.5 (3-84)	0.381
ALP, med. (min-max)	106.5 (38-412)	86 (7-402)	80 (8-412)	0.107
Albumin, mean ± SD	45.23 ± 6.27	45.27 ± 6.97	44.87 ± 6.61	0.967
Proteins, med. (min-max)	76.05 (45-84)	70.95 (54-83)	70.6 (59.7-84)	0.029*
LDH, med. (min-max)	674.5 (199-947)	609.5 (176-1846)	640.5 (128-1420)	0.558
Type of inflammation, n(%) Acute form Chronic form	 17 (51.5%) 13 (22.8%)	 8 (24.2%) 22 (38.6%)	 8 (24.2%) 22 (38.6%)	0.021*
Comorbidities, n(%) There is none Arterial hypertension Obesity Diabetes	 14 (38.9%) 9 (31%) 5 (38.5%) 2 (16.7%)	 15 (41.7%) 8 (27.6%) 1 (7.7%) 6 (50%)	 7 (19.4%) 12 (41.4%) 7 (53.8%) 4 (33.3%)	0.11
ASA score, n(%) 0 1 2 3 4	 7 (50%) 9 (29%) 10 (34.5%) 4 (30.8%) 0 (0%)	 4 (28.6%) 14 (45.2%) 8 (27.6%) 3 (23.1%) 1 (33.3%)	 3 (21.4%) 8 (25.8%) 11 (37.9%) 6 (46.2%) 2 (66.7%)	0.492

Statistical significance is defined as $p < 0.05$

after the operation. A statistically significant increase was recorded in direct bilirubin, as well as in the transaminases AST and ALT, which indicates the presence of postoperative hepatocellular damage. In addition, alkaline phosphatase (ALP) values increased significantly after surgery, which may indicate a cholestatic component or the effect of pneumoperitoneum. On the other hand, total bilirubin, albumin, and total proteins did not show statistically significant changes. Interestingly, LDH values decreased significantly after surgery. These results indicate that laparoscopic cholecystectomy leads to significant but selective changes in biochemical parameters of liver function (Table 2).

By comparing postoperative values of biochemical parameters between groups with different intra-abdominal pressure, no statistically significant difference was found for most of the analyzed parameters. The values of direct and total bilirubin and transaminases AST and ALT, as well as ALP, albumin, and total proteins, were comparable between the groups. However, a statistically significant difference was observed in LDH values, which indicates a possible influence of intra-abdominal pressure on this parameter. However, the absence of significant differences in most biochemical indicators suggests that different levels of intra-abdominal pressure during laparoscopic cholecystectomy do not have a significant effect on postoperative liver function as a whole (Table 3).

Analysis of postoperative biochemical parameters in relation to the ASA score revealed statistically significant differences in certain indicators of liver function. Direct bilirubin values differed significantly between groups, as did AST and ALP values, indicating a possible influence of the general preoperative status of patients on the degree of postoperative liver damage. Also, a statistically significant difference in albumin values was recorded, which may reflect differences in the nutritional and functional status of patients according to the ASA classification. On the other hand, total bilirubin, ALT, total proteins, and LDH did not show statistically significant differences between the groups. These results suggest that the ASA score, as an indicator of the general state of health, can have an impact on certain postoperative parameters of liver function, but not on all analyzed biochemical indicators (Table 4).

DISCUSSION

In this research, the high homogeneity of the examined groups was confirmed in terms of key demographic and clinical characteristics, which represents a significant methodological advantage and enables a reliable

Table 2. *Comparison of preoperative and postoperative values of biochemical parameters*

Parameter	Before surgery	After surgery	p
Direct bilirubin, med. (min-max)	3.1 (1.4-25.3)	3.99 (1.12-6.69)	0.002*
Total bilirubin, med. (min-max)	12.3 (2.6-38.4)	13.05 (4.2-24.4)	0.261
AST, med. (min-max)	26.5 (2-137)	59 (11-112)	0.000*
ALT, med. (min-max)	26 (2-167)	57.5 (16-94)	0.000*
ALP, med. (min-max)	90.5 (7-412)	290 (51-510)	0.000*
Albumin, med. (min-max)	46 (31-58)	44 (31-57)	0.203
Proteins, med. (min-max)	72 (45-84)	71.35 (60-84)	0.237
LDH, med. (min-max)	648 (128-1846)	483.5 (124-809)	0.000*

Statistical significance is defined as $p < 0.05$

Table 3. *Comparison of postoperative biochemical parameters between groups of different intra-abdominal pressure (IAP)*

Parameter	IAP 13 mmHg (n = 30)	IAP 14 mmHg (n = 30)	IAP 15 mmHg (n = 30)	p
Direct bilirubin, mean ± SD	3.95 ± 1.78	3.34 (1.29-6.69)	4.04 ± 1.67	0.991
Total bilirubin, med. (min-max)	12.5 (5-24.4)	11.35 (4.2-23.6)	14.05 (4.5-24.2)	0.739
AST, med. (min-max)	62.5 (14-107)	64.47 ± 30.57	53.5 (13-111)	0.589
ALT, med. (min-max)	53.5 (16-93)	61.3 ± 23.21	52.7 ± 21.87	0.304
ALP, mean ± SD	297.2 ± 149.91	287.5 ± 142.53	268.5 (54-508)	0.727
Albumin, mean ± SD	42.1 ± 7.71	45.87 ± 7.21	43 (31-57)	0.213
Proteins, med. (min-max)	71.5 (60.5-84)	72.58 ± 7.41	72.55 (60.7-84)	0.775
LDH, med. (min-max)	647 (131-809)	324.5 (124-771)	444.5 (138-799)	0.012*

Statistical significance is defined as $p < 0.05$

Table 4. *Postoperative liver function parameters according to ASA score*

Parameter	ASA 0	ASA 1	ASA 2	ASA 3	ASA 4	p
Direct bilirubin	4.28 ± 1.57	5.07 (1.29-6.61)	2.48 (1.12-6.66)	4.12 ± 1.65	4.85 ± 1.62	0.020*
Total bilirubin	12.75 ± 5.35	10.9 (4.2-24)	14.94 ± 6.48	18 (4.7-22.9)	8.86 ± 4.56	0.486
AST	64.29 ± 27.09	74 (11-112)	58.59 ± 30.46	39.85 ± 18.27	37 ± 28.47	0.023*
ALT	66.43 ± 18.51	58 (20-94)	55 (16-93)	45 ± 22.02	57.67 ± 26.95	0.2
ALP	129.5 (51-485)	236 (53-505)	341 ± 132.04	314.92 ± 164.49	220.33 ± 101	0.021*
Albumin	38.93 ± 7.45	46.58 ± 6.95	44 (31-57)	41.46 ± 8.02	54 (33-54)	0.037*
Proteins	73.38 ± 7.70	69 (60-84)	72.59 ± 7.08	74.08 ± 7.5	68.63 ± 5.63	0.425
LDH	491.36 ± 233.56	458 (131-809)	495 (144-799)	493.15 ± 246.35	341.33 ± 232.29	0.745

Statistical significance is defined as $p < 0.05$

comparison of postoperative outcomes. Although differences in total protein and type of inflammation were observed, the absence of differences in age, sex, body mass, and most biochemical parameters indicated that these variables likely did not have a significant confounding effect on the results. A similar approach to the selection of homogeneous groups was applied in other studies examining the impact of pneumoperitoneum on the liver, thus minimizing the impact of external factors and enabling the focus on intra-abdominal pressure as the main investigated parameter (4).

The results of this study demonstrate that laparoscopic cholecystectomy is associated with significant postoperative changes in selected biochemical parameters of liver function, including increases in AST, ALT, ALP, and direct bilirubin levels. These findings represent transient postoperative biochemical changes rather than clinically significant liver injury. This observation is consistent with previous studies suggesting that pneumoperitoneum may reduce hepatic blood flow through portal vein compression and decreased splanchnic circulation, resulting in temporary alterations in hepatocellular metabolism. In the present study, postoperative increases in liver enzymes were observed, followed by spontaneous normalization, supporting the interpretation that these changes are transient and clinically benign (4).

On the other hand, the absence of significant changes in total bilirubin, albumin, and total proteins indicates that the synthetic function of the liver remains preserved despite the biochemical changes. This finding has important clinical significance because it suggests that although biochemical abnormalities occur, they do not reflect actual functional liver damage. Similar results have been described in other studies which emphasized that enzyme changes do not correlate with clinical worsening of liver function, which further confirms the safety of laparoscopic cholecystectomy as a standard procedure (4).

One of the most important findings of this research is the absence of statistically significant differences in most postoperative parameters between groups with different levels of intra-abdominal pressure (13, 14, and 15 mmHg), which indicates that in this range of pressures, no significant additional liver damage occurs. This result partially contradicts some studies that indicate greater harm with higher values of pneumoperitoneum, but it can be explained by the fact that the pressure differences in our research are relatively small and within standard clinical values. This implies that a clinically relevant difference probably exists only at significantly higher pressure values, which were not routinely used in this work (4).

A particularly interesting finding is the statistically significant difference in LDH values between the groups, as well as its overall reduction postoperatively. This result deviates from the expected pattern and is not often described in the literature, which may indicate specific metabolic or hemodynamic changes during surgery and anesthesia. Given that most studies do not analyze LDH as a primary parameter, this finding may represent a potential new field of research and require additional prospective trials with larger samples (4).

The finding of a statistically significant decrease in serum LDH in the early postoperative period, from the preoperative median of 648 U/L to the postoperative median of 483.5 U/L, deserves special attention. This result is inconsistent with most previously published studies that described a transient increase in LDH, along with AST and ALT, after laparoscopic cholecystectomy, which is mainly related to changes in splanchnic perfusion during pneumoperitoneum (5, 6). However, the results of the literature are not completely consistent, as some studies show that LDH after laparoscopic cholecystectomy can remain without significant changes compared to preoperative values (7). In our research, the decrease in LDH cannot be directly interpreted as a specific indicator of

liver regeneration, bearing in mind the non-specificity of this enzyme and the fact that various extrahepatic sources can influence its serum concentration. One of the possible explanations is that elevated preoperative LDH values reflected an inflammatory and metabolic response associated with the underlying disease, especially if we consider that our sample included a significant number of patients with an acute inflammatory process. After resolution of the underlying pathologic process and surgical treatment, the decrease in LDH could reflect the withdrawal of this preoperative systemic response. However, this interpretation remains hypothetical and cannot be confirmed without additional measurements of LDH over a longer postoperative period and without analysis of other indicators of liver inflammation and regeneration.

Additionally, our analysis showed that the ASA score significantly influenced several postoperative liver function parameters, including direct bilirubin, AST, ALP, and albumin, suggesting that the patient's general health status may have a greater impact on postoperative biochemical changes than intra-abdominal pressure alone. This finding is consistent with recent studies indicating that the hepatic response to CO_2 pneumoperitoneum is modified by comorbidities and baseline liver condition, emphasizing that postoperative biochemical changes should be interpreted within the overall clinical context rather than solely in relation to pneumoperitoneum pressure (4, 9).

In addition, recent research confirms that the increase in liver enzymes after laparoscopic cholecystectomy is an almost universal phenomenon, but with clear temporal dynamics and no clinical significance in most patients. In large reviews, AST and ALT elevations are reported to occur in as many as 75–82% of patients within the first 24–48 hours, with spontaneous normalization within a week. These findings directly correspond with the results of our research, where a significant increase in transaminases was also recorded without signs of permanent damage. This further confirms that the observed biochemical changes are part of the expected physiological response to pneumoperitoneum and not an indicator of clinically relevant hepatopathy (10).

Recent studies also indicate the importance of the duration of pneumoperitoneum as a factor that may have a greater impact than the level of intra-abdominal pressure itself. It has been shown that a longer duration of surgery (>60 minutes) leads to a more pronounced increase in AST, ALT, and ALP, which is explained by the prolonged reduction of splanchnic flow and the effect of ischemia-reperfusion. This finding may help in the interpretation of the results of our study, where no significant difference was found between different pressure levels, suggesting that the duration of exposure may be a more important factor than the pressure level itself. This opens up space for additional analyses in which pressure and duration of pneumoperitoneum would be considered in combination as key determinants of postoperative changes (10).

The advantages of this study include a prospective design, clearly defined groups according to intra-abdominal pressure, and relatively uniform demographic characteristics of the subjects, which allows reliable interpretation of the results. Also, the analysis of a number of biochemical parameters provides a comprehensive insight into liver function after laparoscopic cholecystectomy. Compared to other studies, the inclusion of the ASA score as a factor in the analysis is an advantage, which further expands the understanding of the influence of the patient's general condition.

On the other hand, limitations of the study include a relatively small range of intra-abdominal pressure between groups, which may reduce the possibility of detecting differences in the impact of pneumoperitoneum, as well as a relatively limited number of subjects. Also, the lack of long-term follow-up makes it impossible to assess the durability of the observed changes. Nevertheless, the results provide a significant contribution to the understanding of the effects of laparoscopic cholecystectomy on liver function and indicate the need for further research with a wider range of pressures and a larger sample of subjects.

It should be emphasized that this study included only patients operated on at standard values of intra-abdominal pressure (13–15 mmHg), which represent the routinely applied range in our institution. Therefore, the aim was not to compare standard and low pneumoperitoneum but to assess whether small differences within the clinically most commonly used range could affect postoperative biochemical parameters of liver function. The obtained results indicate that there are no significant differences in liver function in this pressure range. Future randomized trials involving lower values of intra-abdominal pressure (6–10 mmHg), which are now increasingly recommended in selected patients, could provide additional information on the potential benefits of low-pressure pneumoperitoneum.

A limitation of this study is that certain intraoperative factors that may influence postoperative changes in liver function parameters, such as duration of surgery, duration of pneumoperitoneum, total CO_2 insufflation time, intraoperative blood loss, anesthetic regimen, and episodes of intraoperative hypotension, were not analyzed. Future

studies should include these parameters to more accurately assess their impact on transient changes in liver enzymes after laparoscopic cholecystectomy. An a priori analysis of sample power was not performed in this study, which is a limitation in the interpretation of negative findings. However, the sample size was determined by the availability of patients who met the inclusion criteria during the study period. The results obtained should be interpreted in the context of the study design, and future prospective studies with a larger number of subjects and the inclusion of additional intraoperative factors should confirm these findings.

Laparoscopic cholecystectomy leads to transient and selective changes in biochemical parameters of liver function, which appear to be clinically insignificant. Variations of intra-abdominal pressure within the commonly used operative range did not result in significant differences in postoperative liver function parameters, supporting the safety of standard pneumoperitoneum pressures. Patient-related factors, including ASA score, may contribute to postoperative biochemical variations; however, further studies are required to determine their independent influence relative to intra-abdominal pressure. These findings highlight the importance of careful preoperative patient assessment and individualized risk evaluation in laparoscopic surgery.

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Author Contributions

Conceptualization, M.K.; Data collection, G.I., A.J., and M.M.; Statistical analysis, S.D.; Final manuscript approval, N.M.and Z.E. All authors have read and approved the published version of the manuscript.

Statement of Ethics

The study was approved by the Ethics Committee of the Clinical Hospital Center of Kosovska Mitrovica (approval No. 3634, issued on June 2, 2025).

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 the article.

Statement of Generative AI Use

No generative AI was used.

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THE IMPACT OF SOCIO-ECONOMIC FACTORS ON THE QUALITY OF LIFE OF EDENTULOUS ELDERLY PATIENTS IN SERBIA

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Tooth loss is a significant global issue, particularly among the elderly, as it impairs essential stomatognathic functions like chewing, swallowing, speech, and aesthetics. Edentulism can also affect nutrition, overall health, and quality of life, leading to functional, psychological, and social challenges. This research aimed to determine whether the type of prosthetic restoration affects patients' overall satisfaction and the extent to which socio-economic factors affect quality of life. The study included 120 patients of both genders and over 60 years of age, who were divided into two groups: patients with conventional complete dentures (Group 1) and patients with complete dentures with mini-implants (Group 2). The respondents completed the Oral Health-related Quality of Life (OHRQoL) questionnaire, which was divided into four parts: socio-demographic data, hygiene habits, changes in oral functioning, and changes in ways, 30 of life related to oral health. Patients with conventional complete dentures had significantly more frequent speech impairment (80.3%), swallowing (47.5%), and chewing (82.0%), compared to patients with mini-implants. The association of speech, swallowing, and chewing disorders with educational level and income was also demonstrated. They were also significantly more stressed, more nervous, more distracted by daily activities, and more uncomfortable than another group of subjects. Patients rehabilitated with mini-implant-supported complete dentures demonstrated higher overall satisfaction levels. The level of education and income have the highest impact on the quality of life of all socioeconomic factors.

Keywords: complete denture, quality of life, mini-implants, socio-economic factors

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INTRODUCTION

Tooth loss is an important problem on a global scale, and it is especially present in the older population. Edentulism is known to impair the basic functions of the stomatognathic system, such as loss or limitation of chewing movements, swallowing, speech, reduction of the lower third of the face, a decrease in vertical dimensions, poor aesthetics, and phonetic issues, mimicry, temperomandibular joint dysfunction, etc. (1,2). Typically, individuals with edentulism prefer foods that are easier to chew, which can compromise their nutritional intake, potentially affecting their overall health. These changes can negatively impact oral health-related quality of life (OHRQoL). As an irreversible condition, edentulism may lead to functional impairment as well as psychological or social disability (3).

The annual frequency of losing ≥ 1 tooth ranges from 1 to 14% in European countries. 30% of the global population between the ages of 65 and 74 is edentulous. Some authors have suggested that, by the year 2020, less than 15% of the population will be edentulous (4). Tooth loss is associated with socioeconomic factors, habits and general medical status of the organism (5).

Serbia is a country with a Human Development Index (HDI) of 0.787 and has an estimated 7 million inhabitants, of which about 60% reside in urban areas. The prevalence of individuals without natural teeth has shown slight variation over the years—10.2% in 2006, 12.4% in 2013, and 11.1% in 2019—as has the prevalence of denture use within this subgroup (79% in 2006, 82.3% in 2013, and 80.1% in 2019). Women who have lost all their natural teeth are more likely to use dental prostheses (83.8%) compared to men (73.4%). Among individuals over the age of 60, the percentage of edentulous persons ranges from 30% to 45%(6).

The objective of each prosthetic treatment is to establish normal functioning of the stomatognathic system. In cases where all teeth are missing, the preferred treatment would be implant placement and fixed restorations. However, this is not always possible. The limiting factors are often poor financial situation, inadequate anatomical conditions or poor general condition of the organism. Considering that there are usually at least two of these limiting factors present in the elderly population, it is clear why the choice of complete denture is the preferred therapy for them (7).

Resorptive changes after tooth loss cause a decrease in the height and width of the alveolar ridge and thus impair the retention and stabilization of the lower complete denture. In cases of unfavourable anatomical conditions, numerous oral-surgical pre-prosthetic surgical procedures are available, like vestibuloplasty, alveolar ridge augmentation, which facilitate the production of an adequate lower complete denture. On the other hand, such procedures are often avoided in the elderly due to the extent of the intervention and the potential systemic complications. Recently, the use of mini-implants (8-13) has proven to be one of the possible solutions for the edentulous patients. Implant-supported dentures offer many benefits such as better retention and denture stabilization, better chewing efficiency, even load distribution, better aesthetics and maintenance of vertical dimensions of occlusion compared to conventional complete dentures.In order to achieve adequate dental rehabilitation for the last 20 years, great attention has been paid to personal attitude and satisfaction of the patient. This has resulted in the development of research involving the measurement of oral health-related quality of life (OHRQOL), aimed at providing information on the impact of the oral cavity on the quality of human life (14).

This study aimed to determine:

1. Whether the type of prosthetic solution applied affects the overall satisfaction of the patient.
2. Whether there is a difference in quality of life depending on the type of prosthetic solution used.
3. To what extent socio-economic factors affect the quality of life of edentulous elderly patients.

METHODS

The research was conducted at the Faculty of Dentistry in Pančevo and included 120 patients of both genders, all over 60 years of age. The participants were divided into two groups: Group 1 consisted of patients with conventional complete dentures, while Group 2 included patients with complete dentures supported by mini-implants. In all participants, the current prosthetic solution had been applied within the last year.

Entry criteria for study participation were:

- Age 60 years or older.
- Complete edentulism in one or both jaws.
- Presence of prosthetic restorations no longer than 12 months.

Exclusion criteria included the following habits:

- Smoking,
- Frequent alcohol consumption.

The implementation of this study was approved by the Ethics Committee of the Faculty of Dentistry in Pančevo.

Only respondents who provided written informed consent for voluntary participation were included in the study.

Participants completed a structured questionnaire divided into four sections, each corresponding to a specific group of variables considered in the research.
The variables included in the study were grouped as follows:

- Applied prosthetic solution.
- Sociodemographic and socioeconomic characteristics (gender, age, place of residence, marital status, educational degree, income.
- Hygiene habits (washing dental prosthesis, removing dental prosthesis at night).
- Changes related to oral functioning (difficulties when eating, difficulties while speaking, feeling of discomfort, pain in the lower jaw, denture stability.
- Changes in the way of life related to oral health (nervousness, discomfort, stress interfering with daily activities).

Statistical analysis
Statistical data analysis was performed using the IBM SPSS Statistics software, version 30. Pearson's Chi-square test was applied as a goodness-of-fit test and for the analysis of contingency tables, to determine whether there were significant differences in the frequencies of non-parametric data for one or two variables.
For parametric data, the ANOVA F-test was used to compare mean values between groups. In addition, Spearman's rank-order correlation, a non-parametric test, was employed to assess the relationship between oral health-related quality of life (OHRQoL) and other examined variables, depending on data distribution.
The level of statistical significance was set at $p < 0.05$.

RESULTS

A total of 50.8% of participants were women (55.7% in Group 1 and 45.8% in Group 2), while 49.2% were men (44.3% in Group 1 and 54.2% in Group 2). The average age of the patients was approximately the same between the groups: Group 1 had a mean age of 67.54 ± 2.90, and Group 2 had a mean age of 67.54 ± 29.07 years. Regarding monthly income, the fewest participants in both groups had an income of up to 15,000 RSD (3.3% in Group 1 and 0% in Group 2), while the largest share had an income of up to 60,000 RSD in Group 2 (44.1%) and up to 30,000 RSD in Group 1 (39.3%) (Table 1).
Further testing related to the distribution of patients relative to the health-related quality of life (HRQOL) showed differences concerning the representation between groups (Table 2).

Table 1. *Demographic and socioeconomic characteristics of respondents*

Table 1.	Total		Group 1		Group 2		Chi2, §F test sign p
	n = 120	(%)	N = 61	%	N = 59	%	
Gender							
Male	59	49.2	27	44.3	32	54.2	0.275/ns
Female	61	50.8	34	55.7	27	45.8	
Average age	67.59 ± 2.94		67.54 ± 2.90		67.64 ± 2.99		§0.849/ns
Place of residence							
Countryside	24	20	10	16.4	14	23.7	0.468/ns
City	57	47.5	32	52.5	25	42.4	
Suburban area	39	32.5	19	31.1	20	33.9	
Degree of education							
Elementary school	9	7.5	4	6.6	5	85	0.189/ns
High school	77	64.2	44	72.1	33	55.9	
Faculty	32	26.7	13	21.3	19	32.2	
Scientific title	2	1.7	0	0	2	3.4	
Marital status							
Married	72	60	38	62.3	34	57.6	0.875/ns
Single	9	7.5	5	8.2	4	6.8	
Divorced	16	13.3	8	13.1	8	13.6	
Widowed	23	19.2	10	16.4	13	22	
Monthly income (RSD)							
up to 15,000	2	1.7	2	3.3	0	0	0.000***
up to 30,000	25	20.8	24	39.3	1	1.7	
up to 45,000	30	25	23	37.7	7	11.9	
up to 60,000	33	27.5	8	13.1	25	42.4	
over 60,000	30	25	4	6.6	26	44.1	

Speech difficulties were reported at a significantly higher rate in Group 1 (80.3%) compared to only 3.4% in Group 2. Swallowing difficulties were also notably more frequent in Group 1 (47.5%) compared to 3.4% in Group 2. A similar trend was observed with chewing difficulties, where 82% of participants in Group 1 reported issues, while only 1.7% did so in Group 2.
Responses to the question "Are you satisfied with the stability of your lower complete denture?" also varied significantly between groups. In Group 1, only 1.6% of respondents were completely satisfied, 68.90% were dissatisfied, and 29.5%were completely dissatisfied. In contrast, responses in Group 2 showed that 17.0% were completely satisfied, 55.90% were dissatisfied, and 27.10% were completely dissatisfied—indicating notably higher satisfaction compared to Group 1.

Table 2. *Distribution of patients relative to oral health-related quality of life (OHRQOL) among groups*

Table 2.	Total		Group 1		Group 2		Chi squere test, p sign
	n = 120	(%)	N = 61	%	N = 59	%	
Do you experience any difficulties while speaking?							
Yes	51	42.5	49	80.3	2	3.4	0.000***
No	57	47.5	11	18	46	78	
Occasionally	12	10	1	1.7	11	18.6	
Do you experience any difficulties while swallowing?							
Yes	31	25.8	29	47.5	2	3.4	0.000***
No	81	67.5	32	52.5	49	83	
Occasionally	8	6.7	0	0	8	13.6	
Do you experience any difficulties while chewing?							
Yes	51	42.5	50	82	1	1.7	0.000***
No	62	51.7	10	16.4	52	88.1	
Occasionally	7	5.8	1	1.6	6	10.2	
Are you satisfied with the stability of the complete lower denture?							
Yes, totally	11	9.2	1	1.6	10	17	0.014*
No	75	62.5	42	68.9	33	55.9	
No, not at all	34	28.3	18	29.5	16	27.1	
Do you remove complete lower denture at night?							
Yes, always	33	27.5	28	45.9	5	8.5	0.000***
Occasionally	45	37.5	27	44.3	18	30.5	
No	42	35	6	9.8	36	61	
Do you experience any difficulties while eating a certain type of food?							
Yes	27	22.5	27	44.3	0	0	0.000***
No	67	55.8	14	23	53	89.8	
Occasionally	26	21.7	20	32.7	6	10.2	
Do you feel pain in the lower jaw?							
Yes	13	10.8	10	16.4	3	5.1	0.034*
No	85	70.9	44	72.1	41	69.5	
Occasionally	22	18.3	7	11.5	15	25.4	
Are you frequently highly stressed in the process of communication?							
Yes	47	39.2	40	65.6	7	11.9	0,000***
No	73	60.8	21	34.4	52	88.1	
Do you frequently think about your denture?							
Yes	32	26.7	29	47.5	3	5.1	0.000***
No	43	35.8	10	16.4	33	55.9	
Occasionally	45	37.5	22	36.1	23	39	
Do you feel uncomfortable because of your denture?							
Yes	22	18.3	21	34.5	1	1.7	0.000***
No	58	48.4	16	26.2	42	71.2	
Occasionally	40	33.3	24	39.3	16	27.1	
Are you nervous because of your complete lower denture?							
Yes	36	30	29	47.5	7	11.9	0.000***
No	62	51.7	11	18	51	86.4	
Occasionally	22	18.3	21	34.5	1	1.7	
Does the wearing of a denture interfere in any way with your daily activities?							
Yes	34	28.4	31	50.8	3	5.1	0.000***
No	67	55.8	17	27.9	50	84.7	
Occasionally	19	15.8	13	21.3	6	10.2	

How often do you wash your denture?							
In the morning and in the evening	46	38.3	25	41	21	35.6	0.848/ns
Only in the morning	2	1.7	1	1.6	1	1.7	
Only in the evening	19	15.8	8	13.1	11	18.6	
After every meal	53	44.2	27	44.3	26	44.1	
When did you get your first complete lower denture?							
Last year	22	18.3	12	19.7	10	17	0.756
3 years ago	65	54.2	34	55.7	31	52.5	
5 years ago	33	27.5	15	24.6	18	30.5	
Are you satisfied with your health?							
Very dissatisfied	2	1.7	2	3.3	0	0	0.000***
Disatisfied	31	25.8	24	39.3	7	11.9	
Neither satisfied nor dissatisfied	29	24.2	19	31.2	10	16.9	
Satisfied	52	43.3	11	18	41	69.5	
Very satisfied	6	5	5	8.2	1	1.7	

Table 3. *Correlation of OHRQOL relative to the socioeconomic health factors in the overall sample*

Table 3.		Group	Age	Gender	Residence	Education	Marital	Income	Income <45000rsd≥
Difficulties while speaking	R	.758**	0.042	0.004	-0.074	.218*	0.091	.610**	.609**
	Sig.	0	0.652	0.965	0.423	0.017	0.324	0	0
	N	120	120	120	120	120	120	120	120
Difficulties while swallowing	R	.540**	0.083	0.01	-0.12	.297**	0.052	.391**	.469**
	Sig.	0	0.366	0.917	0.193	0.001	0.57	0	0
	N	120	120	120	120	120	120	120	120
Difficulties while chewing	R	.787**	-.181*	-0.055	-0.124	.207*	0.169	.580**	.590**
	Sig.	0	0.048	0.548	0.178	0.024	0.065	0	0
	N	120	120	120	120	120	120	120	120
Stability satisfaction	R	0.085	-0.129	-0.073	-0.148	0.023	0.165	-0.085	-0.062
	Sig.	0.358	0.161	0.426	0.108	0.805	0.072	0.358	0.5
	N	120	120	120	120	120	120	120	120
Do you remove complete lower denture at night?	R	.566**	0.005	-0.055	-.242**	-0.039	.209*	.458**	.437**
	Sig.	0	0.956	0.554	0.008	0.671	0.022	0	0
	N	120	120	120	120	120	120	120	120
Difficulties while eating certain types of food	R	0.166	0.044	-0.138	-0.019	0.036	0.045	.303**	.216*
	Sig.	0.071	0.636	0.134	0.835	0.694	0.623	0.001	0.018
	N	120	120	120	120	120	120	120	120
Frequently thinking about denture	R	.264**	-0.076	0.015	-.218*	0.122	.259**	.388**	.348**
	Sig.	0.004	0.41	0.874	0.017	0.186	0.004	0	0
	N	120	120	120	120	120	120	120	120
Uncomfortable feeling	R	0.112	0.052	-.263**	-0.118	0.019	0.066	0.078	0.039
	Sig.	0.225	0.575	0.004	0.198	0.835	0.474	0.396	0.67
	N	120	120	120	120	120	120	120	120
Pain in the lower jaw	R	.235**	-0.051	-0.146	-0.173	-0.022	.182*	.306**	.292**
	Sig.	0.01	0.582	0.11	0.059	0.814	0.047	0.001	0.001
	N	120	120	120	120	120	120	120	120
Nervous	R	0.059	-0.049	-0.152	-0.008	-0.127	-0.031	0.086	0.059
	Sig.	0.521	0.594	0.098	0.928	0.166	0.74	0.352	0.52
	N	120	120	120	120	120	120	120	120

Denture wearing vs. daily activities	R	.295**	-0.049	-0.062	-0.134	.207*	-0.04	.361**	.447**
	Sig.	0.001	0.592	0.502	0.143	0.023	0.665	0	0
	N	120	120	120	120	120	120	120	120
Denture washing frequency	R	0.028	-0.012	0.107	-0.125	0.003	0.02	-0.167	0.005
	Sig.	0.763	0.897	0.247	0.173	0.975	0.826	0.068	0.953
	N	120	120	120	120	120	120	120	120
Health satisfaction	R	.400**	-0.1	-0.142	-0.159	0.024	-0.02	.369**	.352**
	sig	0	0.275	0.121	0.083	0.797	0.829	0	0
	N	120	120	120	120	120	120	120	120

Frequent stress during communication was reported by 65.6% of participants in Group 1, whereas only 11.9% in Group 2 experienced the same issue (Table 2).

The analysis of the relationship between speech, swallowing, and chewing difficulties and socio-demographic characteristics showed no statistically significant correlation with age, gender, place of residence, or marital status.

A significant correlation was found between speech difficulties ($R = 0.758$, $p < 0.0001$), swallowing difficulties ($R = 0.540$, $p < 0.0001$), and chewing difficulties ($R = 0.787$, $p < 0.0001$) in Group 1 compared to Group 2 (Table 3).

A significant correlation was also observed between speech difficulties ($R = 0.218$, $p < 0.017$), swallowing difficulties ($R = 0.297$, $p < 0.001$), and chewing difficulties ($R = 0.207$, $p < 0.024$) in the group with lower education compared to the group with higher education (university degree or scientific titles) (Table 3).

Furthermore, a significant correlation was found between speech difficulties ($R = 0.609$, $p < 0.0001$), swallowing difficulties ($R = 0.469$, $p < 0.0001$), and chewing difficulties ($R = 0.590$, $p < 0.0001$) in the group with an income below 45,000 RSD compared to the group with an income above 45,000 RSD (Table 3).

A significantly higher percentage of participants in Group 1 (47.5%) consider their dentures compared to Group 2 (5.1%), with place of residence showing strong correlation—most commonly among suburban residents (43.6%), then urban (21.1%), and least among rural (12.5%). This is also notable among married and single individuals (33.3%), and those earning below 45,000 RSD (47.4%). Discomfort is significantly associated with female gender.

Wearing dentures interferes with daily activities more in Group 1 (50.8%) than in Group 2 (5.1%), with the strongest correlation among those with elementary or secondary education (33.7%) and low income (52.6%). Lower jaw pain is more frequent in Group 1 (16.4%) than Group 2 (5.1%), especially among divorced individuals and those earning under 45,000 RSD (15.8%).

No significant link was found between denture-related nervousness or hygiene habits and sociodemographics. Self-rated health does not significantly differ by age, gender, residence, education, or marital status; however, higher satisfaction is seen in Group 2 (3.61 ± 0.72) and among those earning over 45,000 RSD (3.56 ± 0.83 vs. 2.89 ± 0.95).

DISCUSSION

The status of oral health has a significant impact on both physical and mental health, which ultimately influences the overall quality of life. This includes a person's appearance, speech, chewing, food perception, social life, and self-esteem (15, 16). According to estimates, 20% of the population in Serbia was over 65 years old in the year 2017 (17).

Our study aligns with previous research by Celebić et al., which reported that more than a third of edentulous patients are not fully satisfied with their complete dentures, primarily due to issues with insufficient stability, retention, and discomfort during mastication. Similarly, in our study, patients with conventional complete dentures expressed significant dissatisfaction with the stability of their prostheses, leading to complaints of difficulties in speaking, chewing, and overall discomfort, which aligns with the findings of Celebić et al. (18). On the other hand, our results showed that patients with mini-implant-supported complete dentures were significantly more satisfied. Patients with conventional dentures were more often stressed, nervous, and unable to perform daily activities, frequently thinking about their dentures compared to patients in Group 2. These findings are consistent with several previous studies. For example, Burns and colleagues found that patients with implant-retained overdentures have greater preference and satisfaction compared to conventional dentures, particularly in terms of comfort and functionality (19). Similarly, long-term studies show that mini implants provide good stability,

minimal bone loss, and high patient satisfaction over extended periods [20,21]. Elsyad et al. demonstrated that the design of overdentures with mini implants significantly reduces supportive tissue loss and enhances stability, which directly affects functional efficiency and satisfaction (20, 22). Additionally, histological and clinical results from a one-year study by Passos and colleagues support the effectiveness of mini implants in lower dentures, with patients showing a high degree of adaptation and satisfaction (23).

This situation can likely be attributed to the better stabilization provided by mini-implants compared to conventional complete dentures (24-26). More stable dentures enable a satisfactory chewing capacity, thereby having a positive impact on patients' quality of life (QoL) (27-30). On the other hand, an unstable denture negatively affects the patient's ability to eat, speak, and smile. Patients with conventional dentures often encounter difficulties while doing housework, socializing, and engaging in leisure activities (31,32).

Our study did not reveal any significant differences in terms of gender, except for the fact that women experienced certain discomfort (33). Silverman et al. (32) suggest that men adapt better to their dentures. These results can be explained by the fact that addressing or alleviating oral problems and improving oral health does not significantly reduce women's concerns. Women generally view complete dentures as a form of handicap, which can hinder socialization and cause discomfort (24).

Married patients tend to express higher overall satisfaction with conventional dentures compared to single patients, although no significant differences were observed (25). Our research showed that married respondents removed their dentures more often at night, while divorced patients experienced more pain, and both married and single patients thought more often about their dentures.

Respondents with higher education experienced fewer difficulties with speaking, swallowing, and chewing compared to those with lower education levels. Dentures interfered more with the daily activities of respondents with elementary and secondary education, which is consistent with the findings of Caglayan (34). This could be attributed to the fact that higher-educated respondents were more likely to have complete dentures with mini-implants, which are more stable and therefore cause fewer problems. Patients with an income lower than 45,000 RSD more frequently reported discomfort while eating, interference with daily activities, pain, and more frequent thoughts about their dentures. This may be explained by the fact that people with lower income are generally more dissatisfied with their dentures (33).

The use of mini-implants has recently become the method of choice for the prosthetic rehabilitation of patients who are unable to receive standard dental implants. The growing body of evidence on the efficacy of implant treatments aimed at increasing the stability of complete dentures and improving patients' quality of life (QoL) is promising (35-37). Mini-implants are highly acceptable, with a success rate of 92.32%, similar to the success rate of therapy using standard implants (8,38). These results indicate that complete dentures retained by mini-implants are an acceptable option, especially for patients with financial limitations or inadequate anatomical structures for standard implant insertion (39,40).

According to Kostić et al., improving oral health and regular dental check-ups would improve the functions of the orofacial system, physical appearance, and self-confidence of the subjects, as well as their social life (41). It is crucial to consider dental implants, particularly for patients who cannot mentally accept being edentulous and for elderly patients receiving their first complete denture, as they may have decreased neuromuscular adaptation.

Within the limitations of this study, the following conclusions have been drawn:

1. Patients with conventional complete dentures report overall lower satisfaction compared to those with complete dentures supported by mini-implants.
2. The selected prosthetic solution significantly affects the stability of the denture, influencing chewing, speaking, daily activities, discomfort, and communication difficulties.
3. Level of education and income have the greatest impact on the quality of life among all socio-economic factors. Patients with higher education levels more easily understand the advantages of using mini-implants for the retention and stabilization of complete denture.

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Author Contributions

Conceptualization, J.A. and J.D.; Methodology, investigation, and data curation, J.A., Ć.A., and P.M.; Formal analysis, P.M., Ć.A., and P.A.; Writing – original draft preparation, J.A., P.M., and J.D.; Writing – review and editing, J.A.; Supervision, J.D. All authors have read and approved the published version of the manuscript.

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This study was approved by the Ethics Committee of the Faculty of Dentistry in Pančevo and conducted in accordance with the Declaration of Helsinki.

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The authors declare no relevant conflicts of interest.

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

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AFMN BIOMEDICINE

CASE REPORT

ARTERY OF PERCHERON INFARCTION PRESENTING AS COMA AND ACUTE-ONSET VASCULAR DEMENTIA: A CASE REPORT

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A rare anatomic variant that arises from the P1 segment of the posterior cerebral artery (PCA) and vascularized thalami is the artery of Percheron (AOP). We present a patient with acute onset of unconsciousness that occurred as a result of bilateral thalamic infarction verified by magnetic resonance imaging (MRI). Bilateral thalamic infarction was the cause of the acute onset of dementia that we followed over the next 30 months. A 54-year-old female was admitted to the Neurological Emergency Unit because of sleepiness that began a few hours after awakening. During the examination, the patient developed a deeper disturbance of the state of consciousness lasting forty minutes with a Glasgow coma score of 3/15. Brain MRI showed symmetric bilateral thalamic infarcts and upper midbrain lesions. Mini-Mental State Examination, which is used to evaluate global cognitive function, was 19 on the sixth day of the disease course. The patient remained with vascular dementia 30 months later. The middle age of the patient, female sex, sleepiness, coma, and vascular dementia are the unique clinical features of our case. A middle-aged female patient with coma and vascular dementia that followed for 30 months has never been reported before.

Keywords: coma, posterior cerebral artery, vascular dementia

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INTRODUCTION

The Percheron artery is a blood vessel that provides vascularization to the paramedian thalamus and the rostral portion of the midbrain, and it arises from the P1 segment of the posterior cerebral artery (PCA) (1). In the literature, this artery was first described in 1973 by the French neurologist Percheron (2). The Percheron artery was observed in 4 to 12 percent of the population (3), while infarction of the Percheron artery appears in 0.1 to 2 percent of all ischemic strokes (4). Approximately 11 percent of all strokes in the vertebrobasilar territory are thalamic strokes (5). The most common symptoms resulting from occlusion of the Percheron artery include disturbance of mental status, vertical gaze palsy, behavioral disorders, speech problems, hypersomnolence, and coma (6). When the paramedian thalamus is affected by a stroke, the dominant symptoms are cognitive impairment and behavioral disturbances (7). The best method for diagnosing bilateral paramedian thalamic infarction is magnetic resonance imaging.

CASE REPORT

A 54-year-old female, a housewife, was admitted to the Neurological Emergency Unit of Clinical Center Kragujevac because of sleepiness that developed a few hours after awakening. The patient had no history of prior illnesses, except that she was an extreme smoker. During the examination, the patient developed a deeper disturbance of the state of consciousness lasting forty minutes with a Glasgow coma score of 3/15. A neurological examination found miotic pupils, which were not reactive to light. She had neither sensory nor motor focal signs. After that, the state of consciousness improved and the patient became drowsy, slow to respond, but adequately responsive to questions, and remained in this state for the next five days. A computerized tomography scan of the brain performed on admission was unremarkable. Digital subtraction angiography showed the occlusion of the Percheron artery-type IIb (Figure 1; Figure 2). After waking up, the patient developed slowed response. Cardiologic examination revealed arterial hypertension. Laboratory examination showed newly registered type 2 diabetes mellitus. During hospitalization, an echocardiogram was done and was described as normal, as well as a 24-hour Holter ECG. Coagulation screening-platelet count, fibrinogen, prothrombin, prothrombin time test and INR (PT/INR), partial thromboplastin time test, antiphospholipid antibodies, D-dimer were normal. Also, thyroid-stimulating hormone, serum folic acid, serum vitamin B_{12} and imunoserological analyses were normal. Computed tomography of the brain performed immediately on admission was normal.

Magnetic resonance imaging of the brain was performed 24 hours after admission and demonstrated the existence of symmetric bilateral thalamic infarct and upper midbrain lesions (Figure 3). Mini-Mental State Examination, which is used to evaluate global cognitive function, was 19/30 on the sixth day of hospitalization. Dominant symptoms were: difficulty paying attention or concentrating, difficulty organizing thoughts, difficulty making plans and communicating them, slowed thinking, memory loss and speech difficulties. During hospitalization, the Mini-Mental

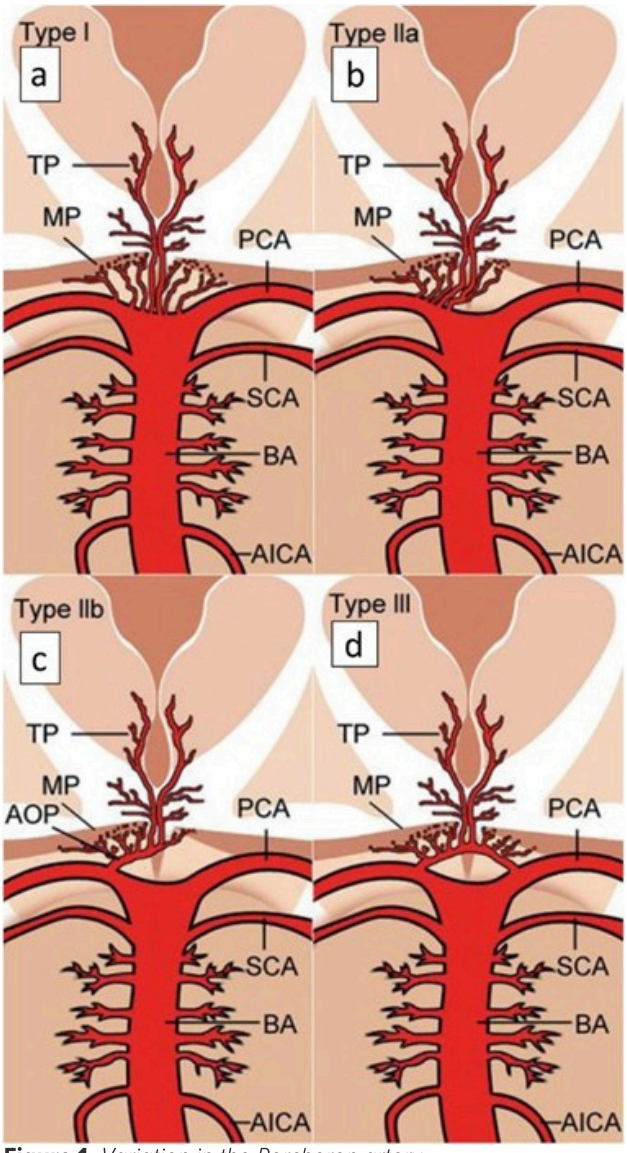


Figure 1. *Variation in the Percheron artery*

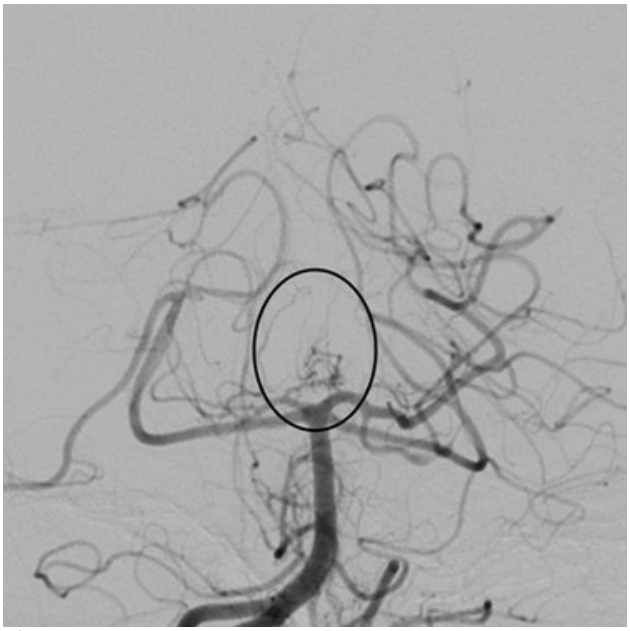


Figure 2. *Occlusion of the Percheron artery-type IIb by digital subtraction angiography*

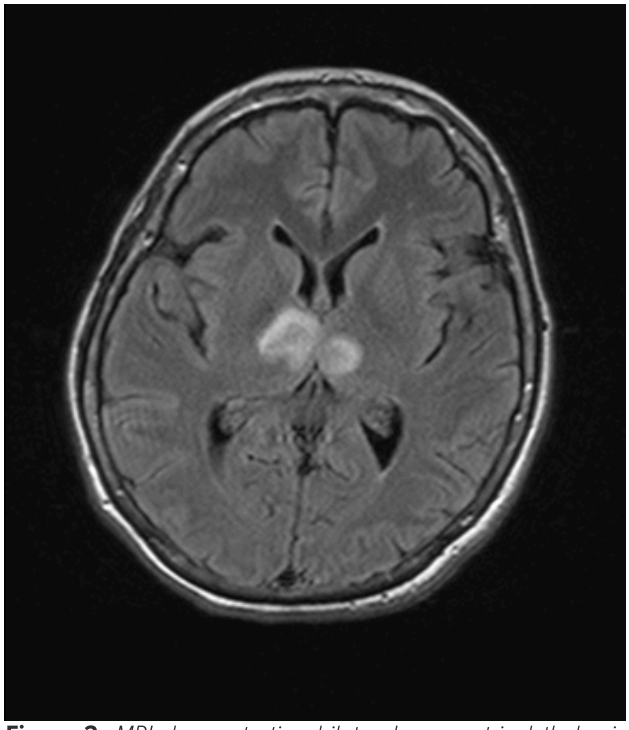


Figure 3. *MRI demonstrating bilateral, symmetrical thalamic infarction*

State Examination was repeated on the twelfth day of hospitalization and was identical to the previous one. On discharge, on the thirtieth day, the Mini-Mental State Examination score was 21/30. The patient was treated with antiplatelet and antihypertensive therapy. The patient was followed clinically, neurologically, neuropsychologically, and radiologically for 30 months. On neurological examination, there was no pyramidal/motor deficit. A brain MRI, 30 months later, demonstrated bilateral thalamic infarction in the chronic phase. Neuropsychological testing Mini-Mental State Examination score was 21/30. The patient demonstrated physical deficits manifested in psychomotor slowness, overall slow motor function, and apathy impacting the ability to complete daily activities. The patient remained with vascular dementia 30 months later.

DISCUSSION

The thalamus is part of the diencephalon, containing nuclei that control nociception, speech, motor and cognitive functions. It also contains nuclei responsible for motivation and behaviour (8). The vascularization of this part of the thalamus is divided into four regions: anterior, paramedian, posterior, and inferolateral (9). The artery that supplies the paramedian part of the thalamus arises from the posterior communicating artery and the P1 and P2 segments of the posterior cerebral artery (10). One of these arteries is the Percheron artery, which is a single blood vessel, which supplies bilateral vascularization of the thalamus. The prevalence of this artery varies from 7 to 11 percent (11). Occlusion of this artery leads to infarction of both paramedian thalami and the midbrain. The highest incidence of infarction in this region is between 60 and 70 years of age, with a slightly higher incidence among men (12). We presented a 54-year-old female patient. The etiology of this disease is multifactorial. Risk factors for ischemic stroke include: cardiovascular risk factors, diabetes mellitus, smoking, alcohol consumption, hyperlipidaemia, and physical inactivity (13). In our patient's history of risk factors, we had newly diagnosed hypertension, diabetes mellitus, and smoking. According to the literature, ischemic stroke in the region of bilateral paramedian thalamus clinically manifests as vertical gaze palsy , memory and behavioural disturbances, confusion, coma, disturbances of mental status, speech disorders, motor deficit, and cerebellar signs (14). In our case, we presented a female patient who had coma and associated vascular dementia manifesting as attentional disturbances, memory impairment and speech problems, psychomotor slowing and apathy which affected her activities in everyday life. The patient had a typical clinical picture of bithalamic infarction. One of the components of the reticular activating system is the thalamus. The intralaminar nuclei of the thala-

mus play the most significant role in transmitting information from the reticular activating system to the cortex (6). Coma is the most severe disorder of consciousness and is characterized by the inability to open the eyes, the inability to speak, and the absence of a motor response (GCS < 6). The diagnostic criteria for acute stroke in the territory of the Percheron artery is coma lasting less than 24 hours, based on a small ischemic lesion in the region of the unilateral or bilateral paramedian thalamus and/or rostral midbrain. In a study that analyzed 30 patients with Percheron artery infarction (PAIC), lesions of the paramedian thalamus and/or rostral midbrain were described in 46.7 percent of cases (15). The dorsomedial and intralaminar nuclei control memory and behavior. Ischemic lesions in the region of the paramedian thalamus lead to disruption of connections between the thalamus and the cerebral cortex and manifest with the following symptoms: amnesia, apathy, and memory disturbances (16). Several cases of thalamic dementia resulting from occlusion of the Percheron artery (AOP) have been described in the literature; however, thalamic infarctions are considered strategic lesions in the etiology of vascular or post-stroke dementia.

Saida et al. (17) presented three patients with ischemic stroke in the thalamic region who manifested hypersomnia, coma, vertical gaze palsy, and neuropsychiatric disorders.

Koutosuraki et al. (18) described a 38-year-old male patient without risk factors whose clinical features included memory impairment, hypophonia, and apathy. The patient was not followed up further. García-Casares et al. (19) reported a 42-year-old male patient who was a smoker and presented with memory and behavioral disturbances; he was followed up for the next six months.

Kouasi and Doumbia-Ouattara (20) described a 51-year-old HIV-positive patient who had a paramedian bithalamic infarction with acute onset of thalamic dementia and who died two months later. The dominant symptoms were amnesia and dysexecutive syndrome.

The patient's middle age and the female sex are some of the unique clinical features of our case. Clinical manifestations of our patient consisted of sleepiness, coma, and thalamic dementia.

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