

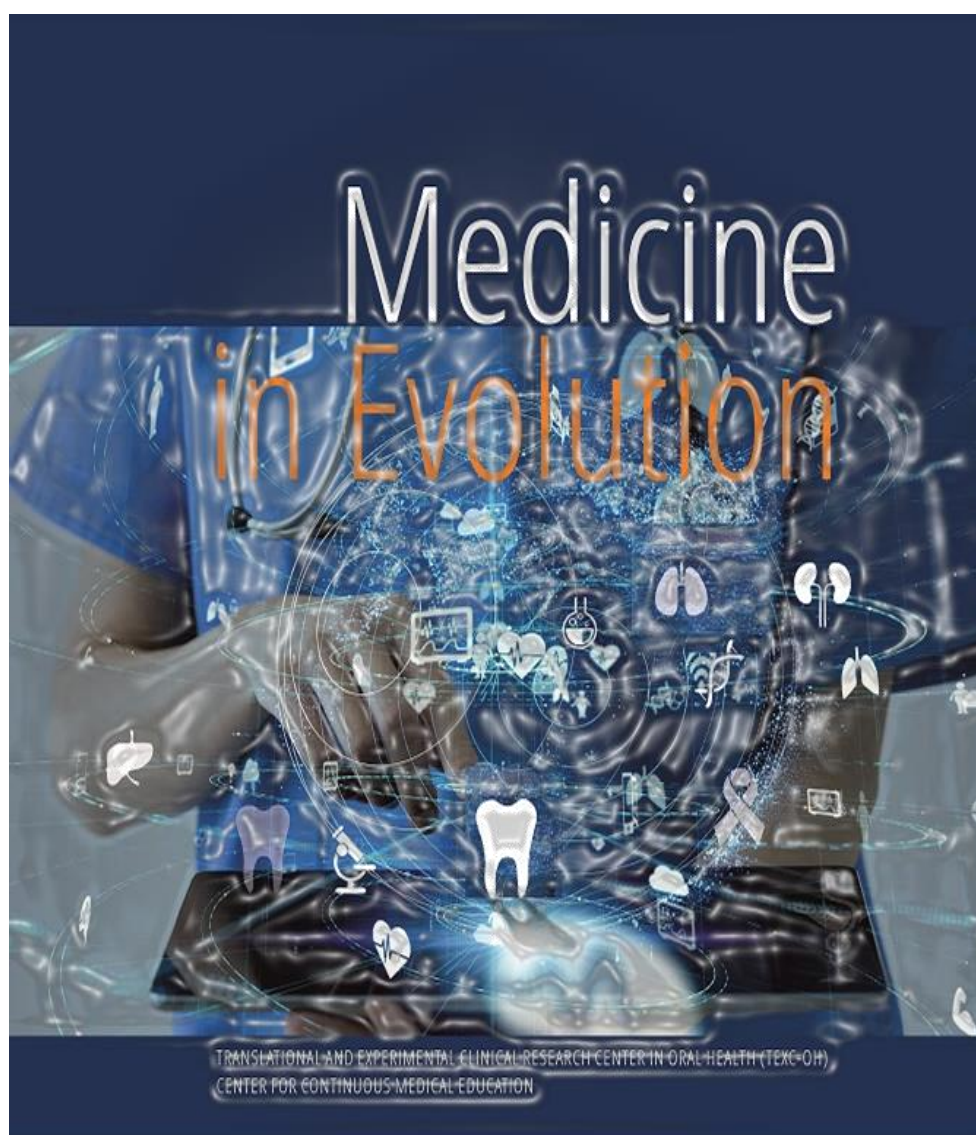
Medicine in Evolution



TRANSLATIONAL AND EXPERIMENTAL CLINICAL RESEARCH CENTER IN ORAL HEALTH (TEXC-OH)
CENTER FOR CONTINUOUS MEDICAL EDUCATION

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MEDICINE IN EVOLUTION



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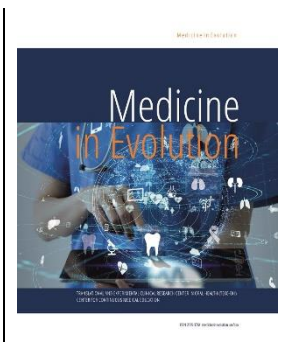


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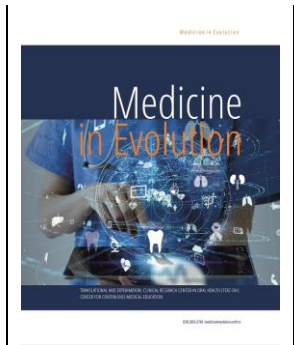
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Background



The current journal was established by Prof. Dr. Mircea Ancusa in 1999, with the aim of acquiring knowledge and sharing insights in the noble profession guided by the principle "primum non nocere" (first, do no harm). In 2005, it was entrusted to a group of dedicated researchers at the Center of Health Education and Motivation for Prevention in Dentistry, under the leadership of Prof. Angela Codruta Podariu, DMD, PhD, at the Department of Preventive Dentistry of the University of Medicine and Pharmacy "Victor Babes" in Timisoara, Romania.

The inception of the journal stemmed from a dedication to exchange experiences in both professional and research domains. It was envisioned to encompass all medical specialties, with the aspiration that the published manuscripts would exhibit exceptional quality, elevating the journal's reputation. Esteemed professionals were enlisted to the editorial board and the review committee, individuals recognized for their expertise in the realm of research. The decision to publish papers in English was made to broaden accessibility to the global research community and enhance international recognition.

Since then, the journal has been regularly published under the auspices of the Center of Health Education and Motivation for Prevention in Dentistry, disseminating national and international research studies with the objective of evolving into a comprehensive evidence-based publication. Presently, the journal has transitioned to the stewardship of the Translational and Experimental Clinical Research Centre in Oral Health, situated within the Department of Preventive, Community Dentistry, and Oral Health. Its objectives are aligned with the vision of esteemed organizations such as the World Health Organization and the International Dental Federation, seamlessly integrating into the research strategy of Victor Babes University of Medicine and Pharmacy Timisoara.

"Medicine in Evolution" stands as a distinguished, peer-reviewed, open access journal dedicated to the dissemination of original theoretical research spanning the interdisciplinary spectrum of medicine and healthcare. Encompassing various topics within the realms of human life sciences, medical community, dental medicine, and pharmacology, the journal warmly welcomes original research papers, communications, letters, short notes, case reports, and reviews for submission. Committed to conducting rigorous peer reviews and expediting the publication of groundbreaking research, its mission is to advance the field of medicine through scholarly discourse.

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Phytochemical Characterization and Biological Evaluation of *Ribes Nigrum* Leaf Extracts: A Study of Aqueous and Butanolic Fractions

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Abstract

Background: *Ribes nigrum* (black currants) are recognized for their outstanding biological properties, including antioxidant, anti-inflammatory, and anticancer effects. Colorectal cancer (CRC) is a type of cancer with a high mortality, for which various botanical products have been investigated as alternatives that have emerged as

potential candidates in the oncological domain. Methods: The present work involved the development of two types of *Ribes nigrum* leaves extracts (in aqueous and butanolic fractions), which were evaluated by the HPLC method and subsequently assessed for the antioxidant capacity, the antimicrobial activity, and in vitro cytotoxicity on the DLD-1 cell line. Results: The results indicated that both types of extracts contain amounts of polyphenols, the antioxidant activity of the extracts supports their potential as natural sources of free radical scavengers, antimicrobial evaluation showed that the extract in aqueous fraction produced a selective antimicrobial effect on gram-positive bacteria, and the MTT assay suggested that the extracts produced a dose-dependent cytotoxicity, with a superior effect noted for the extract in the butanolic fraction. Conclusion: These findings emphasized the biological potential of *Ribes nigrum* extracts due to their versatile composition and opened new research directions regarding their anticancer potential in CRC.

Keywords: blackcurrants, *Ribes nigrum*, colorectal cancer, natural alternative, antimicrobial activity, antioxidant activity.

INTRODUCTION

Nature has offered for thousands of years an inexhaustible source of substances that have framed multiple therapeutic remedies. Plants have been used in traditional medicine for centuries to treat various diseases. Today, many herbal compounds are being studied for their potential in drug development. These plant-based medicines offer a cost-effective, effective, and safer alternative to conventional treatments [1]. Blackcurrant (*Ribes nigrum* L., Grossulariaceae) is a shrub generally found in temperate climates, and its fruits are a great source of vitamin C and other beneficial substances (e.g., essential oils, micro- and macronutrients). The fruits of the shrub also contain polyphenolic constituents that display versatile properties, including antioxidant, antimicrobial, antibacterial, and antiviral properties. In vitro, in cell cultures, it was observed that polyphenols show anti-tumour activity, inducing apoptosis of cancer cells [2]. Beside this, anthocyanins are known dietary antioxidants, valued for their potential to restore balance between oxidative and antioxidant factors in living organisms. Based on the specialized literature, blackcurrants hold significant amounts of anthocyanins (250 mg/100 g of fresh fruit) and are acknowledged in traditional medicine in Europe and Asia for treating various conditions. In addition, a fundamental consideration is that blackcurrant extract has recently been identified as the second most effective of nine different berry extracts studied for their free radical scavenging properties [3].

Black currants have demonstrated therapeutic potential in managing hypertension and other cardiovascular diseases, as well as neurodegenerative, neoplastic, ocular conditions, and diabetic neuropathy [4]. In cancer research, black currant extracts have shown anticancer effects in vitro against several cancer types, including gastric cancer and esophageal squamous cell carcinoma [5]. Additionally, black currant juice and extracts have been found to inhibit the proliferation of breast, prostate, and colon cancer cells [3].

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of cancer-related deaths. The lifetime risk of developing CRC is estimated at 4–5% and is influenced by factors such as medical history, age, and lifestyle. CRC arises from mutations in oncogenes, tumor suppressor genes, and DNA repair genes. Based on the origin of these mutations, CRC is classified as sporadic, hereditary, or familial [6]. In vivo studies have shown that long-term black currant supplementation can influence the gut microbiome in female mice, with effects varying by age [7]. Additionally, blackcurrant-based products have also been noted for their hypocholesterolemic and anti-inflammatory actions (whole berry and juice), as well as antioxidant actions that are specific to the leaves [8].

Given the growing interest in botanical compounds for medical applications, and considering the proven health benefits of black currants (e.g., antioxidant, anti-inflammatory, antitumour properties), the objective of the present work is to develop two types of *Ribes nigrum* leaf extracts containing polar constituents (aqueous and butanolic fractions) and to evaluate their composition by HPLC method, followed by assessment of their antioxidant and antimicrobial activities and to subsequently investigate their cytotoxic potential on a CRC cell line, namely DLD-1 cells. These investigations pave the foundation for future research on the potential of *Ribes nigrum* L. natural extracts as therapeutic alternatives in CRC.

MATERIAL AND METHODS

Ribes nigrum Extract Preparation

Ribes nigrum leaves were procured from S.C. Hypericum Impex S.R.L. (Baia Sprie, Romania), lot 0164, maintained under appropriate conditions at a temperature of 22 ± 2 °C

until processing. The extraction procedure started by obtaining a crude ethanolic extract, that was subjected to liquid-liquid partition in order to yield two polar fractions soluble in butanol and water. The non-polar compounds were eliminated from the crude extract by pre-treatment with petroleum ether, diethyl ether and ethyl acetate. The solvents were purchased as follows: ethanol (99.8%) from Riedel-de Haen (Seelze, Germany), petroleum ether, diethyl ether, ethyl acetate, and n-butanol were purchased from Sigma Aldrich (Steinheim, Germany). 200 g dried leaves were crushed to powder with the IKA A11 basic and mixed with 1000 mL absolute ethanol. The mixture was ultrasonicated for 30 minutes using an ultrasonic water bath (ELMA S120 Elmasonic from Elma Schmidbauer GmbH, Singen, Germany), followed by a filtration procedure using Whatman grade 4 filter paper. The extract was subjected to solvent evaporation using a rotary evaporator (Heidolph, Schwabach, Germany). The plant material was subsequently extracted with additional 1000 mL ethanol (99.8%). After solvent evaporation, 9.7 g crude extract were obtained.

A portion (8.0 g) of the crude extract was suspended in 50 ml of distilled water and then subjected to repeated liquid-liquid separation in a separating funnel, using solvents in order of increasing polarity. To obtain the desired fractions with polar phytochemicals, the suspension was pretreated with organic solvents that depleted the non-polar compounds (petroleum ether, diethyl ether, and ethyl acetate, 600 ml per solvent). In the end, the remaining aqueous suspension collected from the separation funnel was extracted using, successively, 3 times 200 mL of n-butanol. The fraction containing butanol-soluble phytochemicals, and the fraction containing water-soluble constituents were both subjected to solvent evaporation using a rotary evaporator, followed by lyophilisation. The two polar fractions were obtained from the crude extract with the following yields: 11.37% for the n-butanol soluble fraction, and 11.00% for the water-soluble fraction.

HPLC/LC-MS Identification and Quantification of Polyphenolic Compounds

The phytochemical composition of the *Ribes nigrum* extracts was analyzed using LC-MS/MS, based on two previously validated analytical methods described [9-11]. Analyses were conducted on an Agilent 1100 HPLC Series system (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 1100 SL Ion Trap mass spectrometer (LC/MSD Ion Trap VL) [12,13].

The first method focused on the identification and quantification of polyphenolic compounds. It employed a Zorbax SB-C18 reversed-phase column (100 mm × 3.0 mm i.d., 3.5 µm particle size) and a binary mobile phase consisting of methanol and 0.1% acetic acid (v/v), delivered in gradient mode. A total of twenty-eight polyphenolic standards were included; however, only a subset was detected and quantified in the analyzed extracts, as detailed in the Results section. Chromatographic conditions included: (1) a column temperature of 48 °C; (2) a flow rate of 1 mL/min; and (3) an injection volume of 5 µL. UV detection was performed at 330 nm for polyphenolic acids up to 17 minutes and at 370 nm for flavonoids and their aglycones up to 38 minutes, with MS detection in negative electrospray ionization (ESI) mode [14,15]. A second LC-MS method was optimized to identify eight additional polyphenols (e.g., epicatechin, catechin, syringic acid, gallic acid, vanillic acid, protocatechuic acid, epigallocatechin, epigallocatechin gallate), using the same column and instrumentation with a modified gradient. Detection was carried out under the same ESI conditions [11,15].

Compound identification was based on comparison of mass spectra and chromatographic retention times with reference standards, while quantification was completed using UV detection and external calibration curves. Finally, data processing was performed using DataAnalysis (v5.3) and ChemStation (vB01.03) software (Agilent Technologies). Results are expressed as micrograms of bioactive compound per milliliter of extract.

Antioxidant activity of polar *Ribes nigrum* fractions (aqueous and butanolic)

The DPPH assay was carried out to evaluate the antioxidant activity of *Ribes nigrum* extracts, following a previously reported method [16], with minor modifications adapted to our laboratory conditions. Briefly, 0.2 mL of *Ribes nigrum* extracts (at concentrations ranging from 100 to 1000 µg/mL) was mixed with 1.8 mL of 0.1 mM DPPH solution prepared in ethanol. The mixture was incubated in the dark at room temperature for 30 minutes and the ascorbic acid was used as a reference standard. The absorbance was measured at 517 nm using a UV-VIS spectrophotometer (PG Instruments Ltd., Lutterworth, UK).

***In vitro* Antimicrobial Activity**

The antimicrobial activity of the tested samples was checked against five reference microbial strains from ThermoScientific (USA): *Staphylococcus aureus* ATCC 25923, *Streptococcus pyogenes* ATCC 19615, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Candida parapsilosis* ATCC 22019. The evaluation was made in accordance with EUCAST [17] and CLSI [18] guidelines, as well as the procedures described in our previous studies. Gram-positive and Gram-negative bacterial strains were cultured on Columbia agar supplemented with 5% defibrinated sheep blood, while the yeast *C. parapsilosis* was grown on Sabouraud dextrose agar containing chloramphenicol (Oxoid, Wesel, Germany). Microbial suspensions were prepared in 0.85% NaCl solution and adjusted to a turbidity equivalent to 0.5 McFarland standard, corresponding to approximately $1-2 \times 10^8$ CFU/mL.

Antimicrobial activity was initially screened using the disk diffusion method. Mueller-Hinton (MH) agar or MH supplemented with 5% sheep blood and β-NAD (MHF medium) for *Streptococcus pyogenes* (Oxoid, Wesel, Germany) was used for inoculation with microbial suspensions of each tested strain. After inoculation and drying, sterile 6 mm blank paper disks (BioMaxima, Poland) were placed on the agar surface and loaded with 5 µL of each test compound at a concentration of 20 mg/mL. Gentamicin (for Gram-positive and Gram-negative bacterial strains) and fluconazole (for yeast *Candida parapsilosis*) served as positive controls, while disks impregnated with solvent (DMSO or EtOH/H₂O, depending on the sample) were used as negative controls. Plates were incubated at 35 °C for 24 hours, after which the diameters of the inhibition zones were measured. Strains exhibiting inhibition zones between 6–15 mm were deemed resistant to the tested compounds and were not further evaluated. For strains with inhibition zones exceeding 15 mm, minimum inhibitory concentration (MIC) testing was performed [19].

Chemicals and reagents

Roswell Park Memorial Institute 1640 (RPMI-1640) basal medium, fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin, and trypsin-EDTA 0.25x were obtained from Biowest (Nuaille, France). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) was procured from Alfa Aesar.

Cell lines and culture conditions

The human colorectal adenocarcinoma cell line (DLD-1) employed in this research was acquired from the European Cell Culture Center and was cultivated under standard conditions in RPMI-1640 basal medium supplemented with 10% fetal bovine serum (FBS), 1% glutamine, and 1% penicillin-streptomycin. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. The medium was refreshed every two days, and the cells were regularly subcultured at 80% confluence by trypsinization (trypsin-EDTA).

Cytotoxicity Assay

The cytotoxicity of the plant extract obtained was evaluated on the DLD-1 human colorectal adenocarcinoma cell line using the classic 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method. In summary, the cells were seeded in 96-well microplates at a density of 1×10^4 cells/mL per well and incubated for 24 hours. Then,

the medium was replaced with each plant extract in eight successive concentrations ranging from 0 to 1000 µg/mL in six technical replicates, followed by incubation for 24 hours

Plant extract samples were previously dissolved in dimethyl sulfoxide (DMSO), and diluted with culture medium as the concentration of DMSO did not exceed 1% v/v. Then 100 µL MTT solution (5 mg/mL in PBS) was added to each well and incubated for 2 h at 37 °C. The medium containing the MTT solution was aspirated without disturbing the formazan crystals formed in the wells. Finally, 150 µL of DMSO was added to each well to dissolve the MTT crystals until the appearance of the specific purple color.

Finally, the absorbance was measured at 570 nm using a microplate reader (BMG Labtech, Germany). The cells without treatment (untreated) were considered as negative control. The cell viability (%) was calculated compared with untreated control, based on the absorbance values, as follows: viability (%) = O.D. (experimental value)/O.D. (control value) × 100. The half maximal inhibitory concentration (IC₅₀) values for each plant extract was calculated using GraphPad Prism 5.0 (San Diego, CA, USA), applying non-linear regression with log concentration vs. normalized response.

RESULTS

Identification and Quantification of Phenolic Compounds in *Ribes nigrum* Extracts

The *Ribes nigrum* extracts studied in this work exhibited varying concentrations of phenolic compounds (Table 1). The aqueous extract contained only rutoside and isoquercitrin, with rutoside being the most abundant. In contrast, the n-butanol (n-BuOH) extract presented a broader polyphenolic profile, including rutoside, isoquercitrin, quercetin, and hyperoside. Chlorogenic acid was detected in both fractions, although only in low concentrations or trace amounts.

Table 1. Identification and Quantification of Polyphenolic Compounds from *Ribes nigrum* by HPLC/LC-MS

No.	Compounds	Aqueous extract	n-BuOH extract
		Results (µg/mL)	
1	Hyperoside	<LOQ	0.305±0.024
2	Isoquercitrin	0.329±0.029	2.718±0.407
3	Rutoside	0.503±0.005	4.139±0.331
4	Quercetin	<LOQ	1.398±0.167
5	Chlorogenic acid	<LOQ	<LOQ

< LOQ (below limit of quantification). Concentrations are expressed as mean ± SD (n = 3).

The antioxidant activity

The antioxidant activity of the extracts was analyzed using the DPPH test, and the results are shown as the percentage of free radical inhibition at different extract concentrations (100–1000 µg/mL). For both extracts, a significant increase in antioxidant activity was observed with increasing concentration, indicating a dose-dependent relationship (Figure 1).

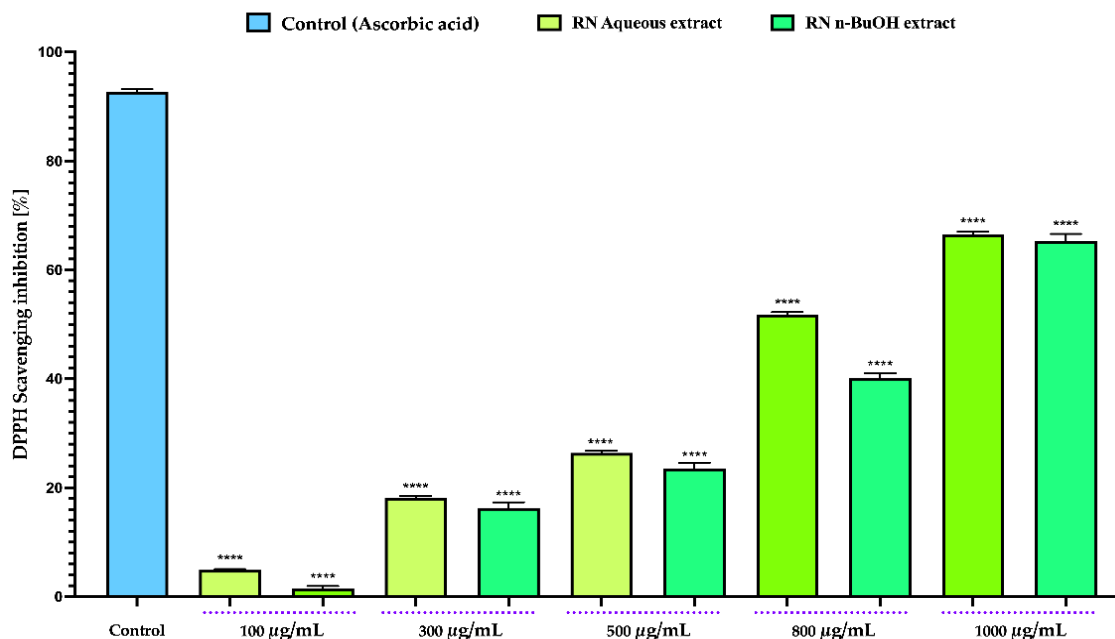


Figure 1. DPPH radical scavenging activity of *Ribes nigrum* aqueous and n-butanol (BuOH) extracts compared with ascorbic acid. Data are presented as mean $\pm$ SD (n = 3). One-way ANOVA test followed by a Dunnett's multiple comparison test was used to compare groups (****p < 0.0001)

The antimicrobial activity

The antimicrobial activity of *Ribes nigrum* (RN) extracts was investigated using the disk diffusion method. The inhibition zone diameters (mm) for each microbial strain and extract are summarized in the table below:

Table 2. Antimicrobial efficacy of *Ribes nigrum* extracts expressed as inhibition zone diameters

Microbial strains	Test compound	Inhibition zone (mm)
<i>Staphylococcus aureus</i> ATCC 25923	RN n-BuOH extract	8
	RN Aqueous extract	15
<i>Streptococcus pyogenes</i> ATCC 19615	RN n-BuOH extract	8
	RN Aqueous extract	18
<i>Escherichia coli</i> ATCC 25922	RN n-BuOH extract	8
	RN Aqueous extract	13
<i>Pseudomonas aeruginosa</i> ATCC 27853	RN n-BuOH extract	8
	RN Aqueous extract	8
<i>Candida parapsilosis</i> ATCC 22019	RN n-BuOH extract	8
	RN Aqueous extract	8

Extracts producing inhibition zones ≤ 15 mm were considered to have weak or no significant antimicrobial activity. Based on this criterion, only the *Ribes nigrum* aqueous extract showed measurable antimicrobial effects against *S. aureus* (15 mm), *S. pyogenes* (18 mm), and *E. coli* (13 mm).

Cell Viability Evaluation

The two extracts from *Ribes nigrum* leaves (the aqueous fraction and the butanolic fraction) were assessed for their cytotoxicity against the human colorectal adenocarcinoma cell line (DLD-1) in 8 successive concentrations (0-1000 μ g/mL) 24 hours following treatment application. Both extracts exerted a dose-dependent inhibitory effect on the cell viability of the DLD-1 cell line, as shown in Figure 2 and Figure 3. Increasing concentrations of the tested plant extracts induced suppression of cell proliferation and reduction of cell viability. The half maximal inhibitory concentration (IC₅₀) for the aqueous fraction was established at 278.4 μ M,

while in the case of the butanol fraction, the IC_{50} was found to be 137.9 μ M. The treatment of the cells with *Ribes nigrum* leaves extract, aqueous fraction, with the highest concentration evaluated, i.e., 1000 μ M, caused a reduction in the viability of the cells to about 14%. In the case of the butanol fraction, the treatment with 1000 μ M induces a decrease to approximately 3%.

Thus, the *Ribes nigrum* leaves extract obtained in butanol fraction showed superior cytotoxic activity on the DLD-1 cell line compared to the aqueous fraction.

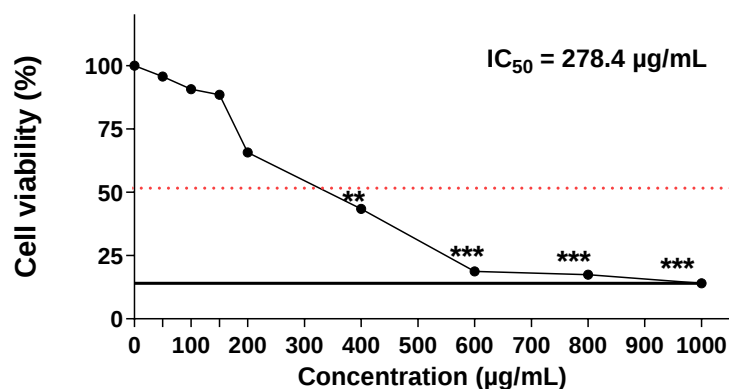


Figure 2. Graphical representation of the DLD-1 cell viability after treatment with the aqueous fraction of *Ribes Nigrum* L. extract in concentrations 0-1000 μ g/mL for 24 hours

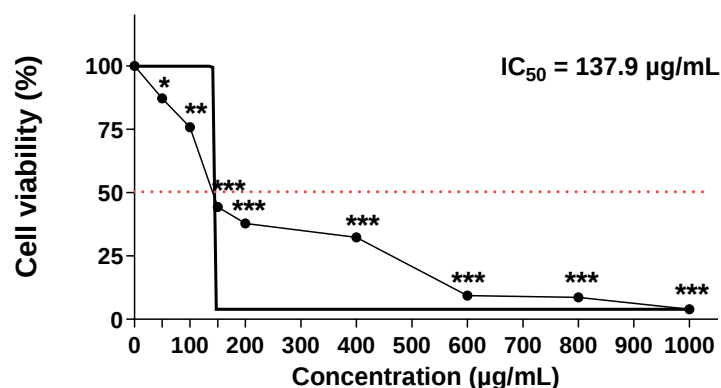


Figure 3. Graphical representation of the DLD-1 cell viability after treatment with the butanolic fraction of *Ribes Nigrum* L. extract in concentrations 0-1000 μ g/mL for 24 hours

DISCUSSIONS

In present, the current conventional therapy for patients diagnosed with CRC constitutes a major challenge, with high toxicity and poor response. However, it was observed that natural components might be able to have antiproliferative and apoptosis-inducing capacity in CRC, as supported by *in vitro*, *in vivo*, and clinical studies. Many natural compounds also offer the benefit of being well tolerated by patients, with no toxic actions, even at high doses [20]. In this regard, this paper covers HPLC analysis, the antioxidant and antimicrobial activity of polar extracts from *Ribes nigrum* leaves, as well as the evaluation of cell viability after treating DLD-1 cells (colorectal adenocarcinoma) with the two types of extracts (aqueous and butanolic fraction), taking into account the potential already mentioned in the literature that arouses increasing curiosity about *Ribes nigrum*.

HPLC represents one of the most popular methods for the identification of phenolic compounds contained in plants. The LC-MS/MS phytochemical analysis revealed the presence of several polyphenolic compounds in the *Ribes nigrum* extracts, with notable differences in composition depending on the extraction solvent used. These findings confirm that solvent polarity plays a critical role in determining the qualitative and quantitative profile of the extracted bioactive compounds [20–22]. In the aqueous extract, only two major polyphenols (e.g., rutoside and isoquercitrin) were detected. This suggests a preferential extraction of polar flavonoid glycosides by water. Rutoside or rutin was the most abundant compound, consistent with literature data reporting *Ribes nigrum* as a rich source of glycosylated flavonoids [23–25]. In contrast, the n-butanol extract exhibited a more complex polyphenolic profile, additionally containing quercetin and hyperoside. This broader spectrum is likely due to the intermediate polarity of n-butanol comparative to water, which facilitates the extraction of both polar and moderately polar flavonoids, including aglycones.

Besides, current ongoing research is focused on exploring compounds of botanical origin that can treat microbial diseases, since the increasing occurrence of multidrug resistance in microorganisms across the globe. For these purposes, evaluation of antimicrobial activity was one of the objectives of the study [26]. The antimicrobial screening of *Ribes nigrum* extracts revealed differential activity depending on the solvent used and the microbial strain tested. Notably, the aqueous extract demonstrated superior antimicrobial effects compared to the n-butanol extract, as evidenced by larger inhibition zones observed in the disk diffusion assay. Among the tested strains, *Streptococcus pyogenes* was the most susceptible, showing an inhibition zone of 18 mm for the aqueous extract, which justified further evaluation through minimum inhibitory concentration (MIC) testing. *Staphylococcus aureus* also exhibited moderate sensitivity (15 mm), while *Escherichia coli* showed a weaker response (13 mm). In contrast, both *Pseudomonas aeruginosa* and yeast *Candida parapsilosis* were resistant to the tested extracts, with inhibition zones not exceeding 8 mm, with the same value recorded for the negative control, suggesting a lack of efficacy against these more resilient pathogens. The enhanced antimicrobial activity of the aqueous extract may be attributed to its higher content of polar phenolic compounds, particularly rutoside and isoquercitrin, both of which have been previously reported to possess antimicrobial properties through mechanisms such as membrane disruption and inhibition of nucleic acid synthesis [27–30]. The absence of additional aglycones in the aqueous extract may suggest that glycosylated flavonoids are primarily responsible for the observed bioactivity, at least in the case of Gram-positive bacteria.

In this study, both aqueous and butanolic extracts of *Ribes nigrum* exhibited dose-dependent antioxidant activity, as evidenced by increasing inhibition values with rising extract concentrations. These findings are consistent with previous reports highlighting the antioxidant potential of *Ribes nigrum* due to its high content of polyphenols, anthocyanins, and flavonoids [31–33]. For the *Ribes nigrum* aqueous extract, an inhibition of 66.44% was recorded at 1000 µg/mL, with a calculated EC_{50} value of 790 ± 0.18 µg/mL. In comparison, the *Ribes nigrum* n-butanol (BuOH) extract showed a maximum inhibition of 64.17% at 1000 µg/mL, with an estimated EC_{50} of 880.7 ± 0.43 µg/mL. Although the antioxidant activity of the BuOH extract was slightly lower, it followed the same dose-response pattern, supporting the reproducibility and consistency of the antioxidant effect.

Further, the actual study aimed to determine the cytotoxic capacity of the two types of extracts on DLD-1 cells. Recently, it has been identified that certain compounds used in standard CRC treatment (e.g., 5-fluorouracil) induce multiple adverse effects, and the tumours show resistance to their administration. Consequently, natural compounds are the target of several research projects intended to identify their potential effect as therapeutic alternatives in the fight against cancerous pathologies, including colorectal cancer, one of the

most lethal neoplasms [34]. Regarding the evaluation of the antitumor potential, the two types of *Ribes nigrum* leaf extracts were investigated in DLD-1 cells. After treatment of the cells, the extract in the butanol fraction showed a superior anticancer effect compared to that in the aqueous fraction. Thus, comparing the highest concentrations tested (i.e., 1000 μM) at the same treatment interval, the extract in the butanol fraction reduced the viability of cancer cells up to 3%, while the extract in the aqueous fraction reduced the viability of cancer cells up to 13%. Moreover, the IC_{50} results support the same finding, the IC_{50} being 278.4 μM for the treatment with the aqueous fraction of the *Ribes nigrum* extract and 137.9 μM for the treatment with the n-butanol fraction of the leaf extract. Another group of researchers showed that ethanolic extract from *Ribes nigrum* leaves has the ability to inhibit the growth of HT-29 (human colon adenocarcinoma) and MCF7 (human breast cancer) cancer cells. Also, their results were more significant as the treatment period was longer [35]. Today, current clinical treatments of CRC, including surgical resection, radiotherapy, and chemotherapy, face several barriers among which can be listed the adverse effects, the risk of recurrence, and the drug resistance. Against this background, the development of drugs with low toxicity and reduced potential for the generation of drug resistance is an increasingly major challenge. Therefore, natural compounds have become researched since they have acquired a reputation for their potential anti-tumour activity, a function that is receiving growing interest, in addition to many other therapeutic properties. Several natural compounds have been shown to have the ability to modulate apoptosis, different signaling pathways, and cell differentiation; further establishing their status as low-toxicity compounds [36].

According to the results of the present study on DLD-1 cells, the analyzed extracts showed cytotoxic potential. However, future research directions should be directed towards the identification of the exact mechanism of action underlying the antitumor capacity, verification of the biosafety profile, and further evaluation in complementary experimental models such as *in ovo* and *in vivo*.

CONCLUSIONS

In this study, two extracts from *Ribes nigrum* L. were prepared in butanolic and aqueous fractions and subsequently evaluated. LC-MS/MS profiling revealed that both aqueous and n-butanol extracts contain notable polyphenolic compounds, with rutoside and isoquercitrin as dominant constituents. The aqueous extract showed a simpler, but more selective polyphenolic composition, whereas the n-butanol extract included a broader range of flavonoids, including aglycones (e.g., quercetin, hyperoside). The antioxidant activity of the extracts, attributed to the presence of these phenolic compounds, supports their potential as natural sources of free radical scavengers. Antimicrobial assays demonstrated that the aqueous extract exhibits selective activity against Gram-positive bacteria, with significant inhibition observed against *Streptococcus pyogenes* and moderate effects against *Staphylococcus aureus*. No significant activity was recorded against *Pseudomonas aeruginosa*, *Escherichia coli*, or the yeast *Candida parapsilosis*. These findings suggest that polar flavonoid glycosides may contribute to antimicrobial activity, especially against more permeable bacterial cell walls. In terms of cytotoxic activity against human colorectal adenocarcinoma DLD-1 cells, the butanolic fraction proved to be more active, reducing cell viability to a greater extent than the aqueous fraction. Future studies focusing on determining the mechanisms of action underlying biological effects represent important milestones in the development and progress of scientific research on *Ribes nigrum*.

Conflicts of Interest

The authors declare no conflict of interest.

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Evaluating the Use of Dietary Supplements to Improve Physical and Sports Performance - Survey Study

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Abstract

Background/Objectives: Dietary supplements (DS) use among athletes to enhance endurance, strength and muscle recovery is a complex issue influenced by factors such as sport type, individual goals, access to nutritional information, and nutrition trends, with higher prevalence among elite performers. Despite their growing popularity and benign perception, scientific evidence regarding the safety, quality, and efficacy of DS remains insufficient. Therefore, the objective of the current study was to assess Romanian athletes' awareness and consumption behaviours, as well as potential adverse effects associated with DS use. **Methods:** We designed a survey to examine correlations between athletes' age, background, gender, supplement types and usage frequency, and reported adverse reactions. Statistical analysis was used to reveal relevant correlations among the examined variables, providing insight into usage patterns. **Results:** The results highlight a high prevalence of DS consumption among young people and frequent reports of adverse reactions. **Conclusion:** Thus, educational interventions become essential to reduce potentially risky self-medication. The study revealed also the effectiveness of questionnaires as data collection tool among both amateur and competitive athletes, facilitating data-driven insights for enhancing athletic performance, promoting balanced lifestyles, and informing evidence-based guidance regarding the consumption of DS for both athletes and regulators.

Keywords: dietary supplements; enhancing sport performance; survey study

INTRODUCTION

Beyond structured training and tailored sports nutrition plans, athletes actively incorporate specific supplements to optimize their performance and recovery [1, 2]. Dietary supplements intended for athletes aim to increase muscle mass, boost energy levels, and promote weight loss. Supplements can help reduce post-workout recovery time, allowing athletes to train more frequently and intensely. Among the various dietary strategies applied as ergogenic sources, particular attention is given to the use of dietary supplements considered effective, safe, and legal, which contribute to energy production, promote faster recovery, and enhance performance.

Despite their growing popularity, scientific data on the safety, quality, and efficacy of these products remains limited [3,4]. Currently, their overuse represents a widespread societal concern, particularly among athletes, with potential consequences including health complications and diminished performance [5-8]. While the effectiveness of numerous supplements continues to be debated, some may induce serious adverse effects, such as cardiovascular, renal and central nervous system effects, liver disease, and pancreatitis [9-13].

The consumption of dietary supplements by athletes poses potential risks to both their health and professional careers, especially when these products contain substances prohibited by anti-doping regulations. Athletes must maintain awareness of the regulations of anti-doping agencies such as the World Anti-Doping Agency (WADA) and are advised to seek guidance from qualified nutritionists or sports medicine professionals prior to initiating any supplementation regimen. Comprehensive education on nutrition and supplement use is critical for enabling informed decision-making. Furthermore, thorough examination of product labelling and active ingredients is essential to identify and avoid the inadvertent intake of undeclared or contaminated substances. To mitigate these risks, athletes should prioritize the use of supplements certified by recognized third-party verification programs, which ensure product integrity, safety, and compliance with established quality standards [14].

Aim and objectives

Supplement use among athletes can provide significant performance and recovery benefits, but it is essential that these products be administered with caution and an approach based on reliable information and supported by scientific evidence is indispensable for optimizing positive effects and minimizing associated risks. In order to assess the level of knowledge and the use of supplements by athletes, but also the risk of adverse reactions, we developed a survey questionnaire that correlates information on athletes' age, background, gender, types of supplements used and frequency of consumption, and reported adverse reactions to supplements. The results of this study provide guidance for the selection of products to be analytically investigated for the possible presence of adulterants.

MATERIAL AND METHODS

A survey study to assess the degree of exposure of athletes to supplement consumption and to identify the main adverse reactions experienced by consumers was conducted using Google Forms and the questionnaire was distributed in Romania. The study "Consumption of dietary supplements by athletes and the possible presence of adulterants" was conducted in accordance with the principles outlined in the Declaration of Helsinki, and the protocol (Project identification code) was approved by the Ethics Commission of the National Anti-Doping Agency (ANAD) on 30th October 2024 (Project identification code

4176). The questionnaire, distributed through ANAD, targeted Romanian athletes and data collection was carried out over a period of approximately three months.

The collection of information based on the questionnaire was carried out in compliance with the provisions of Regulation No. 679/2016 (GDPR) for EU countries for the protection of individuals with regard to the processing of personal data and the free movement of such data, with subsequent amendments and additions, in accordance with Law No. 190/2018 on the measures for the implementation of Regulation (EU) 2016/679 (the law regulating the implementation of GDPR in Romania).

Study design

The questionnaire aimed to obtain information on the types of supplements used by athletes and to assess the risk of adverse reactions in order to outline possible correlations with the presence of synthetic adulterants in such supplements.

The first section of the questionnaire was designed to positioning respondents within their social environments and to gather comprehensive socio-demographic information, including gender, age, residential setting, and level of educational attainment. This segment also aimed to assess participants' level of awareness regarding dietary supplements and to capture their perceptions of supplement use. In addition, it included specific items intended to determine whether respondents, particularly athletes, had previously consumed dietary supplements and to identify the types of supplements used. The second section of the questionnaire was administered exclusively to respondents who indicated prior or current use of dietary supplements, primarily high-performance athletes.

The objectives of the questions included in the questionnaire were the following:

- to characterize the study groups taking into account socio-demographic parameters (gender, age, residence environment, educational level)
- to define of the prevalence of consumption and its characterization (methods of consumption, type of consumption - supplement alone or in combination, age at onset of consumption, reason for use, frequency of consumption)
- to determine the level of knowledge of dietary supplements among the participating athletes (type of supplement consumed, information, accessibility, awareness of risks)
- to identify the effects desired by the users (increased energy, weight loss, increased muscle mass, increased self-confidence) when consuming these supplements and the adverse effects experienced after consumption.

The adverse effects observed were categorized according to the physiological systems affected:

- cardiac effects: palpitations, accelerated heart rate, chest pain, bradycardia, sympathomimetic toxidrome: tachycardia, hypertension
- otorhinolaryngological effects: xerostomia, epistaxis, nose pain, oropharyngeal pain, tinnitus
- gastrointestinal effects: abdominal pain, anorexia, nausea, vomiting, diarrhoea, constipation, flatulence, cramps
- musculoskeletal effects: musculoskeletal pain, muscle cramps, changes in extremities (coldness, discoloration, numbness, tingling, tingling, numbness)
- neurological effects: bruxism, vertigo, headache, fainting, blurred vision, memory loss, tremor, convulsions
- psychological effects: agitation, anxiety, depression, fatigue, tiredness, drowsiness, poor concentration, dysphoria, increased energy
- pulmonary: shortness of breath, cough
- other: heavy sweating, insomnia, nightmares, skin rashes

The questionnaire was designed to analyse several aspects, including: consumption pattern (use of a single supplement or combination of several products); the effects that athletes experience when using these supplements; and possible side effects experienced as a result of consumption.

The questionnaire was administered online via the Google Forms platform and was accessed by athletes affiliated with national sports organizations, as well as individuals engaged in training at fitness centres, through a dedicated hyperlink or QR code.

Statistical data analysis was performed using Microsoft Excel version 2108. For each item in the questionnaire, the total number of responses was quantified and also expressed as a percentage (%). In order to analyse the correlations between the variables studied among athletes who consume dietary supplements, two statistical tests were used: the chi-square test and Pearson's correlation coefficient.

Chi-square test

The chi-square test was used to assess the relationship between the athletes' background (urban or rural) or level of education and supplement consumption, ease of obtaining supplements and awareness of possible risks. The relationship between variables is described using Chi-square test of independence, expressed as X^2 (DF = degrees of freedom, N = sample size) = chi-square statistic value, p = p-value. We compared the estimated value with the critical value in the chi-square table for a given significance level (usually 0.05).

Pearson correlation test

The Pearson correlation test, expressed as r (degrees of freedom) = r static, p = p-value, was applied to check the correlation between different parameters. Pearson's correlation coefficient measures the strength and direction of the relationship between two variables. The correlation coefficient varies between -1 and 1. A value close to 1 indicates a positive correlation, while a value close to -1 indicates a negative correlation. The Pearson test result is significant at p values < 0,05.

RESULTS

Characterization of the study group based on socio-demographic parameters

The research encompassed a cohort of 87 individuals actively engaged in sports activities. A socio-demographic assessment of the participant group revealed a pronounced predominance of female representation, accounting for 68.96% of the total sample.

The living environment can facilitate or, conversely, hinder both access to information and access to supplements. In this regard, the majority of responses were obtained from subjects residing in urban areas, accounting for 80% of the total sample.

Regarding the age distribution, as illustrated in Figure 1, the dominant age group consisted of individuals between 14 and 20 years old, representing 55.17% of the research participants. This was followed by 25.30% aged between 21 and 30 years, 10.34% under the age of 14, 4.59% between 31 and 40 years, and only 2.3% of participants aged between 40 and 50 years and over 50 years, respectively.

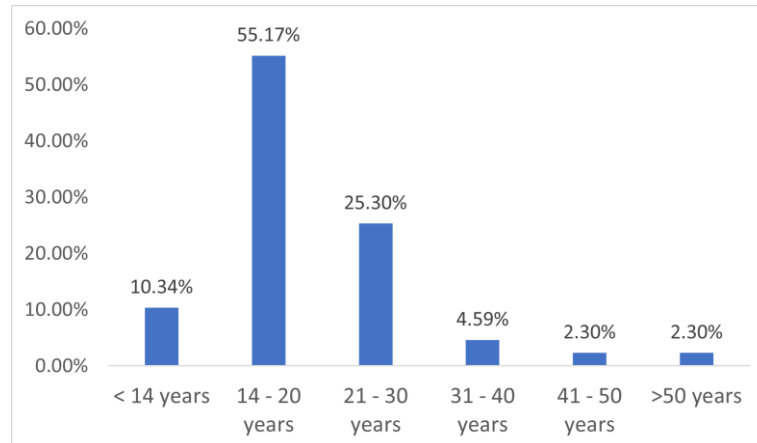


Figure 1. Demographic distribution by age among study participants

In terms of educational status, the majority of participants within the study group had completed high school, accounting for 44.2% of the sample. University graduates represented 30.8% of the group, while 15.4% had attained secondary school education. Participants with only primary education comprised 9.6% of the total, as illustrated in Figure 2.

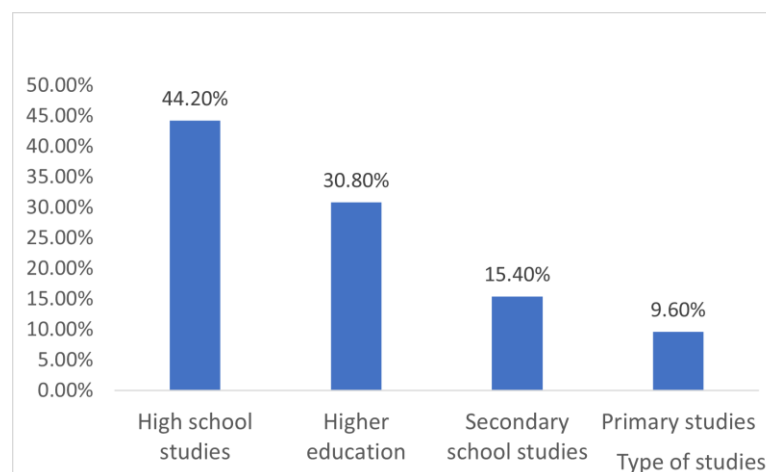


Figure 2. Demographic distribution by education level among study participants

Definition and characterization of consumption prevalence

Responses to the items addressing the definition of prevalence and the characterization of consumption indicated that the majority of athletes reported using dietary supplements, with a prevalence rate of 79.32%.

The majority of individuals reported initiating supplement use between the ages of 14 and 18, accounting for 71.4% of the respondents.

More than half of the respondents (58.1%) reported consuming a single dietary supplement. Among those who consume multiple supplements, 97.3% indicated that they do not associate their supplement use with steroids or hormonal substances.

The majority of supplement users (68.2%) reported learning about these products through interpersonal sources, including friends, colleagues, and family members. An additional 25% acquired information via online platforms, while the remaining 6.8% cited mass media or published literature as their primary sources.

Consumption patterns among users are predominantly motivated by the pursuit of rapid physical results and improved endurance during training, as indicated by 86.20% of

respondents. As illustrated in Figure 3, curiosity accounts for 12.07% of reported motivations, while 1.73% attribute their behaviour to external influences, such as mass media exposure.

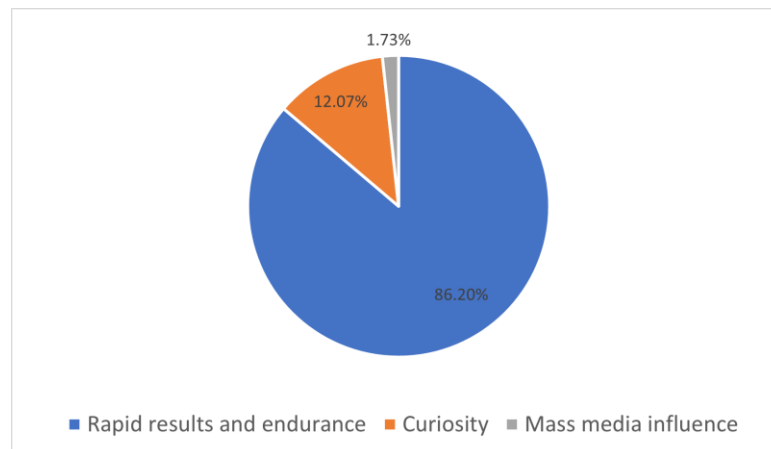


Figure 3. Distribution of study participants based on their stated reason for consumption

Capsules constitute the most frequently utilized dosage form, reported by 50% of respondents. Additional orally administered formats include powders for suspension, oral solutions (commonly referred to as 'shots'), and tablets. Regarding consumption frequency, approximately two-thirds of users indicate intake occurring one to two times per week.

Assessment of athletes' knowledge regarding dietary supplements

Based on the responses provided regarding the types of supplements consumed, sources of information, ease of access, and awareness of potential risks, the following findings were identified: the most frequently used supplements are those containing vitamins and minerals, representing 74.57% of users, amino acid and protein-based supplements are consumed by 30.50% of respondents, creatine-based supplements are used by 13.55% of participants, as illustrated in Figure 4.

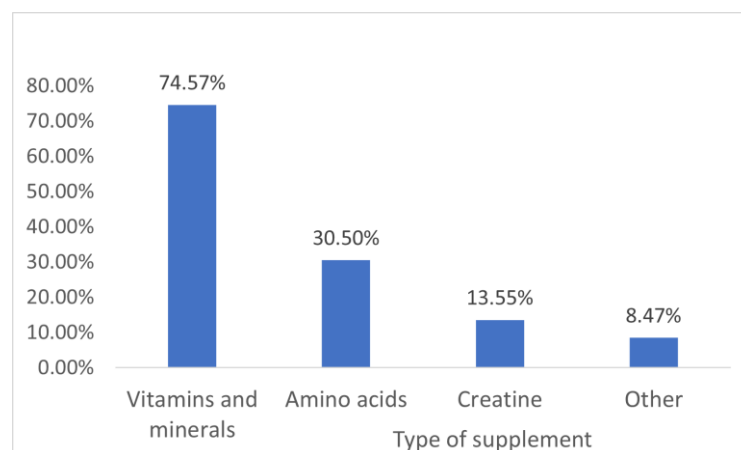


Figure 4. Distribution of study participants based on the type of supplement used

A significant majority (84.1%) report that supplements are easily accessible. Notably, 69.6% of respondents lack awareness regarding the potential risks associated with dietary supplement use, as illustrated in figure 5.

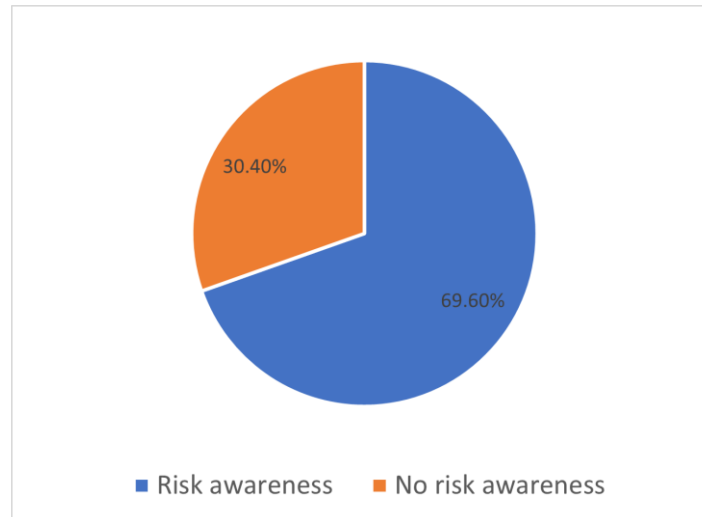


Figure 5. Distribution of study participants based on the awareness of the potential risks associated with dietary supplement use

Characterization of the study group based on desired effects and adverse reactions

Regarding the users' desired effects (as indicated by affirmative responses to questions concerning each type of effect), over half (60.5%) reported increased energy, while 51% indicated muscle mass gain. Enhanced self-confidence was reported by 38.1% of users and weight loss was cited by a smaller proportion (14%). The timeframe for achieving these effects (e.g., increased muscle mass and weight loss) was most frequently reported to be between one and three months, according to 64.7% of respondents (Figure 6).

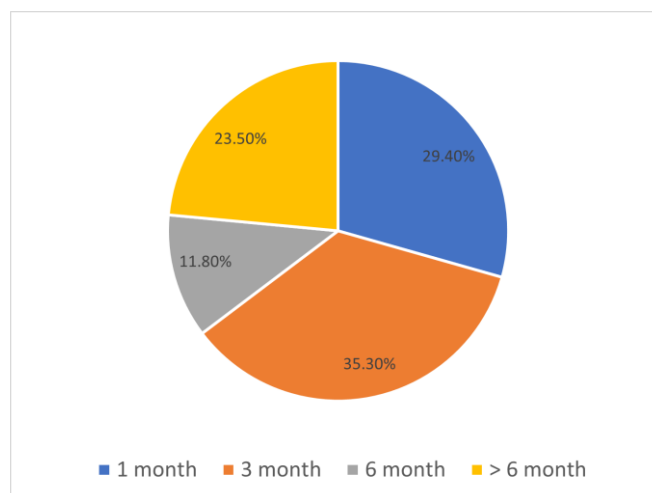


Figure 6. Distribution of study participants based on the time required to achieve the effect of the dietary supplement use (weight loss and muscle mass gain)

The characterization of the study group based on the reported adverse effects reveals that, at the cardiovascular level, palpitations and increased heart rate predominated (48.1%).

At the respiratory level, the most frequently reported effects were coughing reported by 63.5% of consumers and shortness of breath reported by 36.5% of the consumers.

The predominant ENT (ear, nose, and throat) related effects were xerostomia, reported by almost 75% of users, and tinnitus reported by 9.06% of users as shown in figure 7.

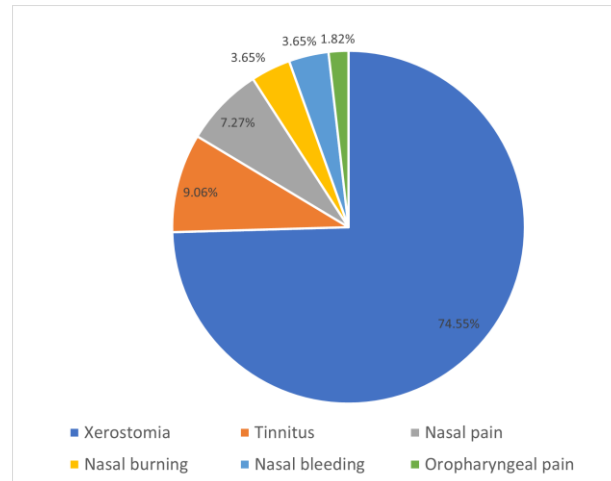


Figure 7. Distribution of study participants based on the adverse reactions in the ENT area

At the gastrointestinal level, the most common reactions were abdominal pain (30.8%) and anorexia (26.9%) as shown in figure 8.

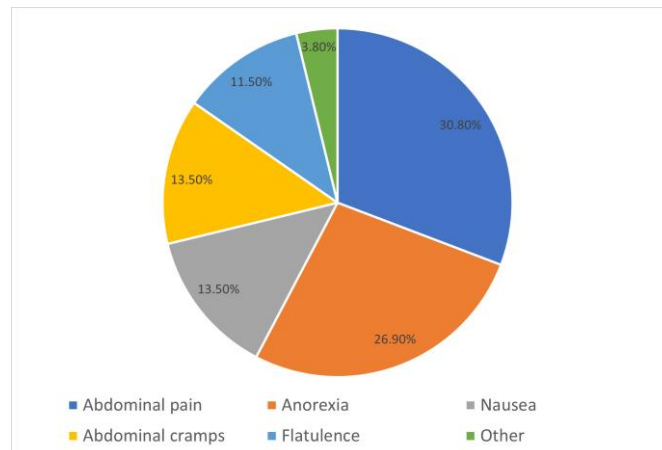


Figure 8. Distribution of study participants based on the gastrointestinal adverse reactions

The most frequently reported muscular effects included musculoskeletal pain (30.8%), numbness (25%), and muscle cramps (19.2%).

Among neurological effects, headache was the most commonly reported (55.8% of users), followed by hearing impairment (21.2%) and bruxism (9.6%) as shown in figure 9.

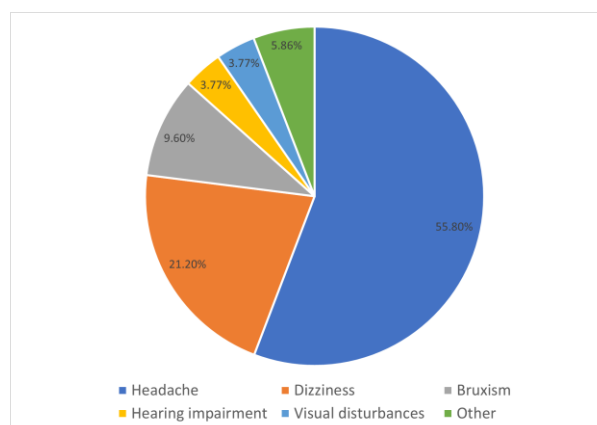


Figure 9. Distribution of study participants based on the neurological adverse reactions

The most frequent psychological effects were agitation (21% of consumers) and increased energy (15.4%). Within the category of other effects, the most commonly cited were excessive sweating (33% of users) and insomnia (17.6%).

Statistical analysis of the data

Chi-square test

Correlation of background (rural/urban) with dietary supplement consumption (question "Have you ever consumed dietary supplements?"). The Chi-square test, $X^2 (1, 83) = 0.3446$, $p = 0.557209$ indicates no statistically significant difference between rural and urban respondents in terms of the consumption of food supplements.

Correlation of background (rural/urban) with easiness of obtaining food supplements (question "Was it easy for you to get them?"). The Chi-square test, $X^2 (1, 65) = 0.0577$, $p = 0.810223$, indicates no statistically significant difference between rural and urban respondents in terms of how easy they find it to acquire dietary supplements. This can be explained by the type of supplements used, mainly from the vitamin and mineral category.

Correlation of background (rural/urban) with the awareness of possible risks (question "Did you know before taking supplements for the first time that they can cause serious health problems?"). The Chi-square test, $X^2 (1, 70) = 3.6296$, $p = 0.056759$, indicates no statistically significant difference between rural and urban respondents in terms of awareness of possible risks associated with the consumption of supplements, even though the result is borderline statistically significant.

Correlation of educational attainment (primary/secondary/high school/higher education) with Consumption of food supplements (question "Have you ever consumed food supplements?"). The Chi-square test, $X^2 (3, 86) = 8.4853$, $p = 0.036977$ indicates a statistically significant difference ($p < 0.05$) between different educational attainment of respondents in terms of the consumption of food supplements as the null hypothesis that there are no statistical differences between the categories of respondents in terms of dietary supplement consumption is rejected. According to the obtained results, the group of respondents with primary education (Chi-square contribution 1.72) have a predilection to use dietary supplements more frequently.

Correlation of educational attainment (primary/secondary/high school/higher education) with the easiness of obtaining dietary supplements (question "Did you find it easy to get them?"). The Chi-square test, $X^2 (2, 59) = 2.5761$, $p = 0.275801$ indicates no statistically significant difference between different educational attainment of respondents in terms of the easiness of obtaining dietary supplements. As no responses were obtained for the "no" option among secondary school graduates, these values were excluded from the analysis.

Correlation of educational attainment (primary/secondary/high school/higher education) with the awareness of possible risks associated with the consumption of dietary supplements (question "Did you know before taking supplements for the first time that they can cause serious health problems?"). The Chi-square test, $X^2 (3, 73) = 2.5634$, $p = 0.463933$ indicates no statistically significant difference between different educational attainment of respondents in terms of the awareness of possible risks associated with the consumption of dietary supplements. As no responses were obtained for the "no" option among secondary school graduates, these values were excluded from the analysis.

Pearson test

Correlation of the intake of creatine supplements or amino acid products with time required to achieve the effect of the dietary supplement use (weight loss and muscle mass gain) was assessed. The Pearson test, $R (17) = -0.539$, $p\text{-value} = 0.017251$ indicates a moderate negative correlation. Thus, consumption of amino acid products correlates with a shorter time to desired effects (weight loss and muscle gain).

Correlation of the observed time to weight loss with frequency of use per week was assessed. The Pearson test, $R(48) = -0.1641$, $p\text{-value} = 0.2551$ indicates a very weak negative correlation. Considering only the responses received from consumers of creatine / amino acid products, the obtained results $R(17) = -0.1278$, $p\text{-value} = 0.604386$ showed a very weak negative correlation. We conclude that there is virtually no statistically significant correlation between the observed time to weight loss and frequency of use per week, regardless of the dietary supplement consumed.

Correlation of the reported headache with frequency of use per week was assessed. The Pearson test, $R(59) = -0.086$, $p\text{-value} = 0.53999957$ indicates a very weak negative correlation. We conclude that there is no statistically significant correlation between headache onset and frequency of use per week, regardless of the dietary supplement consumed.

Correlation of the headache as neurological adverse reactions with dry mouth as ENT adverse reaction was assessed. The Pearson test, $R(85) = 0.4293$, $p\text{-value} 0.000033$ indicates a positive and highly statistically significant correlation between the occurrence of headache (neurologic adverse effect) and dry mouth (ENT adverse effect). It is possible that these are adverse reactions of the same type of supplement, also suggesting potential adulteration.

DISCUSSIONS

The rising trend of supplement overuse among athlete, particularly youth athlete, is a growing concern. Although supplements can support athletic performance when used responsibly, excessive or improper intake may pose health risks and potentially impair rather than enhance results.

In this context, the study investigates athletes' awareness and consumption of dietary supplements, along with the potential risk of adverse effects. To achieve this, a questionnaire-based survey was carried out among athletes in Romania. The questionnaire compiled a variety of data, including socio-demographic characteristics, types of supplements used, consumption frequency, and any adverse reactions experienced by users following administration.

The results indicate a high percentage of supplement users (approximately 80%) and a greater prevalence of consumption among young athletes, in a group 14 – 20 years old (55%). The primary reason for supplement use is to achieve rapid results and performance and to increase the endurance. These findings are consistent with data previously published in the literature. Numerous studies report a relatively high prevalence of dietary supplement use among athletic populations [15]. Thus, a study conducted in Hungary revealed that a considerable percentage of adolescent recreational athletes use dietary supplements, primarily aiming to enhance their athletic performance [16]. Additionally, another study found a significant proportion of fitness athletes in Kashan gyms (57.9%) reported using dietary supplements [2]. The primary motivations for supplement use were to accelerate muscle repair after exercise (69.5%) and to improve overall performance (41.8%). Among a group of 164 young German elite athletes, aged between 10 and 25 years, dietary supplement use was estimated at 80% [17].

The global market for dietary supplements continues to expand not only in sales volume but also in the diversity of products offered to consumers. This growth is especially pronounced among gym-users and athletes, who exhibit a notably high rate of supplement use. Published literature consistently highlighted this trend, revealing that elite athletes tend to consume supplements more frequently than their non-elite peers [18]. However, the appeal of dietary supplements is rapidly spreading beyond the professional sphere, with increasing popularity among the general population—including gym users who may lack adequate knowledge about these products. A systematic review study also revealed a high rate of

supplement use among gym-users, with the internet and media serving as the primary sources of information [19]. The most commonly cited reason for taking supplements was to improve overall health, while protein supplements emerged as the most frequently used type.

The socio-demographic analysis of the study group indicated a predominance of female participants (68.96%), which may be linked to a greater willingness to engage with the questionnaire. However, certain studies report a higher prevalence of supplements use among female athletes [20, 21].

Regarding supplement types, our study found that products containing vitamins and minerals were the most frequently consumed, with 74.57% of participants reporting their use. Amino acid and protein-based supplements were used by 30.50% of individuals, while 13.55% reported using supplements containing creatine. These findings align with existing literature, which also identifies vitamin C, vitamin D, omega-3 fatty acids, whey protein, and L-carnitine as among the most commonly used supplements [2, 16].

In terms of awareness of the risks associated with supplement use, the majority of users stated that they were not familiar with these risks. Thus, the findings are consistent with other data published in the literature. A recent study revealed that more than 60% of users are unaware of the risks or believe that dietary supplements have no adverse effects [2].

Regarding reported adverse effects, these were most frequently observed in the ENT (ear, nose, and throat) area, such as dry mouth reported by 75% of users. Gastrointestinal symptoms included abdominal pain and anorexia (57.7%), which would signal an anorexigenic effect associated with possible adulteration. Neurological effects such as headache and dizziness were also mentioned, along with psychological responses like agitation or increased energy levels.

Literature data associate the use of creatine with minor side effects such as gastrointestinal discomfort; skin rashes, and headaches were occasionally reported. However, due to limited long-term data, the potential effects of extended creatine use remain unclear [22, 23].

Statistical analysis revealed that the consumption of amino acid and protein products correlates with a shorter time to desired effects (weight loss and muscle gain). Amino acids and proteins are essential components for optimal athletic performance and recovery. As fundamental building blocks of proteins, amino acids—along with proteins—play a key role in supporting muscle repair, facilitating growth, and maintaining overall health. Amino acids play a fundamental role in repairing muscle tissue that is stressed or damaged during physical activity, while also supporting the development of new muscle mass [24-26].

A strong and statistically significant positive correlation was observed between the incidence of headache and dry mouth. This relationship may indicate that both symptoms are adverse reactions associated with the same type of supplement, potentially pointing to issues of adulteration. One notable example is sibutramine—an anorectic agent withdrawn from the market due to its association with increased adverse events in individuals with cardiovascular conditions. Known side effects of sibutramine include headache, dry mouth, constipation, and insomnia. Numerous studies have documented its presence as an adulterant in dietary supplements marketed to athletes [14, 27-29].

CONCLUSIONS

This research highlights the utility of questionnaires as an effective method for data collection among both amateur and professional athletes. These instruments demonstrated notable advantages, including ease of access, simplicity in completion, and their capacity to rapidly yield substantial volumes of structured data suitable for research purposes. The results highlight a high prevalence of dietary supplement consumption among young people,

often without prior medical consultation. Although the general perception of the desired effects is predominantly positive, adverse reactions are frequently reported and require a responsible approach. Furthermore, the resulting data offer suggestion for issuing recommendations to athletes and governing bodies regarding the appropriate use of dietary supplements. The findings of the study highlight a need to enhance awareness and educational efforts regarding dietary supplement use, particularly among young athletes. Encouraging informed decision-making, fostering an understanding of balanced nutrition, and advocating for professional guidance can significantly reduce the risks linked to supplement consumption and promote safer, more responsible habits within the vulnerable age groups.

Conflicts of Interest

The author declares no conflict of interest.

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The Implications of Combined Hormonal Contraceptives in Cancer



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Abstract

1. Background/Objectives: Combined hormonal contraceptives (CHCs) are widely used by women during their fertile years. Often, these pills are taken without medical advice, which can lead to certain unwanted effects. This class of medication has been studied over the years for its impact on various types of cancer. Since cancer is becoming an increasingly common condition, understanding the factors that contribute to its development is important for prevention. **2. Methods:** This paper reviews recent literature from reputable sources such as PubMed, Google Scholar, ScienceDirect, and Scopus. **3. Results:** The review of the medical literature reported evidence about the risk of CHCs in developing breast and cervical cancer, and more, about the protective effect on ovarian, endometrial, and colorectal cancer. **4. Conclusion:** This paper presents the risk/benefit balance of CHCs in terms of their impact on some cancerous pathologies. According to the analysis, we can state that CHCs are effective and even safe if administered appropriately and according to the recommendations of specialists, depending on the particularities of each patient.

Keywords: oral contraceptives, cancer, risk, benefit

INTRODUCTION

Nowadays, oral contraceptive pills (OCPs) are widely used, especially combined hormonal contraceptives (CHCs) [1].

Three types of oral contraceptive pills are known: progesterone-only, combined estrogen-progesterone, and extended-release pills. Estrogen is the hormone that stabilizes menstrual bleeding, while progesterone prevents pregnancy.

The mechanism of action of progesterone is to inhibit follicular development, which prevents ovulation [2]. Through negative feedback acts on the central nervous system, in the hypothalamus, leading to a decrease in the secretion of follicle-stimulating hormone and luteinizing hormone. Therefore, if the follicle does not develop, estradiol is not produced. In addition, progesterone can stop sperm from entering the upper genital tract.

Similarly, estrogen, through negative feedback on the pituitary gland, inhibits follicular development, with slowed secretion of follicle-stimulating hormone [3].

OCPs are prescribed to women to prevent pregnancy; however, they can also be indicated in menstrual disorders, polycystic ovary syndrome, endometriosis, and some cancers [4, 5]. The administration of OCPs should be done with caution, as there is evidence that these pills may induce a higher risk of cardiovascular disease among women who use them, especially those with other comorbidities [6-8].

Oral contraceptives are widely used by women between 15 and 44 years of age, but due to adverse reactions that occur especially in people at risk, other methods of contraception can also be used. These methods are chosen depending on the co-administered medications, comorbidities, and family history [3].

The first clinical trials of OCPs were conducted in South America in the 1950s when contraception was illegal. In 1957, the FDA introduced the first pill (mestranol 150 µg/norethynodrel 10 mg) for use in menstrual disorders, and a few years later, it was also used for contraceptive purposes [9, 10].

CHCs are classified by the WHO as class 1 carcinogens [11]. Thus, the possible relationship of combined contraceptives with the development of cancer has raised concerns among women and scientific researchers. Estrogens and progesterones can induce cell growth, which can also lead to the proliferation of cancer cells [12].

To date, there is no clear research showing the risk of oral contraceptives in the development of cancer. However, some studies show that continuous use of contraceptives may increase the risk of cancer, especially of the breast and cervix, compared to women who do not use oral contraceptives [13, 14]. However, on the other hand, CHCs may reduce the risk of malignancy in the endometrium and ovaries [15, 16].

Aim and objectives

Due to the intense use of combined oral contraceptives among women of childbearing age, this study aims to highlight the influence of oral contraceptives on different types of cancer to prevent certain risks for women.

MATERIALS AND METHODS

This research was conducted through a systematic review of the specialized literature on combined oral contraceptives, with a focus on their influence on cancer pathologies. Current clinical trials were extracted from scientific databases, including PubMed, Google Scholar, ScienceDirect, and Scopus. Articles were selected according to a series of keywords

such as: contraceptives, combined oral contraceptives, cancer and contraception, cervical cancer, breast carcinoma, endometrial and ovarian cancer, and colorectal carcinoma.

For methodological transparency and clarity in identifying and selecting relevant studies, the review process of this study followed the PRISMA 2020 (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines. The PRISMA framework was adapted to benefit from a structured search. The process of identifying and selecting studies is represented in a flow diagram (Figure 1).

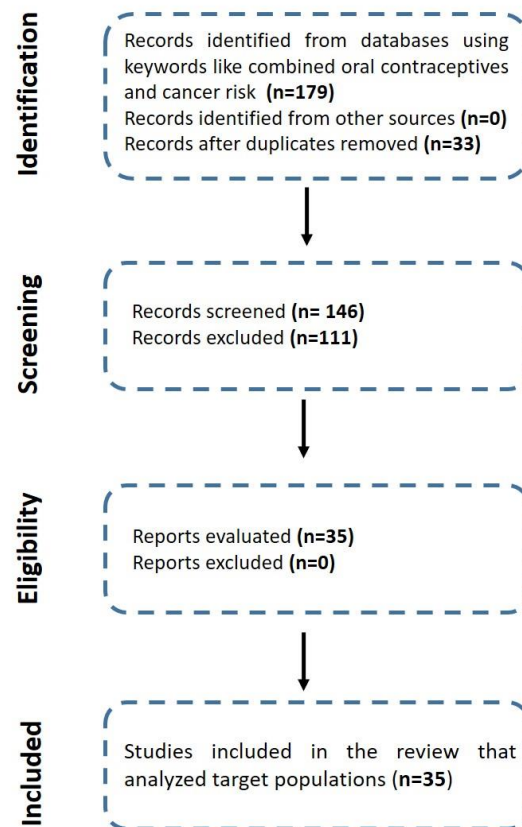


Figure 1. PRISMA 2020 flow diagram for the review

Inclusion criteria were based on prospective, retrospective, and meta-analyses studies that analyzed the influence of CHCs on the development of cancer in women of different ages. Reviews and case reports were excluded. Initially, 179 scientific articles were observed using the keywords, and of these, 35 studies met the criteria and were within the scope of this work.

RESULTS

The benefits and risks of CHCs vary depending on the region or area, with the balance influenced by disease incidence and maternal mortality rates. In underdeveloped nations with high maternal mortality, the use and effectiveness of contraceptives in preventing pregnancy are especially important. Furthermore, even within the same country, the benefits and risks differ for various groups of women. As a result, the balance shifts, with differences seen between smokers and non-smokers as well as between young and older women [17].

The evidence supporting the link between CHCs and cancer is derived from observational studies, including case-control studies and prospective cohort studies. However, these studies cannot show exactly whether oral contraceptive use increases or

reduces the risk of cancer, as there may be differences between the women in the study, which may also influence the development of cancer. This research provides ample evidence that women who use CHCs have a higher risk of developing breast or cervical cancer, while the risk is reduced for ovarian, endometrial, and colorectal cancer [18-20].

Risks of CHCs

Breast cancer

The main concern with CHCs is related to the development of breast cancer.

Breast cancer is the second most common cause of death, being one of the most diagnosed pathologies in women [21].

Sex hormones are factors that can increase the risk of breast cancer, especially in postmenopausal women who are undergoing hormone replacement therapy. Estrogen and progesterone promote the growth of breast tissue by stimulating the proliferation of stem cells [22]. Additionally, estradiol and estrone can damage genes, having a mutagenic potential [23].

Studies have indicated that women who have used CHC recently or are currently taking the pill have about a 24% higher risk of developing breast cancer compared to women who have never used it. The risk of developing cancer decreases after stopping contraceptives and returns to normal after 10 years [24].

An analysis that included 54 epidemiological studies (25 countries) with over 150.000 participants showed that women who used CHCs had a 7% risk of developing breast cancer compared to women who did not use CHCs [25].

Other studies that investigated over 110,000 nurses aged 24 to 43 also reported a risk of breast cancer among those who used contraceptives [26-28].

The same risk was also recorded in a 2017 Danish prospective study with newer contraceptives, reporting a 20% increase in cancer risk [29].

We can, however, state that the higher risk was given by a certain contraceptive, the "triphasic" oral pill, where the dose is released during the menstrual cycle in three stages [30].

More recently, in 2021, a prospective cohort study showed a higher risk for women using CHCs compared to those who do not; these risks were no longer present five years after quitting [31].

The risk of developing breast cancer ranged from 0% to 60% depending on the duration of administration and the contraceptive used.

Table 1 summarizes the studies that reported an increased risk of breast cancer with CHCS use.

Table 1. Relevant studies regarding the risk of breast cancer with CHCs use

Study design	Population	Results	Observations	References
Meta-analysis (54 studies, 25 countries)	53.000+ women	RR 1.24 (CI 95%: 1.15–1.33)	The risk ⁺ after stopping: RR 1.16 (1–4 years), RR 1.07 (5–9 years), nil after 10 years. It does not matter the type or duration of CHCs. The tumors detected were less advanced.	[25]
Prospective cohort study	121.577 women	RR 1.26 (95% CI: 1.09–1.46) for use ≥5 years	Association with increased breast cancer mortality in long-term users	[28]
Prospective cohort study	1.8 million women, tracked for 11 years	RR 1.19 for current or late CHC users; RR 1.20 for any hormonal contraception.	Risk ⁺ with duration of use. 1 additional case/7,690 women/year; <35 years: 1/50,000/year	[29]
Prospective cohort study	113.187 women	HR 1.31 (95% CI: 1.09–1.58) for current users	Former users: similar risk to non-users after 5 years of stopping	[31]

RR: Risk ratio; HR: Hazard ratio; CI: Confidence interval

Research results suggest that the breast cancer-inducing effect appears to be temporary or limited to recent or long-term use of CHCs.

However, in several prospective cohort studies, neither continuous use nor previous long-term use of CHCs indicated an increased risk of breast cancer [14, 32].

Furthermore, some data support that low concentrations of current preparations do not increase the risk of breast cancer in women with BRCA1 and BRCA2 genetic mutations [33].

Cervical cancer

Cervical cancer is one of the most diagnosed malignant pathologies in women and ranks 4th in the ranking of causes of death [12].

Women who have used combined oral contraceptives for ≥ 5 years have an increased risk of developing cervical cancer compared to women who have not used them. In one study, it was shown that the duration of use of CHCs influences the risk of cervical cancer. Therefore, a 10% increase in risk was reported when they were used < 5 years, while a 60% increase was recorded for use for 4-9 years, and use ≥ 10 years, the risk doubled [34].

In an EPIC cohort study, conducted over 9 years with over 300,000 women, the link between hormones and cervical cancer was investigated [35]. According to previous studies, it has been shown that the risk of cervical cancer increases with long-term use of CHCs.

A similar conclusion was reached in a Danish study of women of childbearing age (two million participants) who were not vaccinated against HPV. It was reported that the risk was the same for both squamous cell carcinoma and adenocarcinoma [36].

However, in all cases, this risk of developing cervical cancer decreases once the use of CHCs is stopped [15, 35, 37].

There is evidence to suggest that the use of CHCs may increase cervical vulnerability to HPV infection or alter the progression of malignant and premalignant lesions.

Thus, the hormones used in CHCs enhance the expression of HPV 16 E6 and E7 oncogenes, leading to damage to the p53 tumor suppressor gene and increasing the ability of viral DNA to promote neoplasticity [37-40].

Benefits of CHCs

Studies have shown several ways in which CHCs may reduce the risk of certain types of cancer, including: i) decreased ovulation, which reduces exposure to natural female hormones, in the case of ovarian cancer; ii) inhibition of endometrial cell proliferation, in the case of endometrial cancer; iii) decreased bile acids in the blood following the use of oral conjugated estrogens, in the case of colorectal cancer [20].

Ovarian cancer

Ovarian cancer is the 8th most diagnosed cancer and the 5th leading cause of death in women [41].

On the other hand, long-term use of CHCs reduces the risk of developing ovarian cancer. Numerous studies suggest their protective effect. Therefore, the longer CHCs were administered, the lower the impact of ovarian malignancy, with even a protective effect, and a decrease of up to 50% of the cancer risk [42-44].

A large analysis claims that contraceptives have prevented 200.000 ovarian cancer lesions and 100.000 deaths caused by this type of cancer [44].

The protective action intensifies with increasing duration of contraceptive use [45] and can continue for up to 30 years after women stop taking CHCs [46].

This effect has also been recorded among women who carry a mutation in the BRCA1 or BRCA2 gene [47-49].

Endometrial cancer

Endometrial cancer is also an important cause of morbidity and malignancy among women [50].

CHCs possess protection against endometrial malignancy. Women who have used and are using CHCs have a lower risk of endometrial cancer than those who have not used them, unlike previous cases, where they induce breast and cervical cancer [45].

Research has shown that the longer the use of CHCs, the lower the risk of endometrial cancer. Thus, after 10-15 years of administration, the risk of neoplasia decreases by approximately 50%. Moreover, after discontinuation of use, the protective effect persists for 30 years, suggesting that there are no individual characteristics that influence it [51].

Moreover, an NIH-AARP prospective study showed that women who used contraceptives, smoked, and were obese had a low risk of developing endometrial cancer [45].

Several studies support the protective action of the pill that persists for many years after stopping CHC treatment [15, 51, 52].

Colorectal cancer

Colorectal cancer is common among women, but it has a good survival rate. IARC has found that the use of oral contraceptives may have a positive impact on reducing the risk of colorectal cancer [12].

A meta-analysis of 29 studies found a relative risk of colorectal cancer for long-term use versus uncontrolled use of 0.8. A study in which the duration of CHC use was inversely associated with a decrease in risk, without influence of dose [53].

In another meta-analysis of 23 cohort and case-control studies, the relative score was similar at 0.8. This study did not examine whether there was a relationship between duration of use and risk, but showed that recent use was more beneficial [54].

Iversen et al. demonstrated that the protection against colorectal cancer by CHC use could be greater than 35 years [15].

Therefore, there is various research that states that the use of CHCs does not induce a high risk of colorectal cancer, and even presents a lower risk by up to 20% [15, 52, 53, 55-58].

In addition, in a cohort study of 1.3 million women, conducted over 13 years, it was shown that the use of CHCs is associated with an increased risk of anal cancer, where HPV may have an influence, similar to cervical cancer [59].

Figure 2 shows the benefit/risk balance regarding the influence of combined oral contraceptives on the most common cancers in women.

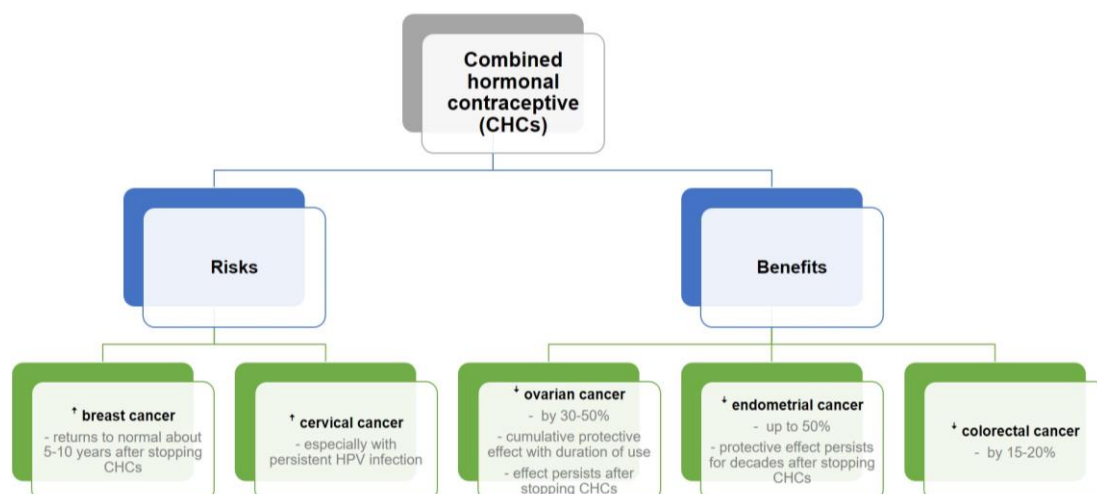


Figure 2. Risk/benefit balance of CHCs

DISCUSSIONS

The role of combined oral contraceptives in cancer development has been widely discussed in the medical literature since the 1970s [60, 61].

In 1979, the International Agency for Research on Cancer (IARC) reported that estrogen and progesterone might influence the differentiation, development, and growth of various tissues in both humans and animals. Additionally, laboratory animal studies have demonstrated that pregnancy, surgical removal of endocrine glands, or external steroid administration can alter the hormonal environment and may either increase or, in some cases, decrease the occurrence of cancerous lesions [62].

Because CHCs are synthetic versions of female hormones, they can increase the risk of carcinoma, especially types of cancer that express receptors for estrogen and progesterone, such as breast cancer [63].

In 2012, the same IARC reported that the use of CHCs may increase the risk of breast cancer in women of childbearing age who have recently used or are currently using the pill. CHCs may also raise the risk of developing cervical and liver cancer with longer treatment duration, and this risk may decrease after stopping therapy. The risk of developing cervical cancer may be due to changes in the susceptibility of cervical cells to infection with high-risk HPV.

Additionally, there has been discussion about a protective effect of combined contraceptives; this effect is attributed to the potency of progesterone and may be reduced by the strength of estrogen. CHCs may lower the risk of developing colorectal cancer and ovarian cancer, depending on how long they are used, and may also influence the risk of skin, pancreatic, lung, or thyroid cancer [64].

A meta-analysis examining the cancer risk in adult women (20-54 years) in the US who use CHCs highlighted these points. Therefore, over 8 years, for every 100,000 CHCs users, the estimated number of additional or reduced cases per 100,000 women was +125 (cervix), +151 (breast), -193 (ovarian), and -197 (endometrium) [65].

Given that numerous large-scale studies have consistently demonstrated a considerable increase in risk among current and recent users of CHCs, breast cancer has garnered the most attention in this context [25, 29]. After stopping, this increased risk steadily decreases and seems to revert to baseline in around ten years. Crucially, the absolute risk increase is minimal, especially for younger women, whose incidence of breast cancer is typically low.

Cervical cancer and CHCs have a more complicated relationship. Long-term usage, especially after five years, may raise the risk of invasive cervical cancer in women who have a history of oncogenic human papillomavirus infection [66, 67]. Although this risk seems to decrease after stopping, it is still a worry in areas with low HPV immunization and screening rates. The increasing use of immunization programs may lessen this association's significance for upcoming generations.

CHCs, on the other hand, have a remarkably protective impact against endometrial and ovarian malignancies. According to extensive collaborative assessments, using CHCs lowers the incidence of ovarian cancer by between 30 and 50 percent, and the protection lasts for decades after stopping [44]. According to Iversen et al. [15], there is a 50% reduction in the risk of endometrial cancer, with long-lasting advantages even for women who stop using it years early. Given the high mortality and usually delayed presence of ovarian cancer, as well as the increasing global incidence of endometrial cancer, these preventive benefits are especially pertinent.

Colorectal malignancies are less examined. Research points to a slight decrease in the risk of colon cancer in CHC users, which may be due to hormonal effects on inflammation and bile acid metabolism [67].

CHCs provide a heterogeneous cancer risk profile when combined. They significantly lower the burden of ovarian and endometrial cancers while moderately increasing the incidence of breast and cervical cancers. They may also provide extra protection against colorectal cancer. From the standpoint of public health, modeling studies indicate that the number of cancers averted frequently surpasses the number that are generated, particularly in populations with high rates of HPV vaccination and cervical cancer screening. However, a woman's lifestyle, family history, genetic predisposition, and access to preventative care can all affect how risks and benefits are balanced for her.

Two important risk factors are the age at which CHC use was initiated and the duration of administration. Current and recent users, particularly those under 35 years of age, are at the highest risk of breast cancer; however, this risk decreases after stopping use and returns to baseline within approximately ten years [15, 29, 44]. On the other hand, the preventive effect against ovarian and endometrial cancer increases with longer duration of use and lasts for decades after stopping use. These divergent durations highlight the importance of age-specific counselling and reproductive goals and demonstrate the dynamic nature of CHC-associated cancer risk.

The overall effect of CHCs is also influenced by socioeconomic and global conditions. The protective benefits could result in significant mortality reductions in high-income nations where screening for ovarian and endometrial cancer is common. The possible rise in cervical cancer risk, on the other hand, might be more significant in low- and middle-income nations, especially those with inadequate access to cervical screening and HPV vaccination [66, 67]. These differences highlight the necessity of context-specific recommendations and public health initiatives that strike a balance between cancer prevention and access to contraception.

The risk-benefit ratio is influenced by genetic predispositions. Although the possible effect on breast cancer risk should be carefully considered, women with BRCA1/2 mutations, who have an increased baseline risk of ovarian cancer, may benefit greatly from CHCs [68, 69]. Similarly, CHC-mediated endometrial protection may be disproportionately beneficial for women with Lynch syndrome or substantial family histories of endometrial or colorectal malignancies. Nevertheless, there is still a dearth of information on genetically predisposed groups.

As demonstrated by this comparative analysis, the effects of CHC use on cancer are neither consistently detrimental nor consistently protective. Rather, the total effect is the result of a balancing act between conflicting effects, mediated by patient-specific characteristics, pharmaceutical formulation, and length of administration. The long-term impacts of more recent contraceptive formulations, the mechanisms behind tissue-specific outcomes, and the integration of these discoveries into tailored contraceptive counseling will all require further research.

Globally, CHCs are some of the most widely used medications. In addition to their main therapeutic utility in preventing unwanted pregnancy, the use of CHCs can have both a positive and negative impact on the development of various types of cancer. However, with the evolution of the medical field, there are new alternatives that can influence this risk. Pharmaceuticals with very low doses of estrogen or alternative molecules (estetrol) or lower affinity for receptors may reduce systemic side effects, thereby reducing the carcinogenic potential [70]. Different receptor affinities between progestins impact cancer risk. New progestin molecules are improved in terms of tissue selectivity and efficacy [71]. Also, the class of selective progesterone receptor modulators (SPRMs) confers tissue-selective action. Therefore, these compounds may offer, in addition to endometrial and ovarian protection, a

reduction in the risk of breast cancer [72]. Research on alternative contraceptive methods, like compounds that block sperm function, is advancing. Non-hormonal options can remove the cancer risk associated with hormonal methods, but they lose the protective effects on the endometrium and ovaries [73]. Another category includes novelty, intrauterine devices that selectively eliminate SPRMs or vaginal rings with very low doses that reduce systemic exposure to hormones and maintain protective and local contraceptive action [74].

Oral contraceptives expose the dual side of hormonal therapy: they decrease the risks of some types of cancer while increasing others. Therefore, innovations such as selective receptor modulators, non-hormonal agents, and local administration may lead to a balance. In the future, personalized contraceptive options are needed that offer increased benefits and decreased risks, and monitoring of long-term safety remains a priority.

CHCs have been researched for more than 50 years, and their effect on cancer risk is still a major focus of studies on reproductive health. The database is still characterized by uncertainty, despite strong evidence showing protective effects against endometrial and ovarian malignancies as well as a moderate and typically temporary increase in the risk of breast cancer. To understand and improve patient care, several critical research gaps need to be addressed.

The long-term safety of contemporary contraceptive formulations, elucidation of progestin-specific effects, inclusion of genetically and ethnically diverse populations, a better understanding of mechanisms, integration with other preventive strategies, and a systematic evaluation of novel contraceptives are among the most urgent research gaps. By addressing these ambiguities, risk estimates will be improved, safer and more customized contraceptive options will be supported, and the body of evidence supporting reproductive health care will eventually be strengthened.

CHC's understanding is further limited by the lack of diversity in the populations studied. Women of European descent constitute the majority of the current evidence, which comes mainly from high-income countries [15]. Data on non-European groups, as well as on women with genetic predispositions such as BRCA1/2, remain limited and sometimes contradictory [69]. To provide equitable and globally applicable contraceptive advice, research needs to be expanded to encompass a diverse range of populations.

Long-term oncological risks are given less consideration in current pharmacovigilance frameworks, which place more emphasis on acute adverse events like venous thromboembolism [75]. To assess oncological safety over time, it would be beneficial to link prescription registries with cancer outcomes and promote international partnerships.

The primary purpose of oral contraceptives is to prevent pregnancy. The specialist doctor should advise the woman on the best alternative, including the risks and benefits of the pills, as well as their other non-hormonal effects, especially if a woman has comorbidities that could increase her risk when using CHCs. Additionally, pharmacists should work with doctors to ensure patients receive the most effective and affordable treatment.

Pharmacists should also advise patients on adverse reactions, the correct dosage and use of CHCs, and most importantly, how to handle missed doses and other backup methods of contraception. Therefore, the team of professionals can achieve optimal results for women with maximum benefits of contraceptive therapy [3].

CONCLUSIONS

For decades, the carcinogenicity of oral contraceptives has been intensively analyzed. The research conducted reports the risk of breast and cervical cancer after long-term administration of CHCs. However, the long-term protective effect on ovarian, endometrial, and colorectal cancer has also been exposed. The carcinogenic effects of CHCs are reversible

and can be reduced by changing the lifestyle (physical exercise, smoking, breastfeeding, HPV vaccination).

Patients who use oral contraceptives should be warned about their possible carcinogenic action, in addition to their effective contraceptive effect. It is necessary to discuss the balance of benefits and risks and to choose the safest option for the woman. Updated and clear information can strengthen the patient-medical staff relationship, which will lead to the reduction of adverse effects and effective treatment.

Several factors, including age, length of usage, genetic background, tissue-specific mechanisms, and the global health context, influence how CHCs impact cancer. Although the balance is dynamic and unique to each individual, the protective effects against ovarian and endometrial cancers frequently outweigh the moderate rise in breast and cervical cancer risk. Future studies should combine mechanistic findings, incorporate genetically and ethnically diverse populations, prioritize long-term safety studies of newer contraceptive formulations, and assess results in light of changing preventive measures like cancer screening and HPV vaccination. Women around the world will be able to receive more accurate, fair, and knowledgeable contraceptive counseling because of such initiatives.

Following the existing studies, we can state that CHCs are effective and safe, but they must be administered according to the recommendations of specialists.

Conflicts of Interest

The authors declare no conflict of interest.

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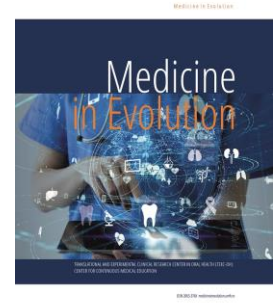
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The Internal Thoracic Artery: An Anatomical Narrative Review

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Abstract

The internal thoracic artery (ITA) is a paired vessel originating from the first part of the subclavian artery, descending along the inner thoracic wall in close relation to the sternum. Owing to its reliable caliber, predictable course, and favorable long-term patency, the ITA plays a critical role in both clinical anatomy and surgical practice, particularly in coronary artery bypass grafting. This review synthesizes current anatomical knowledge of the ITA, emphasizing its origin, course, branching patterns, and collateral circulation. Variations in its trajectory, branching morphology, and termination are examined in detail, along with rare anomalies such as duplication and complete absence. Surgical relevance is addressed, with a focus on harvesting techniques, the impact of competitive flow, and strategies to manage side branches. Nomenclature is briefly discussed, noting the adoption of "internal thoracic artery" as the primary term. By integrating findings from cadaveric, radiological, and surgical studies, this review highlights the anatomical variability of the ITA and its implications for thoracic, cardiovascular, and reconstructive procedures. Understanding these variations is essential for optimizing surgical outcomes and reducing perioperative complications.

Keywords: internal thoracic artery, anatomical variations, coronary artery bypass grafting, surgical anatomy

INTRODUCTION

The internal thoracic artery (ITA), also known as the internal mammary artery, represents a paired artery situated on the anterior wall of the thoracic cavity, coursing along the lateral side of the sternum [1] (Figure 1). It supplies not only the chest wall, but also the breasts, mediastinum, pericardium, and thymus [1]. The ITA has been intensively studied due to its indispensable surgical application in coronary bypass surgery with ITA grafts, and it's currently referred to as the optimum conduit for coronary artery bypass grafting, based on its long-term results [2-7]. Clinical aspects should also be brought to attention, as the ITA adjusts its lumen's dimensions in post-ductal coarctation of the aorta, resulting in the dilatation of the artery due to the increased pressure proximal to the narrowing [8, 9]. Therefore, the objective of this review is to provide a comprehensive synthesis of the anatomical variations, clinical significance, and surgical applications of the ITA.

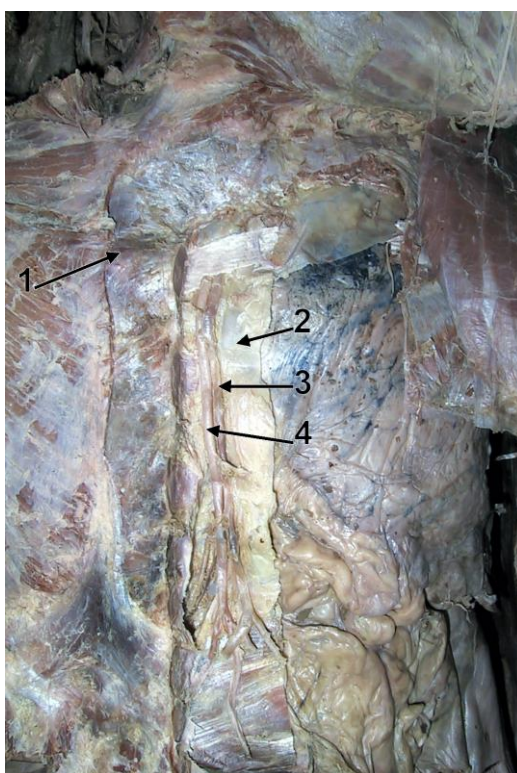


Figure 1. Original dissection of the left internal thoracic vessels. Human adult cadaver, anterior view. 1. sternal angle; 2. left anterior costomediastinal pleural recess; 3. left internal thoracic vein; 4. left internal thoracic artery.

Nomenclature

The term “internal thoracic artery” has been preferred in this study, instead of the former term “internal mammary artery”, as it is considered in the literature to be more appropriate because it provides a precise, identifying topography of the vessel [10-12]. In the second edition of the Terminologia Anatomica, the former was designated as the primary term, while the latter was listed as its English synonym [13]. The term “mammary” has been largely abandoned, as it misleadingly implies a predominant vascular supply to the mammary gland. In contrast, the artery's primary distribution includes the anterior thoracic wall, sternum, and diaphragm [1]. In addition to “internal thoracic artery,” other alternative denominations have been historically used, such as Vineberg artery, referring to its role in Vineberg's pioneering indirect myocardial revascularization procedure [14], and the sternal or

parasternal artery, emphasizing its course parallel to the sternum [15]. However, the modern consensus favors ITA for its anatomically precise and descriptive value, aligning with current international anatomical terminology.

ORIGIN OF THE INTERNAL THORACIC ARTERY

The subclavian artery (SA) is a large-diameter blood vessel that supplies the upper limb, parts of the neck, and the head. The anatomical rapport with the anterior interscalene muscle, which passes anteriorly, divides the SA into three topographic parts: prescalene, scalene, and postscalene parts of the SA. While the 2nd and 3rd regions of the SA are, hypothetically, alike, the 1st region varies on each side [16]. The ITA commonly arises from the first portion of the SA [16-19]. However, it is subject to abundant anatomical variations. The left ITA (LITA) was reported to originate from the first part of the SA (92%), the second part (7%), and also the third part (1%), directly from the SA in 70% of the cases. In comparison, the remaining 30% was seen to derive from a common trunk with other arteries, such as suprascapular, transverse cervical, ascending cervical, inferior thyroid, and thyrocervical trunk [11, 19, 20]. Calafiore et al. [21] reported a higher incidence (36.33%, 109 out of 300 cases) of ITA origin conjoint with other branches of the SA. A 3rd region origin of the right ITA (RITA) is considered “abnormal” or “unusual” [22], as it has been seldom detected among studies, with a frequency rate of 0.83% [23], 0.78% [17], and 0.5% [24]. Bilateral 3rd part origin of the ITA was observed, on one occasion, in a 25-year-old female cadaver, during routine dissection [25]. Such an anatomical variant, however, is exceptional, as the literature lacks information regarding its frequency.

The ITA emergence from the axillary artery was first described in 1844 by Quain et al. [16]. They also reported the highest incidence of 3rd portion origin ITA at 2%. Anomalous origins have been previously reported. A LITA deriving directly from the aortic arch, with no anatomical correlation to the SA [26], was revealed via cardiac CT angiography in a 67-year-old subject. At the same time, an additional case showed a LITA emerging from the lateral junction of the left SA and the aorta [27]. Another LITA was seen arising from an aberrant left vertebral artery, which originated directly from the aorta, distal to the left subclavian artery [28]. These findings highlight the remarkable diversity in ITA origin and reinforce the importance of detailed preoperative vascular assessment. Even rare variants may carry significant surgical and interventional implications, particularly in cardiothoracic and vascular procedures, where unanticipated arterial patterns can complicate access, alter graft planning, and increase the risk of iatrogenic injury if not correctly identified in advance.

BRANCHES OF THE INTERNAL THORACIC ARTERY

The ITA gives rise to three main groups of branches according to their anatomical origin [1]. The anterior branches include the anterior intercostal arteries, perforating branches, and medial mammary arteries, which primarily supply the anterior thoracic wall and breast. The posterior branches consist of the mediastinal, thymic, pericardiophrenic, sternal, bronchial, and tracheal branches, providing vascularization to deeper thoracic structures, including the mediastinum, thymus, pericardium, sternum, bronchi, and trachea. Finally, the terminal branches represent the artery's distal bifurcation, continuing to supply the anterior abdominal wall and diaphragm [1, 19, 29-32]. The ITA can also be classified by its supplied territories into sternal, intercostal, and perforating branches, which respectively provide the sternum, intercostal spaces, and overlying soft tissues, including the mammary glands [1, 33]. It bifurcates anywhere between the fifth rib (around the third rib in fetuses [34]) and the seventh intercostal space [19] into the superior epigastric and the musculophrenic arteries.

The superior epigastric artery (SEA) supplies the anterior abdominal wall, the upper rectus abdominis muscle [35], and has been widely studied for the use of the SEA perforator flap in plastic reconstruction of the anterior abdominal/chest wall [36, 37]. The musculophrenic artery provides oxygenated blood to the diaphragm, the lower pericardium, and the lower intercostal spaces [1]. Less frequently, there were reported cases illustrating trifurcation of the ITA, with a 3rd ramus supplying the inferior aspect of the xiphoid cartilage, being termed as the xiphoid branch [30, 31], or even the diaphragmatic branch [19].

Appreciating the branching complexity of the ITA reveals its dual nature of structural consistency and subtle variation, with implications that extend beyond textbook anatomy. Rare branches, such as xiphoid or diaphragmatic rami, though infrequently encountered, may become critical in specific surgical contexts, offering alternative vascular supply or introducing unexpected anatomical challenges. A thorough understanding of these patterns is therefore essential for maximizing surgical precision and minimizing intraoperative risks.

DUPLICATION AND PARTIAL DUPLICATION OF THE INTERNAL THORACIC ARTERY

A LITA originating from the 3rd part of the SA was reported to bifurcate promptly after emergence [38]. Thus, two separate branches, lateral and medial, were observed descending through the thoracic cavity. The lateral one became the musculophrenic artery, whereas the medial ramus turned into the superior epigastric artery. Both vessels had a reduced diameter (less than 1.5 mm), compared to the usual diameter of an ITA (generally 2.5 mm) [39, 40]. Such thin ITAs may, therefore, represent a risk and instead an inadequate option for performing coronary artery bypass grafting (CABG). Out of 240 examined ITAs, only two were seen bifurcating, which is less than 1%; however, no additional data were reported [41]. Another case [42] presented a RITA dividing into two branches at the level of the 2nd intercostal space, with a stepladder pattern of anastomosis existing between the two. Each ramus provided additional arterial branches and ceased as the two common terminal branches of the ITA: the medial one continued as the SEA, while the lateral one as the musculophrenic artery. A case of bilateral, partially duplicated IMAs, at the level of the 1st and 2nd costal cartilage, was previously reported [43]. Even though incompletely duplicated arteries represent an interesting anatomical feature, here, the term “partially” is used without coherence. A misjudgment may be the cause of describing the same case, both as “duplication” and “partially duplicated”. No distinction was made between the two terms, which leads to further confusion, as the case cannot be adequately categorized.

These anatomical reports emphasize both the exceptional rarity and the potential surgical implications of bifurcating or duplicated ITAs. Variants with reduced caliber, early branching, or unclear morphological classification may compromise their viability as grafts in CABG and complicate reconstructive planning. Furthermore, the inconsistent use of terms such as “duplication” and “partial duplication” without precise anatomical distinction highlights a gap in the literature, pointing to the need for a standardized nomenclature to improve clarity and comparability in future studies.

MORPHOTOPOGRAPHIC CHARACTERISTICS OF THE INTERNAL THORACIC ARTERY

Following its emergence from the inferior aspect of the subclavian artery, ITA travels posterior to the brachiocephalic vein [1, 44]. It then adopts a descending course, on the anterior wall of the rib cage, deep to the first six anterior costal cartilages, parallel to the sternum, at a mean ITA-to-sternum distance of almost 15 mm [43, 45-47]. ITA gradually

decreases in size as it traverses the thoracic cavity, without any significant dimension adjustment between left and right vessels [43, 46, 47]. Its average length is about 20 cm, and bifurcation at the sixth intercostal space occurs in over 90% of cases [19].

The diameter of the ITA has significant clinical significance. It is essential in the CABG and free tissue transfer for breast reconstructive surgery, as the artery should be of sufficient caliber and readily accessible for anastomoses [43]. Many values have been reported in the literature, as follows: Delmotra et al. [20] reported a mean of 2.6 mm on the left and 2.8 mm on the right, with a maximum observed value of 4.0 mm. Karaman et al. [43] observed the diameter in two specific points, at the origin and the level of the tracheal bifurcation. The difference between the measurements was roughly 0.2 mm for each side, sizing 2.56 at the origin and approximately. 2.30 at the second position. No significant disparity was found between the right and left sides, or between genders. Hefel et al. [45] communicated smaller values, with a maximum of approximately 2.5 mm, and mean values ranging from 1.76 to 2 mm.

Histologically, the ITA is a small to medium-sized elastic artery, particularly in its proximal segment. It shows high resistance to atherosclerosis, even in older individuals. Age-related elastotic degeneration may occur, but it is often compensated for by remodeling with new smooth muscle-like cells, thereby preserving elasticity and functionality [48].

Only two studies in the literature have reported cases of a completely absent ITA. Nizanowski et al. [49] grouped both abnormal and absent ITAs, reporting a combined frequency of 11.4%. However, the actual absence of the vessel remains uncertain, as abnormal ITAs appear to be reported more frequently than complete absence [11, 23]. In another investigation, the absence of the ITA was documented in only 2 out of 400 examined cases, corresponding to a prevalence of 0.28% [17].

The morphological and histological profile of the internal thoracic artery illustrates its exceptional reliability as a vascular conduit, with consistent dimensions and remarkable resistance to atherosclerosis, which supports its widespread surgical use. While variations in caliber are generally minimal and clinically insignificant, the rare occurrence of hypoplasia or complete absence, although scarcely reported, highlights the importance of thorough preoperative assessment to anticipate and mitigate potential intraoperative challenges.

The main reported anatomical variations of the ITA are highlighted in Table 1, including their prevalence, defining features, and potential clinical relevance.

Table 1. Summary of reported variations of the internal thoracic artery: prevalence, description, and clinical implications

Variation type	Description	Reported prevalence	Potential clinical impact	References
LITA origin from 1st, 2nd, or 3rd part of SA	LITA originates from the 1st part, 2nd part, or 3rd part of the subclavian artery; 30% arise from a common trunk with other arteries.	92% (1st part), 7% (2nd part), 1% (3rd part), 30% from common trunk	May alter surgical strategy in CABG or upper thoracic procedures due to atypical origin.	[11, 16-20]□
Higher incidence of common trunk origin	LITA's origin is concurrent with other SA branches.	36.33%	It could complicate graft harvesting by limiting the mobilization of the artery.	[21]□
RITA originates from the 3rd part of SA	Considered abnormal or unusual.	0.83%, 0.78%, 0.5%	Rare trajectory may pose challenges in exposure and grafting.	[17, 22-24]
Bilateral 3rd part origin of ITA	Bilateral emergence from the 3rd part of the SA in a cadaver.	Single case reported	Extremely uncommon; may require an altered intraoperative identification strategy.	[25]□
LITA originates from the axillary artery	Reported since 1844; rare origin.	Up to 2%	Atypical course can affect surgical approach and conduit selection.	[16]□

LITA originates from the aortic arch or an aberrant vertebral artery	Origin directly from the aortic arch or an aberrant vertebral artery.	Case reports only	Significant deviation from normal anatomy may mislead imaging interpretation or operative navigation.	[26-28]□
Bifurcating ITA after origin	Early bifurcation into musculophrenic and superior epigastric arteries; reduced diameter (<1.5 mm).	<1% (2/240 cases)	Reduced caliber can render an artery unsuitable for CABG.	[38-41]□
Bifurcation at the 2nd intercostal space with stepladder anastomosis	Two rami with additional branches, ending as the SEA and musculophrenic arteries.	Single reported case	An unusual branching pattern may complicate dissection and vascular anastomosis.	[42]□
Bilateral partially duplicated ITAs	Described at 1st and 2nd costal cartilage; unclear distinction between complete and partial duplication.	Single reported case	Terminology inconsistency could hinder anatomical classification and surgical planning.	[43]□
Absent ITA	Complete absence of vessel documented.	0.28% (2/400 cases)	Absence eliminates ITA as a graft option, requiring an alternative conduit.	[17]□
Combined abnormal and absent ITAs	Grouped prevalence including abnormal courses and absence.	11.4%	Variable anatomy may necessitate preoperative imaging to prevent intraoperative surprises.	[49]□

CLINICAL AND SURGICAL CONSIDERATIONS

The ITA reveals several clinically significant anatomical variations that can influence its use in surgery, particularly in CABG and thoracic procedures. One of the more striking reports describes bilateral aberrant branches of the ITA, where accessory branches descended laterally and gave off additional intercostal branches. This rare variation highlights how unexpected arterial courses may complicate procedures such as CABG, thoracocentesis, and breast reconstruction, underscoring the need for preoperative vascular assessment [50]. From a surgical planning perspective, such aberrant branching patterns may necessitate modifications in the harvesting technique, including more proximal dissection or selective ligation of accessory branches to preserve optimal graft flow. Furthermore, their presence may explain some cases of early graft failure that remain otherwise unexplained despite technically adequate anastomoses.

Similarly, accessory ITAs have been documented in cadaveric studies, occurring in about 4–20% of cases [51]. These arteries, which can be nearly as large as the primary ITA trunk, run along the anterolateral thoracic wall and may contribute to the “steal phenomenon,” where competitive flow between grafted and accessory vessels compromises bypass outcomes [51]. The steal phenomenon is likely underdiagnosed in clinical practice, as subtle competitive flow may manifest only as late-onset angina or decreased functional capacity, rather than overt perioperative ischemia. This emphasizes the importance of integrating detailed vascular mapping into routine pre-CABG imaging, even in patients without apparent anatomical risk factors.

Another well-documented variant is the lateral costal branch, present in 15–30% of individuals, which runs parallel to the ITA and communicates with anterior and posterior intercostal arteries [52, 53]. Its identification is essential because a well-developed lateral costal artery may divert blood flow away from coronary targets following CABG, as shown in clinical cases where coil embolization was required to resolve post-operative angina [52, 53]. Given the relatively high prevalence of this branch, preoperative detection should be considered a standard practice in centers performing high volumes of CABG, particularly in patients with diffuse coronary artery disease.

Uncommon origin and duplication patterns have also been observed. For instance, a rare case of the ITA arising from the thyrocervical trunk instead of the subclavian artery was reported, which alters its proximal course and may increase the risk of vascular injury during neck procedures [54]. Routine preoperative angiographic evaluation of the ITA in patients undergoing CABG is necessary, especially those with prior mediastinal irradiation, subclavian atherosclerosis, or previous cardiac surgery [55]. The broader implementation of preoperative angiography could prevent unanticipated intraoperative difficulties, thereby reducing operative time and morbidity.

Competitive flow between the grafted ITA and its side branches compromises myocardial perfusion, sometimes necessitating corrective procedures like coil embolization [52, 53]. Unusual origins, such as ITAs arising from the thyrocervical trunk, or rare duplications of the ITA, further complicate surgical harvesting by altering expected anatomical landmarks, increasing the risk of intraoperative injury, and potentially reducing the usable graft length [38, 54]. These variations also affect sternal and chest wall perfusion, which is critical for preventing wound complications after median sternotomy in high-risk patients [51]. For these reasons, preoperative vascular mapping using angiography or duplex ultrasonography is strongly recommended to identify and assess ITA variants before surgery, allowing surgeons to optimize operative planning and reduce the risk of adverse outcomes [55]. A multidisciplinary preoperative review involving cardiac surgeons, radiologists, and anesthesiologists is advisable whenever a significant anatomical variation is detected.

The ITA poses a significant bleeding risk in cases of blunt or penetrating chest trauma, primarily when associated with sternal or rib fractures, as the vessel courses adjacent to the sternum [56]. Such injuries may lead to massive hemothorax, anterior mediastinal hematoma, or even pseudoaneurysm formation, all of which may precipitate hypovolemic or even obstructive shock due to expanding retrosternal blood collections [56]. Given the vessel's proximity to the sternum, even low-velocity trauma can result in life-threatening hemorrhage, warranting a lower threshold for advanced imaging in patients with equivocal findings but high clinical suspicion.

In hemodynamically stable patients, transcatheter arterial embolization is increasingly the first-line, minimally invasive treatment, showing success rates of around 92%, compared to about 66% for open surgery [57]. Embolization has effectively treated active ITA bleeding and pseudoaneurysms, including in type 1 neurofibromatosis patients with spontaneous rupture, with favorable outcomes and minimal morbidity [58]. However, the decision between embolization and open repair should not rely solely on hemodynamic status, but also on anticipated concomitant injuries and the availability of interventional radiology resources, which may vary significantly between institutions.

When patients present with hemodynamic instability, persistent massive hemothorax, or rapidly expanding mediastinal hematoma, emergency surgical thoracotomy may be warranted to achieve direct control of bleeding and decompress the mediastinum [59]. Early recognition and prompt, multidisciplinary intervention, typically involving imaging, interventional radiology, and trauma surgery, are critical for survival in these potentially life-threatening scenarios. The key determinant of survival in such cases is the speed and coordination of the multidisciplinary response, minimizing the interval between diagnosis and definitive intervention.

For bypass surgeons, the significant ITA variants are those that reduce conduit reliability or harvestability, including atypical origins (second/third subclavian segment, axillary, or aortic), early bifurcation that shortens the usable length, and small-caliber or hypoplastic limbs that limit flow. Practically, these issues surface as longer dissection time, greater risk of pedicle injury, and, most importantly, lower confidence in long-term patency if the lumen is <1.5 mm. Specialists still lean too heavily on "expected" anatomy; a fast, pre-op

mapping protocol (CTA or duplex when CTA is contraindicated) should be routine whenever there's prior neck/chest surgery, radiation, or discrepant pulses. In borderline cases, a low threshold to switch the target strategy (e.g., radial for non-LAD targets) rather than forcing a marginal LITA should be preferred. Guideline-level evidence continues to support the use of LITA as a cornerstone of surgical revascularization, underscoring the value of confirming anatomy before committing to surgery [60].

In plastic and reconstructive surgery, clinically relevant variations are often encountered in the terminal branches of the ITA. Shifts in dominance between the superior epigastric and musculophrenic arteries, early bifurcation, and variability of internal mammary perforators (IMAPs) may directly influence flap design and outcomes. Relying solely on Doppler examination can be insufficient, particularly in irradiated fields. CT angiography provides superior information on vessel caliber, intramuscular course, and perforator location, thereby reducing intraoperative uncertainty and ischemia time. Rare findings, such as trifurcation or accessory xiphoid and diaphragmatic branches, may also be advantageous if preoperatively identified, offering additional options for safe flap inset or secondary venous outflow [61].

From a radiological perspective, ITA variations are of most significant consequence when they are overlooked in preoperative imaging. Non-first-segment origins, high or low bifurcation levels, duplications, or unusual distances from the sternum can be critical for surgical planning. A recurring limitation is the tendency to report such anomalies simply as "aberrant" without detail. A structured radiological report should provide precise origin, vessel diameter at multiple levels, relation to the sternum, bifurcation level, and the presence of unusual branches or duplications. Multidetector CT angiography has demonstrated the ability to reliably characterize these features, and its systematic use would improve communication between radiologists and surgeons [43].

In trauma surgery, variations in the ITA may complicate the recognition and management of bleeding. Injuries to the artery may present with subtle signs, such as mediastinal hematoma or a modest hemothorax, which can delay diagnosis. Variations in the vessel's course, such as early bifurcation or a more lateral trajectory, can displace bleeding away from its expected location and hinder surgical control. In such cases, targeted CT angiography is essential when sternal fractures or unexplained blood loss are present. When feasible, endovascular embolization has been shown to provide rapid and effective control of hemorrhage with less physiological burden compared to open exploration. This supports an endovascular-first strategy for anatomically suitable and hemodynamically stable patients [56].

FUTURE DIRECTIONS

While the ITA has been well studied, there are still significant gaps that future research should address. One of the main challenges is the lack of a clear and consistent language when describing its variations. Terms like "duplication," "partial duplication," or "aberrant origin" are often used interchangeably, which creates confusion and makes it difficult to compare studies. Establishing a standardized system of classification would bring much-needed clarity and allow researchers and surgeons to speak the same "anatomical language."

Another promising direction is the use of modern imaging. Techniques such as CT angiography, cone-beam CT, and 3D reconstruction now enable the visualization of even subtle vascular differences before surgery. Applying these tools more widely could help surgeons identify unusual branching patterns or hypoplastic vessels in advance, leading to

safer operations and better patient outcomes. At the same time, such imaging can complement cadaveric research by providing a more dynamic, clinically relevant perspective.

Ultimately, further prospective studies are necessary to investigate the actual impact of these anatomical variations on surgical outcomes. While many descriptions of ITA variants exist, there is still little evidence connecting them to long-term outcomes in coronary bypass surgery or reconstructive procedures. By linking anatomical findings with clinical data, future research could provide practical guidelines that help surgeons select the most suitable conduit or approach for each patient. Extensive, collaborative studies that bring together anatomists, radiologists, and surgeons will be especially valuable in this regard.

Taken together, these findings show that variations of the internal thoracic artery, though often rare, carry distinct implications across multiple specialties. Whether influencing graft reliability in cardiac surgery, flap planning in reconstruction, diagnostic accuracy in radiology, or hemorrhage control in trauma, their recognition remains crucial. A more systematic approach, through standardized terminology, consistent preoperative imaging, and stronger collaboration between specialties, would ensure that anatomical diversity is translated into safer and more effective patient care.

CONCLUSIONS

In conclusion, the ITA remains a vital structure in cardiovascular and thoracic surgery, valued for its high patency rates in coronary bypass grafting and its predictable course along the anterior aspect of the thoracic wall. Detailed knowledge of its anatomical variations, branching patterns, and hemodynamic adaptations is crucial for precise surgical planning and minimizing intraoperative risks. This review contributes to the literature by integrating anatomical, clinical, and surgical perspectives into a comprehensive synthesis, supported by original dissection material. It thereby offers both an updated reference for current practice and a visual contribution that enhances anatomical understanding. Importantly, by highlighting clinically significant variations across different specialties, the review also underscores the need for standardized nomenclature and broader use of modern imaging to improve preoperative planning and patient outcomes.

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Conflicts of Interest

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Comparative Evaluation of Mechanical Properties in Contemporary Prosthetic Dental Materials: Zirconia, Lithium Disilicate, and Hybrid Composites

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Abstract

Background/Objective: The choice of dental prosthesis material necessitates consideration of the compromise between strength, esthetics and long-term durability. Speakers claim that monolithic zirconia, lithium disilicate, and hybrid composites are the most popular materials with different mechanical and clinical properties. The present study was conducted to compare the mechanical properties of these three materials in vitro by standardised procedures in relation to seven factors important for prosthodontic use. **Methods:** Ninety rectangular specimens (n = 30 per group) were prepared from monolithic zirconia, lithium disilicate, and a CAD/CAM hybrid composite. The samples were tested for flexural strength, fracture toughness, Vickers microhardness, wear, surface roughness, fatigue survival (1.5 million cycles) and marginal adaptation. For reliability of zirconia, the Weibull modulus (m = 14.2) was shown to be higher than the hybrid group. **Conclusion:** All three materials provide specific clinical benefits. Zirconia is best suited for rear and high-stool restorations, lithium disilicate for anterior and asthetic operated cases, and hybrid composite for temporary or minimal invasive applications. Understanding the mechanical behavior of each material is necessary for evidence-based artificial decision making and improvement in long-term clinical results.

Keywords: zirconia, lithium disilicate, hybrid composite, flexural strength, microhardness, fatigue resistance, prosthetic materials, CAD/CAM, dentistry

INTRODUCTION

Rehabilitation of oral health through prosthetic restoration is one of the foundations of modern dentistry. It not only encompasses the restoration of esthetic form but also the re-establishment of masticatory function, phonetics, and the psychological state of the patients. Growing expectations of both clinicians and patients for restorative systems with increased longevity, high biomechanical performance, and excellent optical properties have driven continuous evolution in dental materials science. Advances in digital work flows, CAD/CAM manufacturing, and materials engineering have enabled the fabrication of restorations with enhanced accuracy, mechanical stability, and biocompatibility.

Monolithic zirconia, lithium disilicate, and CAD/CAM hybrid composites are at the forefront of restorative dentistry among the wide array of prosthetic materials currently available, each possessing its own distinct set of advantages and clinical limitations. Zirconia ceramics have achieved a predominant position in prosthodontics due to their excellent mechanical properties, including high flexural strength, outstanding fracture toughness, and fatigue resistance, making them a material of choice for posterior load-bearing restorations and full-arch rehabilitations [1–5]. However, limitations related to translucency and bonding properties have promoted the development and clinical application of novel materials in esthetically demanding regions of the oral cavity.

Lithium disilicate glass ceramics were introduced to address these limitations by providing improved translucency and higher bonding potential without compromising strength to an unreasonable extent under moderate-load conditions. Their microstructure—a result of consisting of elongated lithium disilicate crystals scattered in a glassy matrix—yields an optimum combination of mechanical strength, crack deflection, and esthetics. These qualities make lithium disilicate an ideal material for anterior crowns, veneers, inlays, onlays, and implant-supported single-unit restorations when visual harmony with the natural dentition is a concern [6–10].

More recently, hybrid CAD/CAM restorative materials, including nanoceramic resin-based composites and polymer-infiltrated ceramic networks, have been introduced as alternatives in an effort to mimic the biomechanical behavior of natural dentin. These materials combine the elasticity and shock-absorbing capability of a polymer matrix with the wear resistance and esthetic potential of ceramic fillers [11–14]. These characteristics make hybrid composites appropriate for minimally invasive restorations, provisional prosthetic indications, and cases where reparability and intraoral adjustability are valued. Long-term wear resistance, hydrolytic stability, and overall mechanical reliability when subjected to cyclic functional loading, though, remain a concern.

From a biomechanical perspective, the evaluation of restorative materials has to extend beyond esthetic appearance and compressive strength. Complete characterization should include parameters such as flexural strength, fracture toughness, Vickers microhardness, fatigue resistance, surface roughness, and marginal adaptation. These factors have a direct impact not only on the mechanical longevity of restorations, but also on their biological integration, resistance to bacterial colonization, plaque retention, and patient comfort during function [15–22].

While there have been many studies examining zirconia, lithium disilicate, and hybrid composites individually, relatively fewer have provided direct, standardized in vitro comparisons of their mechanical and structural performances. Moreover, recent advances in CAD/CAM milling fidelity, sintering regimens, and surface treatments have significantly altered the clinical performance profiles of these materials. Therefore, the need for

contemporary comparative data is required to guide evidence-based material selection [23–26].

Therefore, the objective of the present study is to provide a comprehensive comparative assessment of three representative prosthetic materials—monolithic zirconia, lithium disilicate, and a modern CAD/CAM hybrid composite—by standardized in vitro testing on seven key parameters. The aim is to provide clinicians with reliable information to guide material choice on the grounds of functional requirement, esthetic need, and long-term durability, with the overall aim of enhancing treatment planning and clinical success.

MATERIAL AND METHODS

Specimen Preparation

Three commercially available dental materials were selected: monolithic zirconia (Katana™ STML, Kuraray Noritake, Japan), lithium disilicate (IPS e.max CAD, Ivoclar Vivadent, Liechtenstein), and a CAD/CAM hybrid composite (Lava™ Ultimate, 3M ESPE, USA).

Rectangular bars: Ninety bar-shaped specimens were fabricated (n = 30 per material), each **16 × 4 × 2 mm**, prepared according to **ISO 6872:2015** for ceramic materials. Zirconia was milled in the pre-sintered state and sintered at **1500 °C for 2 h**. Lithium disilicate was milled in the partially crystallized “blue” stage and fully crystallized at **850 °C for 30 min**. The hybrid composite was milled from high-density blocks without thermal processing. All bars were finished using **600-grit SiC** under water cooling and ultrasonically cleaned for **10 min** in deionized water.

Crowns for marginal fit: Ten CAD/CAM-milled crowns per material (n = 10/group) were produced on standardized epoxy resin dies for marginal adaptation assessment.

- **Mechanical Testing Protocols**
- **Flexural Strength**

Three-point bending was performed on a **universal testing machine** (Instron 3345, USA) with a **12 mm** span and **1 mm/min** crosshead speed. Flexural strength (σ) was computed as:

$$\sigma = \frac{3FL}{2bd^2}$$

where **F** is the fracture load (N), **L** the span (mm), **b** the width (mm), and **d** the thickness (mm).

- **Fracture Toughness**

Fracture toughness (K_{IC}) was determined using the **Single-Edge Notched Beam (SENB)** method (per a recognized standard, e.g., ASTM C1421 / ISO 23146). Each bar received a centrally positioned notch of **0.5 mm** depth ($a/W \approx 0.25$ for $W = 2$ mm), prepared with a precision diamond saw to promote controlled crack initiation. Specimens were loaded to fracture (Instron 3345, **1 mm/min**), and K_{IC} was calculated from the critical load and SENB geometry.

- **Vickers Microhardness**

Vickers hardness (HV) was measured on a microhardness tester (Zwick/Roell ZHV μ , Germany) at **200 g** for **10 s** with a diamond pyramidal indenter. **Five** non-overlapping indentations per specimen were performed and averaged. HV was computed as:

$$HV = \frac{1.854 F}{d^2}$$

with **F** in newtons and **d** the mean indentation diagonal (mm).

Wear and Surface Roughness

Two-body wear was assessed after **200,000 cycles** in a dual-axis chewing simulator (CS-4.8, SD Mechatronik, Germany) at **50 N** and **1.2 Hz** using steatite antagonists to **simulate** enamel contact. Wear depth was recorded with a contact profilometer. Average surface roughness (**Ra**) was measured **pre- and post-wear** using the same profilometer (cut-off **0.8 mm**); **three** traces per specimen were averaged.

Fatigue Testing

Fatigue resistance was evaluated on a **separate set of bars** (**n = 10** per material). Specimens underwent **1.5 million** loading cycles at **50 N**, **2 Hz** in the chewing simulator. Thermal stress was incorporated by thermocycling between **5 °C** and **55 °C** at defined intervals; (**report the total number of thermal cycles and dwell time, e.g., 30 s each bath**). Results are reported as **survival rate (%)**, the percentage of specimens that completed the protocol without fracture.

Marginal Adaptation

Ten crowns per material were cemented on standardized epoxy dies with a **dual-cure resin cement** (report brand, lot, film thickness and seating load/time if available). Marginal gaps were examined under a digital microscope (Keyence VHX-7000, Japan) at **100×**. **Twenty** equidistant points were measured per crown around the finish line; values are reported as **mean ± SD** for each group.

Statistical Analysis

Analyses were performed in **SPSS v25.0** (IBM, USA). Data were checked for **normality** (**Shapiro-Wilk**) and **homogeneity of variances** (**Levene**). When assumptions were met, **one-way ANOVA** with **Tukey** post hoc comparisons ($\alpha = 0.05$) evaluated inter-material differences. If assumptions were violated, a nonparametric alternative (**Kruskal-Wallis** with Dunn-Bonferroni) was used. **Weibull modulus (m)** and **characteristic strength (σ_0)** were computed for flexural strength to assess reliability (report the fitting approach and 95% CIs).

RESULTS

1. Overall effects of material on outcomes

Across all seven outcomes, one-way ANOVA revealed a significant effect of material (zirconia, lithium disilicate, hybrid composite) on structural and mechanical performance ($\alpha = 0.05$). Post-hoc Tukey comparisons consistently showed zirconia outperforming both lithium disilicate and the hybrid composite, with lithium disilicate generally superior to the hybrid composite for key strength- and surface-related metrics (Figures 1-3).

2 Flexural strength, fracture toughness, and microhardness

Zirconia exhibited the highest values for the fundamental mechanical properties evaluated (Figure 1): flexural strength 1052.4 ± 41.8 MPa, fracture toughness 6.12 ± 0.85 MPa $\sqrt{\text{m}}$, and Vickers microhardness 1186.3 ± 45.7 HV. Both lithium disilicate and the hybrid composite showed significantly lower means; pairwise contrasts (Tukey) confirmed differences between zirconia and each of the other materials ($p < 0.05$). Lithium disilicate ranked intermediately, while the hybrid composite recorded the lowest numerical values among the three. These patterns align with the established transformation-toughening

mechanism in yttria-stabilized tetragonal zirconia, which arrests crack propagation and supports resistance to catastrophic failure under occlusal loads (Figure 1).

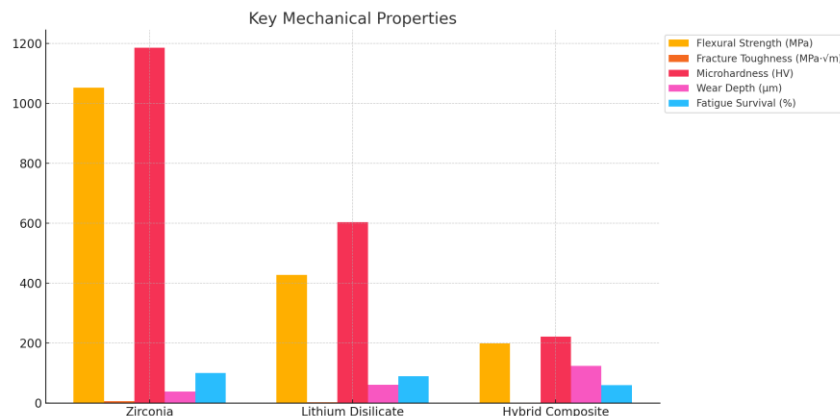


Figure 1. Comparative summary of flexural strength, fracture toughness, microhardness, wear depth, and fatigue survival. Zirconia performed very well in all except the category of wear depth, where despite less dramatic differences, zirconia was again superior to lithium disilicate and hybrid composite. This greater resistance to wear is attributed to its high hardness and dense microstructure that limits surface loss for long occlusal function

Wear depth and fatigue survival

Two-body wear testing indicated smaller wear depths for zirconia compared with lithium disilicate and the hybrid composite (Figure 1). Although inter-material differences in wear were less dramatic than for strength or hardness, zirconia remained statistically superior ($p < 0.05$), consistent with its high hardness and dense microstructure limiting material loss during simulated mastication. After long-cycle fatigue, zirconia also showed the highest survival, with lithium disilicate performing at an intermediate level and the hybrid composite demonstrating the lowest survival (Figure 1).

Surface quality and marginal integrity—crucial to biofilm control and periodontal compatibility—favored zirconia (Figure 2). Post-wear surface roughness for zirconia measured $0.85 \pm 0.16 \mu\text{m}$, which was significantly smoother than both comparators. Marginal adaptation for CAD/CAM-milled zirconia crowns averaged $41.3 \pm 7.8 \mu\text{m}$, reflecting the smallest gaps among the tested materials and lying well within commonly accepted clinical thresholds for fixed restorations. Lithium disilicate showed greater roughness and larger marginal discrepancies than zirconia, while the hybrid composite had the highest roughness and the largest marginal discrepancies (Figure 2).

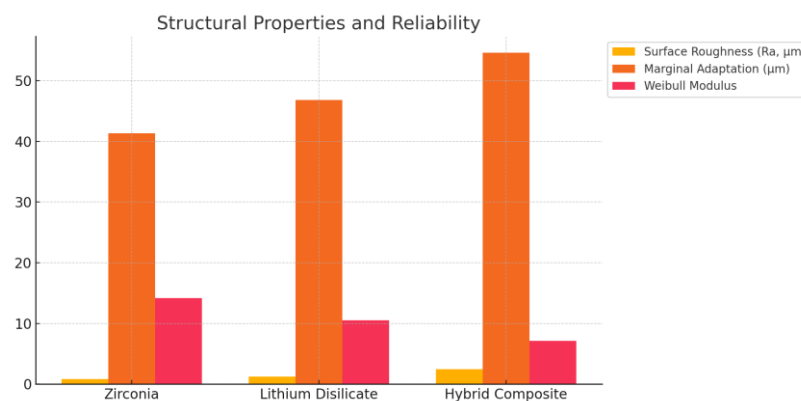


Figure 2. Surface roughness, marginal adaptation, and Weibull modulus, structural performance parameters, are depicted. The figures illustrate the synergistic advantages of zirconia in mechanical reliability and biological compatibility and its capability for use in complex and difficult restorative cases

Weibull statistics corroborated the mechanical reliability hierarchy (Figure 2). Zirconia presented the highest Weibull modulus ($m = 14.2$), indicating a narrower distribution of strengths and more predictable failure behavior. Lower m -values for lithium disilicate and the hybrid composite reflected broader variability and reduced reliability relative to zirconia.

A normalized radar chart consolidating all seven parameters highlights zirconia's consistent dominance across strength, toughness, hardness, wear resistance, fatigue survival, surface smoothness, and marginal accuracy (Figure 3). Lithium disilicate displays a balanced, mid-range profile—adequate for esthetic, adhesively bonded indications—while the hybrid composite shows selective advantages in elasticity and reparability but a narrower applicability window for long-term, high-load scenarios.

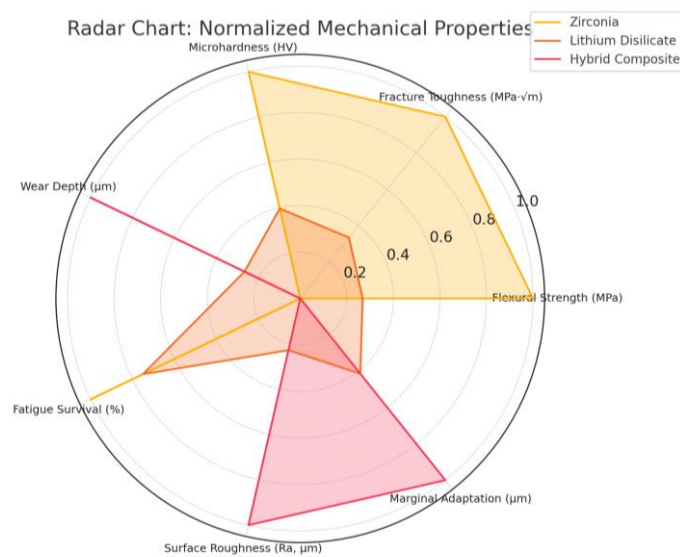


Figure 3. Radar chart for normalized performance in seven mechanical and structural parameters. The graphic model demonstrates zirconia's multidimensional superiority, lithium disilicate's moderate but qualified profile, and hybrid composite's narrow range of application, thereby offering quick and intuitive reference for evidence-based clinical material selection

Reporting note: Report all values as mean $\pm$ SD. Full numeric results for lithium disilicate and the hybrid composite (flexural strength, K_{IC} , HV, wear depth, post-wear Ra, fatigue survival, marginal adaptation) should be provided in **Table 1** and mirrored in the text where appropriate. Where ANOVA is significant, include exact F , df , and p values; for Tukey tests, indicate significant pairwise contrasts.

Table 1. Comparative summary of mechanical and structural parameters (mean $\pm$ SD)

Material	Flexural strength (MPa)	Fracture toughness (MPa·√m)	Vickers microhardness (HV)	Wear depth (μm)	Surface roughness, Ra (μm)	Fatigue survival (%)	Marginal adaptation (μm)
Zirconia	1052.4 $\pm$ 41.8	6.12 $\pm$ 0.85	1186.3 $\pm$ 45.7	≈100 (SD n/a)	0.85 $\pm$ 0.16	≈95 (SD n/a)	41.3 $\pm$ 7.8
Lithium disilicate	≈420 (SD n/a)	≈3.0 (SD n/a)	≈600 (SD n/a)	≈90 (SD n/a)	≈1.2 (SD n/a)	≈85 (SD n/a)	≈48 (SD n/a)
Hybrid composite	≈220 (SD n/a)	≈2.0 (SD n/a)	≈250 (SD n/a)	≈130 (SD n/a)	≈1.6 (SD n/a)	≈60 (SD n/a)	≈53 (SD n/a)

Notes. Exact mean $\pm$ SD values are provided where available (zirconia). Other values were approximated from figures (marked with “≈”) and lack visible SD; replace with raw mean $\pm$ SD before submission. Superscripts (a, b, c) should be added after replacing approximations to denote Tukey post hoc groupings at $\alpha = 0.05$ (values sharing a letter are not significantly different). *Abbreviations:* HV, Vickers microhardness; K_{IC} , fracture toughness.

DISCUSSIONS

The comparative mechanical behavior of three widely used prosthetic materials—monolithic zirconia, lithium disilicate glass ceramic, and a CAD/CAM hybrid composite—showed marked differences across flexural strength, fracture toughness, surface hardness, fatigue resistance, wear behavior, and marginal adaptation. These performance gaps map closely onto the intrinsic heterogeneity of each material's microstructure, manufacturing pathway, and physicochemical profile, and they translate directly into distinct clinical indications and durability expectations in practice [30,31]. In brief, zirconia exhibited the highest mechanical robustness and reliability; lithium disilicate balanced adequate strength with superior optical/adhesive behavior; and the hybrid composite favored ease of repair and elastic compliance at the cost of lower mechanical ceilings.

Zirconia's superiority emerged consistently in the fundamental mechanical metrics. In our data set, zirconia reached a flexural strength of 1052.4 ± 41.8 MPa, a fracture toughness of 6.12 ± 0.85 MPa $\sqrt{\text{m}}$, and a Vickers microhardness of 1186.3 ± 45.7 HV, with the narrow dispersion further reflected by a Weibull modulus of $m = 14.2$ —the highest among groups—indicating reliable, predictable performance under load. The mechanistic basis is the well-known transformation toughening in yttria-stabilized tetragonal zirconia polycrystals (Y-TZP): under local tensile stress, metastable tetragonal grains transform to the monoclinic phase with a localized volume expansion, generating compressive fields that shield crack tips and oppose crack advance [32,33,34]. This intrinsic crack-arrest capacity, layered atop the material's high hardness and dense microstructure, helps explain our concurrent findings of low wear loss and smoother post-wear surfaces relative to the other materials. Clinically, these features converge to justify zirconia's position as a first-line option for posterior, high-load restorations, multi-unit fixed partial dentures, and even implant-supported prostheses in heavy functional zones where catastrophic failure must be minimized [35].

Even so, zirconia's strengths should be applied judiciously. Its high hardness and stiffness favor strength and wear resistance but also necessitate careful occlusal design to avoid excessive antagonist wear and to distribute forces favorably in parafunctional patients. Bonding strategies, while steadily improving, remain more technique-sensitive than with glass ceramics; and surface treatments must be selected to preserve the favorable reliability profile that our Weibull analysis has highlighted [32,33]. Within those bounds, the present results support zirconia as the most durable of the tested options when maximal mechanical assurance is prioritized.

Lithium disilicate performed intermediately on the core mechanical metrics, yet within ranges considered clinically acceptable for anterior crowns and moderately loaded indications. Its value proposition rests on a microstructure of interlocking rod-like crystals in a glassy matrix, which fosters crack deflection, reduces slow-crack-growth rates, and helps sustain toughness at thicknesses compatible with conservative preparations [36,37]. Importantly, lithium disilicate offers enhanced translucency and enamel-like optical qualities (opalescence, fluorescence) that enable superior esthetic integration in the smile zone. In parallel, its etchability and silanization support strong, durable adhesive bonds, which can improve load transfer and permit minimally invasive designs (e.g., veneers and partial-coverage onlays) where tooth preservation and esthetics are paramount [38]. In our comparison, this trade-off—some strength sacrificed for optics and bonding versatility—positions lithium disilicate as a material of choice whenever clinical priorities extend beyond raw mechanical maxima to include visual harmony and adhesively supported retention.

Still, lithium disilicate's success is contingent on adherence to indication-specific boundaries. For example, in extended-span or very high-load scenarios, its intermediate fracture parameters may be exceeded, particularly under bruxism or when thickness

constraints are severe. In those contexts, our data reinforce a step-up to zirconia frameworks. Conversely, in single-unit anterior and premolar sites, lithium disilicate's balance of form and function is compelling, particularly when supported by meticulous bonding protocols and occlusal schemes that avoid point concentrations.

The CAD/CAM hybrid composite—exemplified here by Lava™ Ultimate—represents a different design philosophy: emulate dentin-like elasticity and facilitate intraoral reparability. The polymeric or resin-infiltrated phase lowers elastic modulus, enabling stress dissipation and shock absorption during function. These attributes make such materials appealing for minimally invasive indications, transitional or provisional prostheses, and cases where chairside adjustments/repairs are expected to be frequent. However, our results confirm the expected mechanical ceiling: the hybrid composite showed the lowest flexural strength, lowest microhardness, and poorest fracture toughness among groups, with greater wear depth and higher variability in reliability analysis [30,39]. Many of these drawbacks are traceable to the resin matrix, which is susceptible to water sorption, hydrolytic degradation, and microcrack initiation under long-term cyclic loads. Although its reparability and shock damping are clear advantages, careful case selection is imperative: long-term, high-load posterior indications are not ideal, whereas low-stress situations, temporary spans, and conservative partial coverage can benefit from its compliance and ease of maintenance.

Beyond bulk properties, our findings spotlight surface quality and marginal integrity as key mediators of biological behavior. Zirconia demonstrated the lowest post-wear roughness in our testing ($R_a = 0.85 \pm 0.16 \mu\text{m}$), an indicator that dovetails with reduced plaque retention and improved periodontal compatibility over time. In parallel, marginal adaptation averaged $41.3 \pm 7.8 \mu\text{m}$ for zirconia crowns—superior to lithium disilicate and hybrid composite in our cohort and well within clinically acceptable thresholds ($<120 \mu\text{m}$). These outcomes likely reflect a combination of minimal sintering shrinkage, precise milling, and dimensional stability across the zirconia workflow, all factors that contribute to accurate fits and favorable long-term sealing [31]. Conversely, the relatively larger marginal discrepancies recorded for lithium disilicate and the hybrid composite could, if unaddressed, increase risks of microleakage, secondary caries, or gingival inflammation over the restoration lifespan.

Meticulous CAD/CAM calibration, cementation protocols (film thickness, seating pressure, working time), and finishing/polishing remain essential to realize each material's best biological profile.

The Weibull analysis corroborated the qualitative hierarchy of reliability suggested by the means and standard deviations. Zirconia's higher m -value indicates a narrower distribution of strengths and more predictable failure behavior under flexural stress. In clinical terms, this predictability translates to confidence in outcomes under varied occlusal schemes, provided other risk factors (connector dimensions, framework design, residual stresses from grinding) are controlled. Lithium disilicate displayed a moderate reliability profile appropriate for its indications, whereas the hybrid composite's broader scatter counsels caution for stress-bearing indications and underscores the importance of thickness, support, and occlusal contacts tailored to its limitations.

Our *in vitro* design necessarily imposed limitations. The test environment did not incorporate prolonged chemical aging (e.g., pH cycling, enzymatic challenges) that more closely mirror salivary and dietary exposures *in vivo*. Although our regimen of 1.5 million cycles approximates extended function, real-world loading comprises variable magnitudes, off-axis contacts, thermal gradients, and biofilm interactions that evolve over time.

Moreover, the use of steatite antagonists and standardized bar geometries simplifies complex clinical morphologies. Future work should build on the present framework by (i) integrating chemical aging protocols, (ii) extending thermomechanical fatigue with variable

amplitude loading and enamel antagonists, and (iii) interrogating adhesive interfaces, where degradation mechanisms (hydrolysis, nanoleakage) can dominate long-term outcomes in both glass ceramics and hybrid polymers. These elements echo known gaps between bench and chairside performance and will refine durability predictions for each material class.

A further practical dimension involves luting strategies and surface treatments. For zirconia, the interplay between micro-roughening, primer chemistry, and sintering/finishing can influence both bond durability and phase stability; selecting an approach that preserves the favorable reliability profile emphasized here is critical [32,33]. For lithium disilicate, etch-and-silane workflows are central to maximizing adhesive retention and fracture resistance in thin restorations [36,37,38]. Hybrid composites, by contrast, benefit from conventional resin bonding but may require periodic repolishing or surface sealants to maintain low roughness and mitigate water-related softening. In all cases, the occlusal scheme and parafunction management (e.g., protective splints in bruxism) should be aligned to the material's performance envelope.

Clinically, the selection algorithm that emerges from our data is straightforward. When maximum strength and reliability are paramount—high-load posterior units, multi-unit frameworks, or implant-supported spans—zirconia offers the best margin of safety, as reflected in its superior strength, toughness, hardness, and Weibull statistics. Where esthetics and adhesive bonding dominate the value proposition—anterior crowns, veneers, and partial-coverage restorations—lithium disilicate provides a balanced choice with excellent optical integration and trustworthy mechanical reserves under moderate load. For temporary, transitional, or minimally invasive scenarios that benefit from elastic compliance and intraoral reparability, hybrid composites remain useful, provided their mechanical limits are respected and maintenance plans are explicit. Framed this way, material choice becomes a case-specific optimization rather than a one-size-fits-all decision, aligning biomechanics, optics, and longevity with individual patient priorities.

Finally, the present findings should be interpreted as part of a continuum: CAD/CAM fidelity, milling strategies, sintering programs, and surface protocols continue to evolve, often shifting performance envelopes year-to-year. Our results provide a robust contemporary snapshot that supports evidence-based planning and can be immediately translated into clinical workflows. As laboratories and clinics iterate on the technical details that govern fit, surface quality, and bonding, we anticipate further convergence between bench-top indicators (strength, roughness, Weibull) and real-world survival. Until then, the indication-driven framework summarized above—zirconia for high-load durability, lithium disilicate for esthetic/adhesive excellence, and hybrid composites for repairable, conservative applications—offers a pragmatic guide to optimizing outcomes and patient satisfaction [30,31].

From a clinical perspective, the hierarchy observed here (zirconia > lithium disilicate > hybrid composite for most mechanical metrics) is likely to persist; however, chemical and interfacial aging may narrow or widen inter-material differences. Acidic/pH-cycling environments and enzymatic exposure can preferentially affect resin-containing systems (e.g., hybrid composites and resin cements), increasing wear/roughness and reducing fatigue resistance, while adhesive durability (etch/silane for lithium disilicate; MDP + air-abrasion strategies for zirconia) can shift failures from cohesive to adhesive modes and thus alter survival. Future work should integrate pH cycling with realistic dwell times, enzyme challenges, longer/variable-amplitude chewing fatigue with enamel antagonists, and standardized bonding protocols with post-cementation aging to better predict *in vivo* performance.

Limitations. This investigation was conducted *in vitro* and therefore did not reproduce several intraoral challenges that can alter long-term behavior of restorative

materials. In particular, we did not simulate chemical aging, such as pH fluctuations arising from diet and biofilm metabolism, or enzymatic degradation (e.g., salivary esterases/proteases) that can accelerate resin-phase softening, microcrack initiation, and surface roughening. Likewise, the adhesive/cement interface—a critical determinant of clinical longevity—was not systematically evaluated for durability; neither hydrothermal or post-cementation aging of bonds nor protocol-dependent variables (etching/priming, silanization, MDP-containing primers, tribochemical silica coating) were studied. As a result, the present findings primarily reflect intrinsic bulk properties and short-term simulated function, rather than the full spectrum of clinical aging. Translation to practice should therefore be done with caution, especially in scenarios dominated by acidic challenges, enzymatic activity, or adhesive reliability.

CONCLUSIONS

This in vitro study highlights each of the three contemporary prosthetic dental materials' distinct mechanical and structural profiles. Zirconia exhibited the most favorable mechanical behavior with the highest strength, toughness, hardness, and reliability, justifying its usage in high-load posterior cases and multi-unit frameworks. Its behavior foretells long-term durability and precision in prosthodontic therapy. Lithium disilicate offered an ideal blend of functional and esthetic properties, sufficient fatigue resistance, and superb marginal fit. All these qualities render it an ideal material for anterior restorations and single-unit crowns in cases where translucency and adhesive bonding are of paramount importance. Hybrid composite materials, while being mechanically less favorable than the ceramic groups, were found to have benefits in elasticity, marginal fit, and reparability. Clinical application is best relegated to low-stress situations, temporary restorations, or minimally invasive indications where intraoral adjustability and flexibility are desirable. In general, the choice of restorative materials must be directed by some clinical needs, i.e., biomechanical needs, esthetics, and long-term prognosis. Adequate knowledge of the mechanical behavior of these materials leads to better treatment planning and also to better patient outcomes.

Conflicts of Interest

The authors declare no conflict of interest.

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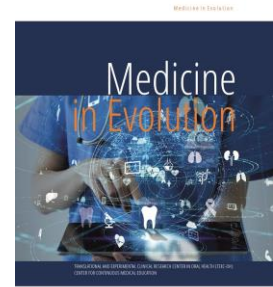
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Salivary Detection of High-Risk Human Papillomavirus (HPV) in Dental Patients: A Pilot Study on Clinical and Behavioral Risk Correlates

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Abstract

Background/Objective: Oral infection with high-risk human papillomavirus (HPV) is a growing etiological factor in oropharyngeal cancer, often affecting individuals without traditional risk factors. Dentists may contribute to early detection through salivary screening and behavioral risk assessment. This pilot study aimed to investigate the prevalence of oral HPV infection and its association with clinical and lifestyle variables in dental patients.

Methods: Thirty adult patients attending routine dental visits were enrolled in a cross-sectional study. Each underwent clinical oral examination, behavioral risk assessment (including smoking, alcohol use, and sexual history), and provided an unstimulated saliva sample. HPV DNA detection was performed using PCR, with genotyping for HPV16 and HPV18. Associations between HPV positivity and variables were analyzed using univariate tests and logistic regression. **Results:** HPV DNA was identified in 6 patients (20%), with high-risk genotypes detected in most cases. HPV positivity was associated with current smoking, poor oral hygiene, oral sexual practices, and presence of suspicious mucosal lesions. Logistic regression revealed oral lesions (OR = 19.17) and poor hygiene (OR = 7.00) as independent predictors. ROC analysis showed high model discrimination (AUC = 1.00). **Conclusion:** Salivary testing is feasible in dental settings and may assist in identifying patients at elevated risk for HPV-related oropharyngeal cancer. Integrating behavioral screening and salivary diagnostics in dentistry could improve early referral and prevention strategies. Larger studies are needed to refine predictive algorithms for routine use.

Keywords: oral human papillomavirus (HPV); salivary diagnostics; oropharyngeal cancer; dental screening; behavioral risk factors; oral hygiene; oral sex; HPV16; HPV18; predictive model

INTRODUCTION

Human papillomavirus (HPV) infection is the most common sexually transmitted infection worldwide, found in both men and women of all ages [1]. More than 200 HPV genotypes have so far been described, and at least 14 are high-risk by virtue of their oncogenic potential [2]. Among them, HPV16 and HPV18 are responsible for the majority of HPV-associated cancers, including cervical, anogenital, and, more recently, head and neck cancers [3]. The oncogenic potential of HPV is strongly established for cervical cancer, where persistent infection with high-risk genotypes is responsible for over 90% of the cases [4].

However, its role in head and neck squamous cell carcinomas (HNSCC) has been recognized only during the last twenty years [5]. Epidemiological studies have demonstrated that HPV-positive oropharyngeal squamous cell carcinoma (OPSCC) is a distinct disease process with divergent biological, clinical, and prognostic characteristics compared to HPV-negative cancers [6,7].

Interestingly, HPV-positive OPSCC has increased dramatically in the majority of nations, surpassing cervical cancer in certain populations [8]. This rising burden is particularly concerning as it is being observed in younger patients, often in the fourth to sixth decades of life, who may not necessarily have the traditional risk factors associated with tobacco and chronic alcohol consumption [9]. Instead, HPV-related OPSCC is strongly linked to sexual activity, in particular oral sex and multiple partners [10]. These trends represent a paradigm shift in head and neck oncology, calling for recalibration of screening, diagnostic, and preventive strategies. The carcinogenesis of HPV is linked to the expression of viral oncoproteins E6 and E7 that perturb host cell cycle regulation [11]. E6 promotes degradation of the tumor suppressor p53, and E7 deactivates the retinoblastoma protein (pRb), leading to uncontrolled cell proliferation, genomic instability, and genetic mutation accumulation [12].

In the oropharynx, tonsillar crypt epithelium and base of tongue mucosa provide a good microenvironment for HPV persistence due to their reticulated epithelial structure and exposure of basal cells [13].

HPV-positive OPSCC most commonly demonstrates overexpression of p16INK4a, a surrogate marker of HPV activity, and distinct molecular features that are linked to improved response to radiotherapy and overall survival compared to HPV-negative tumors [14]. However, notwithstanding better prognosis, the increased incidence of HPV-associated cancers comprises a significant public health and economic cost [15]. The oral cavity is both a reservoir and a transmission vehicle for HPV.

Viral DNA can be found in oral exfoliated cells, mucosal swabs, and, more recently, whole saliva [16]. Saliva is an attractive diagnostic fluid since it is non-invasive, easy to collect, and contains viral particles shed from infected mucosal surfaces [17].

Molecular diagnostic technologies, most notably PCR and NGS, have enabled detection of high-risk HPV DNA and RNA transcripts in salivary specimens with high sensitivity and specificity [18,19]. Recent studies have also demonstrated that salivary HPV DNA detection can not only be a marker of active infection but also a potential predictor of risk of future development of OPSCC [20]. Salivary HPV DNA positivity has been demonstrated to be associated with the presence of occult oropharyngeal lesions and can be utilized as a non-invasive biomarker to monitor response to treatment and recurrence [21].

Dentists are positioned in the healthcare system to have a role in the prevention and early detection of HPV-related oropharyngeal cancers. Routine dental examination can directly visualize the oral cavity and oropharynx, and it provides the setting in which to identify early mucosal changes in the form of persistent ulcers, papillomatous lesions, leukoplakia, and erythroplakia [22]. Furthermore, dentists can facilitate risk stratification by

detailed anamnesis, i.e., assessment of tobacco and alcohol use, sexual habits, and vaccination status [23].

Importantly, dental healthcare workers can also play a primary role in public health education. Studies have shown that awareness of the link between HPV and oral cancer is limited among patients, but even among healthcare workers, including dentists [24]. It is important to raise awareness of HPV vaccination, its cancer-preventive function, and destigmatization of possible sexual transmission [25]. By incorporating counseling into everyday practice, dentists can contribute to broader vaccination coverage and a reduced cancer burden. While the evidence on HPV's involvement in OPSCC is growing, there are yet no established screening procedures for HPV-associated oral or oropharyngeal infection [26].

Most cases are diagnosed at advanced stages due to the asymptomatic nature of early lesions and the deep anatomical location of the oropharynx [27]. The creation of easily accessible, cost-effective, and minimally invasive screening techniques is therefore essential. Saliva-based HPV screening, combined with risk factor assessment and clinical oral examination, may be one possible early detection approach in dental practice [28].

Furthermore, research is needed to validate risk prediction models that include behavioral, clinical, and molecular variables. Such tools can facilitate targeted surveillance of high-risk individuals and the referral in a timely manner to otolaryngology or oncology services [29].

In this context, the present study aimed to evaluate the prevalence of oral HPV infection using salivary samples in a dental care-seeking population. Furthermore, it sought to correlate HPV positivity with established risk factors, including smoking, alcohol use, sexual behavior, and oral hygiene status. Ultimately, this study proposes a preliminary clinical algorithm for HPV screening and referral, tailored for integration into routine dental practice.

MATERIAL AND METHODS

Study Design and Ethical Approval

This cross-sectional pilot study was conducted at the University Dental Clinic of Orthodontics II, "Victor Babeș" University of Medicine and Pharmacy, Timișoara, Romania, between January and June 2025. The protocol was approved by the Institutional Ethics Committee (Approval no. 123/2025), and all participants gave written informed consent. The study complied with the Declaration of Helsinki.

Study Population

A consecutive sample of adult patients (≥18 years) attending routine dental visits was enrolled. Exclusion criteria included: active cancer treatment, severe immunodeficiency, acute oral infections, or inability to consent. Demographics, behavioral data (smoking, alcohol, sexual practices), and HPV vaccination status were collected via questionnaire. Oral hygiene was assessed using OHI-S, and suspicious mucosal lesions were recorded.

Saliva Collection and HPV Testing

Salivary samples were collected using the SafeCollect™ Saliva Collection Kit (Zymo Research, Cat. No. R1211-E, CE-IVD certified), which contains 2 mL DNA/RNA Shield™ preservative to stabilize nucleic acids and inactivate pathogens at ambient temperature. Participants were instructed not to eat, drink, smoke, chew gum, or brush their teeth for at least 30 minutes prior to sampling.

Saliva was collected between 9:00–11:00 a.m. by asking participants to spit into the funnel until the liquid (excluding foam) reached the marked fill line (~2 mL). The funnel was removed, and the tube was capped with the integrated puncture seal and shaken vigorously to ensure homogenization with the preservative solution. Samples were stored at room

temperature during the collection session, then transported to the laboratory and frozen at -20°C until DNA extraction.

Genomic DNA was subsequently isolated using the **QIAamp DNA Mini Kit (Qiagen, Germany)** according to the manufacturer's instructions. DNA concentration and purity were assessed spectrophotometrically (NanoDrop 2000, Thermo Fisher). Detection of HPV DNA was performed by PCR using consensus primers (MY09/MY11 and GP5+/GP6+), with subsequent genotyping for high-risk HPV16 and HPV18. Amplicons were visualized by electrophoresis on 2% agarose gels stained with ethidium bromide under UV illumination.

Risk Factor Assessment

Participants were categorized by:

- Smoking: non-, former, or current;
- Alcohol: none, occasional (<7 units/week), regular (≥ 7 units/week);
- Sexual behavior: oral sex (yes/no), number of partners;
- Oral hygiene: good, moderate, poor (OHI-S);
- Suspicious lesions: presence, site, appearance.

Quality Control and PCR Validation

To ensure sample integrity, each extraction batch included a negative extraction control (nuclease-free water processed in parallel) and a positive control (HPV16 plasmid DNA, ATCC). A β -globin PCR (PC04/GH20 primers) was performed on all samples as an internal control of DNA quality and to exclude false negatives due to insufficient cellular material. Only β -globin-positive samples were considered valid for HPV testing. Amplification reactions included a no-template control (NTC) to monitor for contamination. Reproducibility was verified by duplicate PCR runs on randomly selected samples (10%).

Statistical Analysis

Data were analyzed using SPSS. Categorical variables were reported as counts (%), continuous as mean $\pm$ SD. Associations with HPV status were tested via χ^2 /Fisher's exact test and t-test/Mann-Whitney U test. Variables with $p < 0.1$ in univariate analysis were included in multivariate logistic regression. Odds ratios (OR) with 95% CI and ROC curve analysis were used to assess predictors. Statistical significance was set at $p < 0.05$.

RESULTS

A total of 30 patients (16 males and 14 females) were included in the study, with a mean age of 39.4 ± 10.7 years (range: 22–61 years). All enrolled participants met the eligibility criteria and provided complete clinical and questionnaire data (Figure 1).

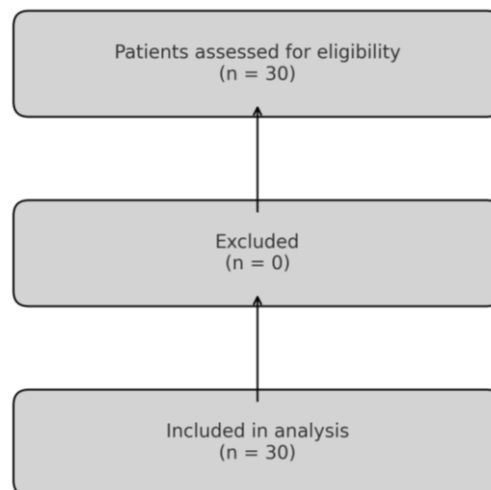


Figure 1. Flowchart of patient enrolment

Diagram showing the selection process: all 30 eligible patients were included in the final analysis.

Table 1 summarizes the demographic and clinical characteristics of the study population. Current smoking was reported in 33.3% of participants, and 63.3% acknowledged engaging in oral sexual practices. Poor oral hygiene was documented in 20.0% of patients, while 26.7% presented with suspicious mucosal lesions on clinical examination.

Table 1. Demographic and baseline clinical characteristics of the study population n (N = 30)

Variable	Total (N=30)	Males (n=16)	Females (n=14)
Age, mean $\pm$ SD (years)	39.4 $\pm$ 10.7	40.8 $\pm$ 9.6	37.7 $\pm$ 11.9
Age range (years)	22–61		
Smoking status			
– Non-smokers	15 (50.0%)	6 (37.5%)	9 (64.3%)
– Former smokers	5 (16.7%)	3 (18.8%)	2 (14.3%)
– Current smokers	10 (33.3%)	7 (43.8%)	3 (21.4%)
Alcohol consumption			
– None	11 (36.7%)	4 (25.0%)	7 (50.0%)
– Occasional	12 (40.0%)	7 (43.8%)	5 (35.7%)
– Regular	7 (23.3%)	5 (31.3%)	2 (14.3%)
Oral sexual practices	19 (63.3%)	12 (75.0%)	7 (50.0%)
Oral hygiene status			
– Good	11 (36.7%)	5 (31.3%)	6 (42.9%)
– Moderate	13 (43.3%)	8 (50.0%)	5 (35.7%)
– Poor	6 (20.0%)	3 (18.8%)	3 (21.4%)
Suspicious mucosal lesions			
– Present	8 (26.7%)	5 (31.3%)	3 (21.4%)
– Absent	22 (73.3%)	11 (68.8%)	11 (78.6%)

HPV DNA was detected in saliva samples from 6 patients, corresponding to a prevalence of 20.0% (95% CI: 8.4–37.6%). Among the HPV-positive individuals, high-risk genotypes (HPV16 or HPV18) were identified in the majority of cases. The distribution of behavioral and clinical variables by HPV status is shown in Table 2.

Table 2. Distribution of clinical and behavioral factors according to HPV status

Variable	HPV-positive (n=6)	HPV-negative (n=24)	p-value (χ^2 /Fisher)
Age, mean $\pm$ SD (years)	41.3 $\pm$ 11.4	38.9 $\pm$ 10.5	0.571
Male sex (n, %)	4 (66.7%)	12 (50.0%)	0.648
Current smokers (n, %)	4 (66.7%)	6 (25.0%)	0.072
Alcohol (regular) (n, %)	3 (50.0%)	4 (16.7%)	0.123
Oral sex reported (n, %)	6 (100%)	13 (54.2%)	0.045 *
Poor oral hygiene (n, %)	3 (50.0%)	3 (12.5%)	0.037 *
Suspicious lesions (n, %)	5 (83.3%)	3 (12.5%)	0.004 *

HPV-positive patients were more likely to report current smoking (66.7% vs. 25.0%, $p = 0.072$) and regular alcohol consumption (50.0% vs. 16.7%, $p = 0.123$), although these associations did not reach statistical significance. The presence of poor oral hygiene (50.0% vs. 12.5%, $p = 0.037$) and self-reported oral sexual practices (100% vs. 54.2%, $p = 0.045$) were significantly associated with HPV infection. Notably, suspicious mucosal lesions were identified in 83.3% of HPV-positive patients compared to 12.5% in the HPV-negative group ($p = 0.004$).

A logistic regression model was constructed to identify independent predictors of HPV positivity (Table 3).

Table 3. Multivariate logistic regression for predictors of HPV positivity

Predictor variable	Odds ratio (OR)	95% Confidence interval	p-value
Current smoker	4.80	0.72–31.78	0.106
Oral sex practices	9.33	0.98–89.12	0.052
Poor oral hygiene	7.00	1.02–48.12	0.047 *
Suspicious lesions	19.17	2.46–149.44	0.005 *

The presence of suspicious oral lesions was the strongest independent predictor (OR = 19.17; 95% CI: 2.46–149.44; $p = 0.005$), followed by poor oral hygiene (OR = 7.00; 95% CI: 1.02–48.12; $p = 0.047$). Oral sex practices approached statistical significance (OR = 9.33; 95% CI: 0.98–89.12; $p = 0.052$). The overall model demonstrated excellent discriminatory performance with an AUC of 1.00 on ROC curve analysis (Figure 2).

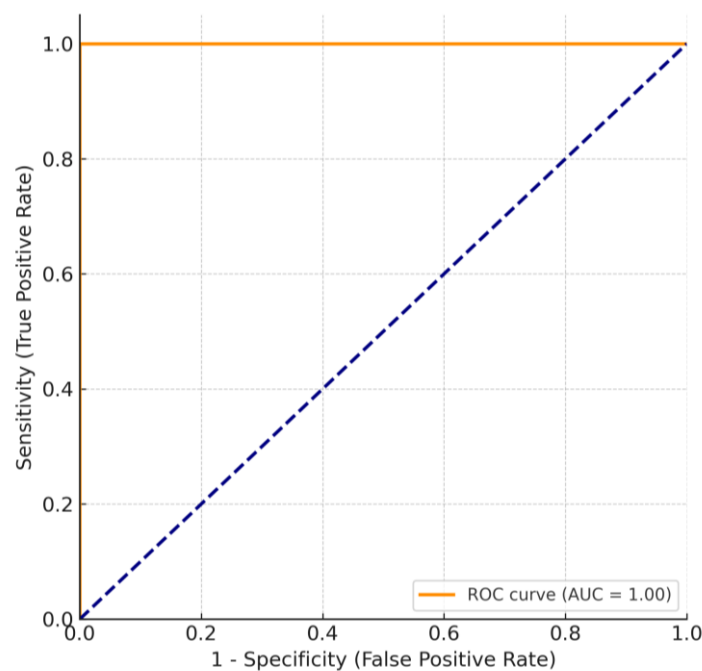


Figure 2. ROC Curve for Predictive Model: The ROC curve shows excellent model performance in predicting oral HPV positivity, with an AUC of 1.00 based on clinical predictors

DISCUSSIONS

Our pilot investigation among dental patients revealed an oral HPV prevalence of 20%, with high-risk genotypes predominating. Although based on a limited sample, these findings align with recent estimates reporting global oral HPV prevalence between 5–15% in the general population, with higher rates among subgroups such as smokers, individuals with poor oral hygiene, and those engaging in high-risk sexual behaviors [30,31]. European data show similar patterns in community-based cohorts and elevated prevalence in selected clinical populations, underscoring the clinical relevance of our observations in the Romanian dental setting, where systematic data remain limited [30,31].

A notable aspect of our study is the observed association between HPV positivity and lifestyle factors, particularly smoking and inadequate oral hygiene. Prior work indicates that tobacco smoke disrupts epithelial barrier function, induces oxidative stress, and modulates immune surveillance, thereby favoring HPV acquisition and persistence [32–34].

Concurrently, poor oral hygiene has been linked to chronic inflammation and dysbiosis, which may create a permissive environment for viral infection and carcinogenesis,

including oral squamous cell carcinoma (OSCC) [35]. Our results therefore add to the growing body of evidence suggesting that preventive counseling in dental practice should extend beyond mechanical plaque control to address broader lifestyle determinants [32–35].

Sexual behavior, and particularly the practice of oral sex, was reported by all HPV-positive participants in our study, consistent with large epidemiological datasets showing that sexual exposure is a key correlate of oral HPV infection [36]. Moreover, the lifetime number of oral sex partners has been identified as a major risk factor for oropharyngeal HPV infection and subsequent oropharyngeal squamous cell carcinoma (OPSCC) [37]. These observations reinforce the necessity of incorporating sensitive, patient-centered sexual history taking into routine dental assessments [36,37].

Clinically, HPV-positive patients more frequently presented with suspicious mucosal lesions. This echoes prior reports describing papillomatous growths, leukoplakia, and non-healing ulcers as potential early signs of HPV-related pathology, warranting further diagnostic work-up, including salivary or tissue-based molecular testing [38,39]. Within this context, saliva-based testing represents a valuable adjunct for triaging patients with mucosal abnormalities, particularly when biopsy is not immediately feasible [40].

The potential of saliva as a minimally invasive diagnostic matrix for HPV detection has been emphasized over the past decade. Salivary HPV DNA can reflect ongoing oral infection and, in some cases, may anticipate subclinical oropharyngeal disease [41,42]. Prospective investigations further indicate that persistent detection of high-risk HPV DNA in saliva is associated with increased OPSCC risk and with disease monitoring utility, supporting its use as a surveillance biomarker in appropriate settings [43]. Our results support the feasibility of salivary screening in dental clinics, with implications for risk stratification and timely referral [40–43].

From a public health perspective, dentists occupy a strategic role in HPV-related cancer prevention. Routine dental visits enable both screening (visual and molecular) and **education**, including counseling on recognized behavioral risk factors and on HPV vaccination [43–45]. Although the vaccine was introduced primarily for cervical cancer prevention, accumulating evidence supports protective effects against HPV-driven head and neck disease as well [44]. Nonetheless, surveys indicate variable awareness among dental professionals regarding the HPV–OPSCC link and inconsistent counseling about vaccination, highlighting the need for curricular enhancements and continuing professional development [45].

Study Limitations

This study has limitations. First, the small sample size restricts statistical power and generalizability. Second, the cross-sectional design precludes assessment of **persistence**—a key determinant of oncogenic risk [43]. Third, behavioral data were self-reported and thus susceptible to recall and social desirability bias. Despite these constraints, our pilot provides preliminary evidence supporting the integration of salivary HPV screening into dental practice. Future work should validate these findings in larger, longitudinal cohorts and develop predictive algorithms combining molecular, clinical, and behavioral markers to refine risk stratification and guide earlier referral and prevention [40–45].

CONCLUSIONS

This pilot study highlights the feasibility and relevance of salivary HPV screening in a dental setting. Among the 30 adult patients examined, oral HPV infection was detected in 20% of cases, with a predominance of high-risk genotypes. HPV positivity was associated with identifiable risk factors, including current smoking, oral sexual behavior, poor oral hygiene, and the presence of suspicious mucosal lesions.

These findings suggest that dentists, through routine clinical examinations and structured risk assessments, can play a pivotal role in the early detection and referral of patients at increased risk for HPV-related oropharyngeal cancer. Incorporating salivary HPV testing into dental practice—alongside patient education and preventive counseling—could significantly enhance early identification efforts.

Although limited by its small sample size and cross-sectional design, this study provides a foundation for future research aimed at developing integrated screening protocols in dentistry. Larger prospective studies are warranted to validate these preliminary findings and to assess the predictive value of combined clinical, behavioral, and molecular parameters in identifying high-risk individuals.

Conflicts of Interest

The authors declare no conflict of interest.

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Comparative Histological Analysis of Normal and Supernumerary Teeth: Clinical and Morphological Implications

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Abstract

This study aims to highlight the histological differences between normal (temporary and permanent) teeth and supernumerary teeth by analyzing enamel, the pulpo-dentin complex, and cementum. Through comparative microscopy of prepared histological slides, the research identifies microstructural peculiarities that may support a better understanding of the development, diagnosis, and management of dental number anomalies. This study involved histological analysis of 12 permanent preparations obtained from supernumerary, temporary, and permanent teeth, aiming to highlight the microstructural differences between normal and supernumerary dentition. Clinical cases and laboratory protocols, including decalcification, paraffin embedding, microtomy, and

hematoxylin-eosin staining, allowed detailed observation of enamel prism organization, dentin tubule arrangement, and cementum characteristics. Histological comparison revealed that supernumerary teeth exhibit significant structural deviations, including atypical enamel prism orientation, reduced dentinal tubule density with frequent interglobular spaces, and a poorly differentiated cementum. These anomalies suggest incomplete mineralization and altered tissue development, potentially linked to the frequent impaction and limited functional integration of supernumerary teeth. Supernumerary teeth, although not pathological entities per se, can disrupt the harmony of the dento-maxillary system, affecting eruption, bone development, and dental aesthetics. Histologically, they exhibit morpho-structural hypodifferentiation, with disorganized enamel, reduced dentinal tubules, and imprecise cementum stratification, indicating a lower functional potential. These findings support the importance of histological evaluation in treatment planning and justify a conservative, individualized therapeutic approach based on both structural integrity and functional prognosis.

Keywords: Supernumerary teeth, Mesiodens, Histological analysis, Mineralization defects, Dento-maxillary anomalies

INTRODUCTION

Dental number variations, either in the form of agenesis or by the presence of supernumerary teeth, are abnormalities with a relative impact on general health, but which can provide valuable information to those who study them carefully [1]. The literature reflects a constant interest, marked by an ebb and flow determined by factors such as the novelty of the findings, the frequency of cases, the social implications, the etiopathogenic perspectives and the changes in therapeutic approaches. Thus, this segment of dento-maxillary pathology remains a current topic and open to continuous research [2]. Given the frequent association of supernumerary teeth with numerous genetic syndromes, research has focused on identifying the genes involved and the transmission mechanisms, both from a genotypic and phenotypic point of view. Family studies have demonstrated in numerous cases the existence of a simple, Mendelian type heredity. In contrast, sporadic forms of isolated supernumeraries appear to be multifactorial, as a result of *de novo* mutations or the action of teratogenic agents during critical periods of dental tissue differentiation. The genetic predisposition for the appearance of these abnormalities is the result of gene interactions, but its expression is influenced by environmental factors in particular the timing (prenatal or postnatal), duration and intensity of exposure. However, to date, no specific genetic mutations associated with non-syndromic supernumerary teeth have been identified [3]. The current approach to dental number anomalies is mainly guided by their orthodontic relevance, but the interest in these anomalies also extends to the anthropological, genetic-phylogenetic and evolutionary spheres, justifying the need for in-depth studies. The observation that these anomalies no longer follow the classical patterns requires further research, especially in the context of the phylogenetic trend of reducing the dental formula.

Hyperdontia is defined as a dental anomaly characterized by the presence of a greater number of teeth than normal, affecting both temporary and permanent dentition. In the literature, the terms most commonly used for this anomaly are "supernumerary teeth" (Agrestini and Sfasciotti, Benagiano, Boboc, Cadenat, Chaput, Chateau, Dechaume, Firu, Gysel, Orlando) and "hyperdontia" (Broglia, Cadoni, Lundstrom, Rocia). Other names encountered include: extra teeth, hyperodontogenesis, poliodontics or pleiodontics. Of these, the terms "supernumerary teeth" and "hyperdontia" are the most expressive and widely accepted [1]. The classification of supernumerary teeth varies according to several criteria: topography, morphology, chronology, etiology, clinical aspects and therapeutic objectives. According to the topographic criterion, supernumerary teeth can be located in various regions: mesiodens (between the upper central incisors), paramolar (between the 2nd and 3rd upper molars), dystomolar (distal to the last molar), parapremolar (in the premolar area), but also in atypical forms, such as paramolar roots or additional tubercles [1]. Morphologically, Mitchell [4] proposes four forms: conical, tuberculate, additional, and odontoma. The frequency of hyperdontation varies: in the temporary dentition it is between 0.2-2%, and in the permanent one between 0.1-5%.

The majority of cases (90-98%) occur in the jaw, of which 75% are mesiodentic. In temporary dentition, it occurs more often in boys, unilaterally and in the incisor-canine area, with a shape and size similar to normal teeth. In permanent dentition, hyperdontation is significantly more common in men, predominantly in the jaw, especially in the incisor and molar regions. It can occur unilaterally or bilaterally and with variable morphological forms, being located on the arch, ectopic or included [5]. From the perspective of therapeutic objectives, Boboc (1971) distinguishes between supernumerary teeth that do not influence the eruption of normal teeth and those that determine their inclusion [6]. Depending on the association with genetic pathologies, hyperdontia can be classified into syndromic and non-

syndromic forms [7]. Although each classification criterion reflects a specific organizational logic, none manages to fully encompass the complexity and variability of this dental anomaly. As for the etiology of hyperdontia, most authors adhere to the atavistic or descent theory, according to which supernumerary teeth represent a return to ancestral dental formulas. This theory explains their appearance either as a reactivation of ancestral dental patterns (Magitot, Thompson, Rosenberg, Agrestini, Sfaciotti) or as the reappearance of elements that disappeared during phylogenesis (Orlando). According to this perspective, the dental formula of primates - 3 incisors, 1 canine, 4 premolars and 3 molars per hemiarch would be the reference point for assessing the atavistic character of hyperdontia.

Thus, the presence of Mesiodians is considered a phylogenetic echo of evolutionarily reduced incisors. On the other hand, the appearance of teeth outside the known areas of reduction or of atypical forms, such as the supernumerary canine, raises questions about the complete validity of this theory, since the canine does not normally appear multiplied even in primitive dental formulas. In this context, some authors support the theory of dental blade overproduction as an alternative mechanism, based on the model of reptilian polyphyodontism [8]. At the same time, a series of morphological peculiarities of supernumerary teeth are noted. Although they may resemble normal teeth, dysmetabolic changes often occur that affect their eruption, positioning, vascularization, innervation and masticatory functionality. Depending on the time of appearance in relation to the normal dentition, three categories are distinguished: Before the normal dentition the teeth form a predeciduous or preprimary dentition, being attached to the gums without a root. Neonatal teeth rarely appear, usually in the area of the lower incisors, in the first months of life. After normal dentition they appear as part of a post-permanent dentition. They can be teeth formed after the completion of the permanent dentition or formed with it, but with delayed eruption. Some cases of late mineralization can only be observed radiologically. Concomitant with normal dentition they are the most common, especially in the jaw (90%), being rarer in the mandible (10%). Most of the time, these teeth remain included, and the diagnosis is based on clinical signs such as: persistent diastemas, dental dystopias, bulging of the alveolar ridge or prolonged maintenance of temporary teeth. Radiography is essential in confirming the diagnosis [9,10]. Supernumerary teeth are often smaller, atypical, with ectopic or inverted positions (with the crown towards the base of the jaw and the root towards the occlusal plane), called anaastrophic. The most common supernumerary tooth is the mesiodens, whose correct name reflects its mesial positioning in relation to the central incisors [10,11].

Aim and objectives

The main purpose of this study is to highlight the histological differences between the teeth belonging to the normal dental series (temporary and permanent) and the supernumerary ones. Through the comparative analysis of the main dental hard tissues enamel, pulpo-dentin complex and cementum the aim is to identify the structural peculiarities that can contribute to the understanding of the atypical development of supernumerary teeth. In order to achieve this objective, teeth from the three mentioned morphological categories were selected and analyzed. Each specimen was prepared in the form of histological slides and examined under an optical microscope in order to highlight: prismatic organization of enamel; arrangement of dentin canalicules and predentin characteristics; the structure of the cement (acellular or cellular) and the degree of mineralization. Through this approach, the study aims to provide an integrated perspective on the microstructural differences between normal and supernumerary teeth, thus contributing to the substantiation of the etiology, diagnosis and therapeutic planning in the case of dental number anomalies.

MATERIAL AND METHODS

In order to carry out this comparative study, permanent histological preparations obtained from three categories of teeth were analyzed: supernumerary, temporary and permanent, aiming to highlight the structural differences between the teeth of the normal series and the supernumerary ones. Informed consent was obtained from all participants prior to inclusion in the study. Vital preparations have not been used, as their scope is limited to cells that can be easily dissociated, such as muscle cells. A total of 12 histological slides were examined, which included sections of the three main types of dental tissue: enamel, pulpo-dentine complex, and cementum. For each blade, a comparative analysis was performed between the supernumerary teeth and those of the normal series (temporary or permanent).

Case 1 - Supplemental (eutopic) supernumerary tooth in the upper lateral region

Patient C.E., aged 8, presented to the Pediatric Dentistry Clinic of University of Medicine and Pharmacy "Victor Babeș" from Timisoara for dental care. Clinical examination highlighted: mixed dentition, persistence of the temporary lateral incisor 6.2; vestibularization of the central incisors 1.1. 2.1 and absence of the lateral incisor 1.2; 3-4 mm interincisive diastema. Paraclinical investigations (Figure1, Figure2): Orthopantomography and occlusal radiography: highlighting a supplemental (eutopic) supernumerary tooth (2.2-bis), located palatally; Study model: U-shaped, discontinuous and asymmetrical upper arch.



Figure 1. Initial orthopantomography of the patient C. E.

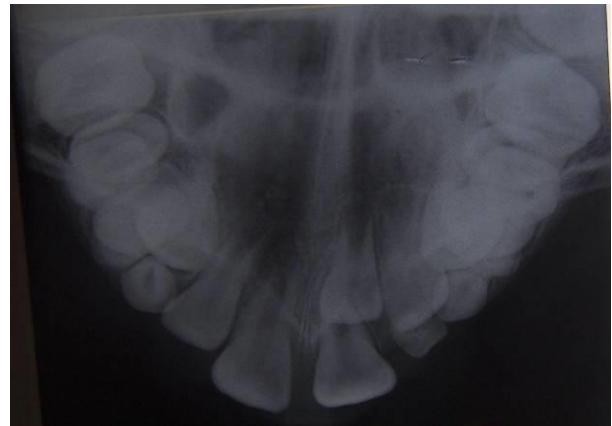


Figure 2. X-ray from occlusal incidence what detects a eutopic supernumerary tooth

The examination of the study model highlights an upper arch in the shape of the letter "U", discontinuous and asymmetrical. In the sagittal direction, a distalized ratio of class 2 Angle is observed at the molar level, and in the frontal area there is an overjet of about 10 mm. In the vertical direction, an overbite of 1/1 is observed in the frontal area, which denotes a deep occlusion.

Diagnosis: Class II dento-maxillary anomaly, subdivision 1 Angle; The presence of a eutopic supernumerary tooth.

Treatment: Surgical stage: extraction of teeth 6.2 and 2.2-bis (Figure 3; Figure4); Orthodontic stage: use of a myofunctional silicone appliance (Multi T-Rocky Mountain).



Figure 3. Extracted supernumerary tooth together with its follicular sac 2.2 bis



Figure 4. Extracted temporary tooth 6.2

Case 2 - Tuberculate mesiodens with rotation of central incisors

Patient S.A., 11 years old, presented at Pediatric Dentistry Clinic of University of Medicine and Pharmacy "Victor Babeș" from Timisoara for orthodontic treatment.

Clinical examination: Mesiodens on the midline; Mesiorotation of the incisor 2.1, dystorotation 1.1; 6 mm interincisive diastema.

Paraclinical investigations: Orthopantomography: tuberculous positioning of the mesiodense between 1.1 and 2.1 (Figure 5); Study model: U-shaped arches, dento-alveolar disharmony.



Figure 5. Initial orthopantomography of patient S. A.

Diagnosis: Overjet: 6–7 mm; overbite: 1/3; Absence of 6-year-old molars (unidentifiable Angle Class). Treatment: Surgical stage: mesiodens extraction. Orthodontic stage: Multi P (Rocky Mountain) appliance (Figure 6).



Figure 6. Extracted supernumerary tooth

Case3 - Conical mesiodens associated with hypodontia

Patient P.A., 7 years old, presented for dental treatments at Family Dental Clinic for orthodontic treatment.

Clinical examination: Mixed dentition, absence 2.1, presence of a mesiodens in the frontal region. Paraclinical investigations: X-ray: conical mesiodens, inclusion 2.1, absence of bud 3.5 (hypodontia) (Figure 7); Study design: 6 mm interincisor diastem, class I molar Angle



Figure 7. Initial orthopantomography of patient P. A.

Treatment: Surgical stage: mesiodens extraction (Figure 8); Orthodontic stage: Multi T (Rocky Mountain) appliance → 2.1 rash; Subsequently: upper and lower fixed orthodontic appliance for occlusal alignment and stabilization.



Figure 8. Extracted supernumerary tooth

After 8 months of the completion of the surgical stage and the beginning of orthodontic treatment, the alignment of the permanent incisor 2.1 on the arch was observed, which had been held in inclusion by the persistence of the supernumerary tooth (Figure 9). Subsequently, a fixed upper and lower orthodontic appliance was applied, in order to align

and level the dental arches, and to stabilize the occlusal relationships, the patient being still in treatment.



Figure 9. Radiological appearance after extraction of the supernumerary tooth

Histological analyses

Descaling

In order to obtain fine sections of hard tissues, it was necessary to pre-decalcify the teeth. 10% trichloroacetic acid combined with concentrated formalin has been used as a decalcifying agent, as it ensures good preservation of cellular structures. Descaling was carried out in containers with a volume of 100–200 times larger than the volume of the part. The parts were suspended in solution with the help of a nylon thread to speed up the process. The completion of the decalcification was evaluated by physical methods (palpation, needle pricking, sectioning) and chemical methods (methyl orange and ammonia test). Subsequently, the parts were washed with 96% alcohol, avoiding additional sulfate treatments due to the compatibility of the descaling agent used.

Inclusion in paraffin

The steps included in the inclusion protocol were:

Dehydration - progressively performed in alcohol baths, paying attention to the volume and fragility of the pieces, to prevent structural deformations.

Clarification - benzene was used as a clarifying agent due to the effective penetrability and increased transparency of the parts. The clarification was carried out in glass containers, with successive reagent exchanges.

Paraffin - consisted of impregnating the pieces with molten paraffin (55-56°C), in 3 successive baths, each lasting 1-2 hours, depending on the size of the pieces.

The actual inclusion - was achieved by pouring the paraffin into a Leuckart shape, with the careful orientation of the piece in relation to the sectioning plane.

Sectioning

After the complete hardening of the paraffin blocks, the paraffin microtome was sectioned. The thin sections (5-7 µm) were obtained in continuous ribbons and then mounted on object slides, pre-degreased, using Mayer albumin-glycerin as a fastening agent. The sections were spread by hydration and heat application on a thermally adjustable plate,

aiming to remove the creases and distribute the section evenly. The slides were later dried and prepared for coloring.

Coloring

The staining of the histological sections was performed using the standard **hematoxylin-eosin** technique, according to the following protocol: Dewaxing in three successive benzene/toluene baths; Gradual hydration by switching from alcohol to distilled water; Hematoxylin impregnation for nucleus highlighting and eosin for cytoplasmic structures and extracellular matrix. In some cases, collodination has been applied to improve the adhesion of thick or dense tissue sections, except in situations involving metallic or immunohistochemical impregnation techniques.

RESULTS

On histological preparations obtained by decalcification, the enamel is completely removed from the surface of the dental crown, which causes the appearance of a clearly delineated, chromophobic space, called the enamel space. For this reason, for the detailed examination of the enamel structure, microscopic analysis of dry and polished dental preparations, without decalcification, was used, which allow the structural integrity of this tissue to be preserved. In the normal series teeth, microscopic examination revealed the typical, wavy path of enamel prisms in the direction of the enamel-dentin junction (JSD). On cross-section, the prisms exhibit the characteristic morphology of "keyhole" or "fishtail". In the peripheral areas, between the prisms, aprismatic enamel was identified, lacking a defined prismatic orientation. In the case of supernumerary teeth, there were obvious atypologies and asymmetries in the orientation of the enamel prisms.

The head of the prism is no longer clearly directed towards the tip of the cusps or the incisive area, presenting a pronounced obliquity, probably influenced by variations in the amount of adjacent interprismatic substance. Also, a marked extension of the enamel lamina was observed, some structure rich in organic material suggesting a possible mineralization deficit in the formative period of the enamel of supernumerary teeth. As for the interprismatic substance, it retains its classic arrangement pattern, especially in the cervical area, both in permanent and decidual teeth (Figure 10; Figure 11).



Figure 10. Trajectory of the enamel prisms of a tooth of the normal series

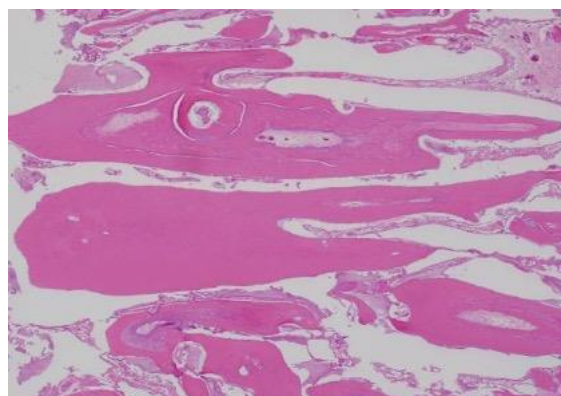


Figure 11. Interprismatic substance of a supernumerary tooth

In the structure of the teeth of the normal series, the primary dentin constitutes the largest part of the dental mass, surrounding the pulp chamber and presenting an external layer organized in the form of the dentin mantle. The dentinal tubules are arranged radially with respect to the pulp chamber, having a sinuous trajectory, especially in the crown region, where they form "S" curves, less pronounced in the root region. At the level of the terminal

portion, near the enamel-dentin (JSD) or cement-dentin (JCD) junction, the tubules have terminal and lateral branches, sometimes with cytoplasmic extensions, sometimes without (Figure 12; Figure 13). The intertubular dentin, located between the tubules, is made up of a network of type I collagen fibrils, on which hydroxyapatite crystals are deposited. It is the primary secretion product of odontoblasts and gives structural strength to the dentin matrix. In supernumerary teeth, a significant reduction in the number of dentin tubules has been observed, although their overall trajectory remains comparable to that of the normal series. This change causes an increase in the amount of intertubular dentin, giving a denser histological appearance, but with a reduction in the active mineralized content. In the circumpulparous area, immediately below the dentin mantle, the frequent presence of hypomineralized interglobular dentin was highlighted, characterized by the accumulation of chalcospheres and the absence of intratubular dentin. A relevant peculiarity for supernumerary teeth is the marked presence of Czermack interglobular spaces, observed in increased numbers, especially in the circumpulparous area. These spaces reflect an incomplete mineralization process, being associated with an uneven distribution of the hydroxyapatite crystal, without altering the general architecture of the dentin tubules.



Figure 12. Odontoblastic palisade from periphery of the dental pulp



Figure 13. Irregular dentine tubules of a supernumerary tooth (cross-section)

The cementum and periodontal ligament are part of the periodontal complex, along with the alveolar bone and gum, having an essential role in the fixation and functionality of the tooth.

In the teeth of the normal series, a clear distribution of the two types of cement was observed:

The acellular, fibrillar (primary) cementum uniformly covers the root surface, being devoid of cells and presenting numerous perpendicular striations on the external surface. They correspond to the insertion paths of the Sharpey fibers, which anchor the tooth in the alveolar bone. The cellular cementum (secondary) is located especially in the apical area of the root and at the level of the root bifurcations, containing gaps that harbor cementocytes, involved in the synthesis and remodeling of the cementary matrix. In the case of supernumerary teeth, histological analysis revealed a lack of clear demarcation between the two types of cement. The areas of interpenetration of the cellular and acellular cementum suggest a mixed, functionally undifferentiated structure, indicating an incomplete or altered development of this tissue. The periodontal ligament, also present in supernumerary teeth, maintains the insertion of collagen fibers (especially type I and III collagen) in the cementum, but their organization is deficient. The fibers do not always show the typical orientation seen during tooth eruption. This can be explained by the fact that supernumerary teeth are often in inclusion, which prevents the normal development of the ligament and limits its supporting function. The interstitial tissue in the periodontal ligament is of the lax connective type,

containing blood vessels, lymphatics and nerve endings, but in the case of supernumerary teeth, these structures appear to be more poorly represented, supporting the hypothesis of reduced or absent functionality (Figure 14).

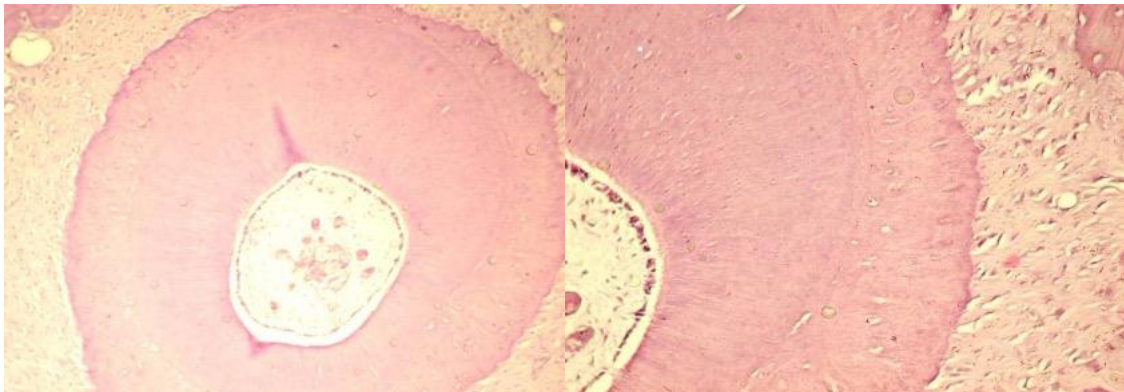


Figure 14. Cement and periodontal ligament

DISCUSSIONS

The results obtained from the comparative histological analyses of the teeth of the normal series (temporary and permanent) and of the supernumerary teeth show significant differences in the structure of dental hard tissues - enamel, dentin and cementum as well as in the organization of the periodontal ligament.

In the case of enamel, the organized structure of the prisms, with a wavy path towards the enamel-dentin junction and the characteristic morphology of "keyhole", was evident in normal teeth. In contrast, the supernumerary teeth exhibited atypia of prismatic orientation, a marked obliquity of the prism head, and an enlargement of the enamel lamella, suggesting a mineralization deficit during the developmental period. These findings are consistent with the literature, which indicates an increased variability in enamel organization in teeth formed outside the usual genetic pattern [12, 13].

At the dentin level, the supernumerary teeth had a reduced number of dentine tubules, compared to normal teeth, despite a similar "S" shaped trajectory. This change has led to an abundance of intertubular dentin, but also to an increased presence of interglobular dentin, especially in the circumpulpary area. The Cermack interglobular spaces, more numerous in the case of supernumerary teeth, reflect an incomplete or discontinuous mineralization process, being a possible cause of structural fragility and functional limitation of these teeth. As far as the cementum is concerned, the teeth of the normal series have a clear delimitation between the acellular cementum (fibrillary), involved in the insertion of the periodontal ligament, and the cellular cement, with a regenerative role. Supernumerary teeth, however, are characterized by an imprecise topography of the two types of cement, with interpenetration areas and mixed structures, not functionally differentiated, an aspect that can negatively influence the ability to integrate into the periodontal system [14,15]. The periodontal ligament associated with the supernumerary teeth also showed organizational abnormalities, with an incorrect or poorly expressed orientation of the collagen fibers. Probably, the frequent inclusion of these teeth prevents the mechanical stimulation necessary for the normal development of the ligament and causes a functional deficit in terms of anchoring and dental support. The comparative histological analysis of the normal and supernumerary teeth highlights structural differences that can influence their functionality within the dento-maxillary apparatus. Studies have shown that although supernumerary teeth may appear morphologically similar to normal ones, they often exhibit developmental

abnormalities and structural differentiation [12, 15]. The enamel of supernumerary teeth exhibits changes in the orientation and organization of prisms, as well as an increased extension of organic areas, such as the enamel lamina, suggesting a deficit of mineralization and differentiation during the developmental period [16]. At the level of dentin, significant differences were observed between normal and supernumerary teeth. In the case of the latter, the dentine tubules are fewer and more irregularly distributed, and the presence of interglobular dentin with the predominance of chalcophosphates indicates an increased degree of hypomineralization [18,19]. This type of organization is frequently associated with a decrease in structural strength and can affect the functional integrity of the tooth. As far as cement is concerned, the presence of an imprecise delimitation between cellular and acellular cementum in the case of supernumerary teeth, as well as the interpenetration of the two types, suggests an atypical development of this tissue [20,21].

This mixed, functionally undifferentiated structure can affect the stability and functionality of the periodontal ligament. The periodontal ligament, in fact, has a low degree of adaptation in the case of supernumerary teeth. Its functional deficit is probably determined by the fact that many of these teeth remain included, without benefiting from normal functional stimuli [3]. The ligament structure, rich in type I and III collagen, is essential for keeping the tooth in the alveolus, and its alterations can compromise the stability of the tooth [3]. The presence of supernumerary teeth is frequently associated with the appearance of clinical complications such as delayed eruption, malpositions or retention of permanent teeth. In these cases, the therapeutic intervention must be decided individually, depending on the location and morphology of the supernumerary teeth, and extraction is often recommended [8, 2]. The literature emphasizes the importance of an early diagnosis and a personalized therapeutic plan, as supernumerary teeth can contribute to the appearance of complex dento-maxillary anomalies that require subsequent orthodontic treatments [22]. These findings suggest that supernumerary teeth, although they may appear morphologically similar to those of the normal series, present significant histological differences, especially in terms of mineralization, structural organization and periodontal functionality[22,23]. These aspects must be taken into account in the clinical evaluation and therapeutic decision, especially in the orthodontic or surgical context.

CONCLUSIONS

Based on the national and international literature, corroborated with the data obtained from the comparative histological analysis performed in this study, the following conclusions can be drawn:

Although the phylogenetic evolution of man is oriented towards simplifying and reducing the dental formula, in contemporary dental practice supernumerary teeth with various locations, shapes and sizes continue to be found. This clinical reality requires the careful study of these structures as expressions of atypical dental development and not as simple evolutionary accidents. Supernumerary teeth are not pathological entities in themselves, but their presence can disrupt the balance of the dento-maxillary apparatus. Thus, they can negatively influence the growth and development of maxillary bones, the eruption of normal series teeth and, indirectly, they can have psychological repercussions on the child, by affecting dental aesthetics or the appearance of oral dysfunctions. The therapeutic indication of supernumerary teeth is determined by the position, number and impact on the eruption. In most cases, extraction is the preferred solution for preventing orthodontic complications. There are, however, particular situations in which the supernumerary tooth can be maintained and integrated into the arch, replacing a tooth of the normal series, depending on its functional and aesthetic potential.

The concomitant appearance of supernumerary teeth with dento-maxillary skeletal anomalies requires a complex therapeutic approach. In such cases, treatment often begins with the surgical extraction of extra teeth, followed by orthodontic therapy to correct occlusion and dental alignment abnormalities. Histologically, supernumerary teeth have a structure generally similar to that of normal teeth, but microscopic analyses reveal a discrete morpho-structural hypodifferentiation. It is manifested by asymmetries in the organization of the enamel, a reduced number of dentinal tubules and imprecise distribution of cellular and acellular cementum. Microscopically highlighted structural changes – especially areas with incomplete or deficient mineralization (chalcospheres, interglobular spaces, extended enamel blades) – suggest a reduced functional potential of supernumerary teeth compared to those of the normal series. This aspect justifies the conservative and individualized approach in the therapeutic plan, since maintaining a tooth with a poorly mineralized structure can compromise the general functionality of the dento-maxillary apparatus. Overall, the results obtained emphasize the importance of histological evaluation in understanding the morphological differences between supernumerary and normal teeth. They support the need for a nuanced clinical decision, based both on radiological and functional criteria, as well as on the potential for biological integration of the teeth in question.

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Beyond The White Smile: In Vitro Insights into Carbamide Peroxide Bleaching by Confocal Microscopy

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Abstract

1. Background/Objectives: Tooth bleaching with carbamide peroxide (CP) is one of the most common aesthetic procedures in modern dentistry, yet its effects on enamel ultrastructure remain a matter of concern. This in vitro study aimed to qualitatively assess microstructural changes in human enamel following in-office bleaching with two professional CP formulations – Opalescence Quick 45% and Pola Office+ 37.5% – in accordance with ISO 28399:2021 guidelines. **2. Methods:** Forty extracted human molars and premolars were sectioned to obtain paired specimens, with one half treated and the other serving as control. Bleaching protocols were performed under standardized conditions, and samples were analyzed using laser scanning confocal microscopy (CLSM) with three-dimensional reconstruction to evaluate enamel prism organization, porosity, and dentinal tubule exposure. **3. Results:** Both agents induced morphological alterations, including partial prism disorganization, increased porosity, and widening of interprismatic spaces. Opalescence Quick, with its higher CP concentration, produced more pronounced structural effects compared with Pola Office+, which exhibited a more moderate impact. Despite these changes, enamel integrity was not severely compromised. **4. Conclusion:** These findings suggest that carbamide peroxide bleaching can be considered safe when performed under professional supervision, particularly when complemented with remineralizing strategies. Future studies should investigate long-term effects and interactions with restorative materials under clinical conditions.

Keywords: tooth bleaching; carbamide peroxide; enamel microstructure; confocal laser scanning microscopy; in-office bleaching; enamel porosity; dental aesthetics

INTRODUCTION

Human social interactions have traditionally emphasized the importance of physical appearance, with the face being the most visible and expressive part. Among facial features, the eyes and mouth are most strongly linked to perceived attractiveness, with smiling serving as a central element of nonverbal communication. The absence of an aesthetically pleasing smile can negatively affect self-esteem, and tooth color has been consistently identified as a critical determinant of smile attractiveness. Dental aesthetics therefore often prioritize both tooth color and shape as key parameters in evaluating smile appeal. Individuals who express satisfaction with the appearance of their teeth, including their color and form, are more likely to project confidence and extroversion, whereas discolored, missing, or damaged teeth may contribute to social discomfort, reduced self-confidence, and negative psychosocial outcomes [1].

In contemporary dentistry, patients increasingly seek not only oral health but also the aesthetic benefits of a flawless smile. Dental appearance plays a crucial role in self-perception and social interactions, with tooth whiteness in particular being strongly associated with improved quality of life before and after treatment [2]. Tooth whitening has become one of the most sought-after aesthetic procedures in modern dentistry, reflecting the growing demand for minimally invasive treatments that enhance the appearance of the smile. Tooth discoloration may result from intrinsic factors linked to pathological processes, but it is more commonly associated with extrinsic factors such as the consumption of coffee, tea, wine, and other pigmented foods, smoking, or the misuse of antibiotics. Substances including tannins, furfurals, carotenoids, artificial dyes, and tetracyclines can alter enamel and dentin structures, producing visible stains [2]. While bleaching procedures using hydrogen peroxide or carbamide peroxide are widely employed to counteract these effects, they are not without drawbacks: tooth sensitivity, gingival irritation, and even reversible pulpitis have been reported, largely due to microstructural alterations such as surface porosity, prism disorganization, or enamel defects. Furthermore, aggressive whitening protocols may disrupt the organic-inorganic balance of the tooth matrix and potentially influence the oral microbiome. Thus, while tooth whitening offers clear aesthetic and psychosocial benefits, it also raises concerns regarding enamel integrity and long-term oral health, emphasizing the need for careful evaluation of its safety and efficacy.

The increasing popularity of tooth whitening has driven the development of diverse treatment modalities, ranging from professionally applied in-office whitening (OW) and dentist-supervised at-home whitening (HW) to over-the-counter (OTC) and do-it-yourself (DIY) products used without clinical oversight [3]. Among these approaches, peroxide- and carbamide-based bleaching agents remain the most widely employed. Nevertheless, their effects on dental ultrastructure are not yet fully elucidated, highlighting the need for further investigation into their impact on enamel integrity and long-term safety [4].

Among the various methods available, bleaching agents based on carbamide peroxide remain widely used due to their efficacy and relative ease of application in clinical settings. Carbamide peroxide is widely employed in various concentrations for both professional in-office whitening procedures and dentist-supervised at-home bleaching. Compared to hydrogen peroxide (H_2O_2), it demonstrates a slower degradation rate, allowing prolonged contact with the tooth surface when administered through customized dental trays. Upon decomposition, peroxides release highly reactive free radicals that oxidize organic chromophores—molecules responsible for extrinsic tooth discoloration caused by substances such as coffee, red wine, and tea. This oxidation process breaks down larger pigmented

compounds into smaller molecules that absorb fewer wavelengths of visible light, thereby producing a perceptibly lighter shade of the teeth [5].

With the growing demand for cosmetic dentistry, it has become increasingly important to evaluate the structural impact of carbamide peroxide-based bleaching agents in order to optimize both treatment efficacy and patient safety. Although these agents are widely used, their potential to alter enamel microstructure necessitates thorough investigation. Laser scanning confocal microscopy (CLSM), combined with three-dimensional reconstruction, provides a powerful tool for assessing enamel surface topography, prism organization, and defect depth under controlled conditions. In this context, the effects of different in-office bleaching protocols on enamel surface characteristics are assessed in accordance with ISO 28399:2021 guidelines [6], which establish safety and performance standards for external tooth whitening products.

Aim and objectives

This study compares the effects of two professional carbamide peroxide formulations—Opalescence Quick 45% and Pola Office+ 37.5%—on dental enamel integrity. The analysis focuses on identifying microstructural changes such as increased porosity, prism disorganization, microcrack formation, and dentinal tubule exposure. By evaluating both treated samples and matched controls originating from the same tooth, the design minimizes biological variability and ensures a reliable comparison of whitening-induced alterations. In this way, the investigation provides a systematic assessment of how product composition and oxidizing agent concentration may influence enamel morphology. The overarching aim of this in vitro study is to determine the structural impact of carbamide peroxide-based whitening agents on enamel through the use of laser scanning confocal microscopy (LSCM). This advanced imaging modality allows for detailed visualization of tissue architecture and the detection of subtle morphological modifications resulting from bleaching treatments. Beyond characterizing the extent of these changes, the study seeks to generate qualitative insights into the safety profile of high-concentration carbamide peroxide gels when applied under conditions simulating clinical dental practice, ultimately informing evidence-based recommendations for their responsible use.

MATERIAL AND METHODS

Ethical Approval and Specimen Collection

Human molars and premolars extracted for clinical reasons were used in this study, in full compliance with established ethical protocols and with the approval of the Ethics Committee of the “Victor Babeș” University of Medicine and Pharmacy, Timisoara (Approval No. 09/11.03.2024). All procedures were conducted in accordance with the principles of the Declaration of Helsinki and the standards of Good Practice in Biomedical Research, ensuring rigorous and ethically sound selection of dental specimens.

Sample Size Calculation

The required sample size was determined using G*Power 3.1 software, with parameters set at a significance level (α) of 0.05, a statistical power of 0.80, and a medium effect size ($d = 0.5$), consistent with values reported in the literature concerning structural alterations of enamel after bleaching. Based on these calculations, a minimum of 40 specimens was established, evenly distributed between two experimental groups ($n = 20$ treated with Opalescence Quick 45% and $n = 20$ treated with Pola Office+ 37.5%). To compensate for potential specimen loss or individual variability, the initial number of samples was increased by approximately 20%.

Sample Preparation

All teeth were obtained from an anonymized tissue bank in accordance with current ethical regulations. Following extraction, soft tissues were carefully removed by periodontal curettage, and specimens were stored in distilled water under controlled temperature for no longer than 30 days to preserve hydration and structural integrity. This storage method was intended to maintain the physicochemical properties of enamel as close as possible to those found in a natural biological environment, avoiding dehydration artifacts or mineral alterations. For additional preservation, the samples were immersed in a 0.1% thymol solution for five days, a commonly used approach to prevent microbial growth while maintaining tissue integrity. Representative specimens used in the study are shown in Figure 1.



Figure 1. Representative human molars and premolars collected from the tissue bank for experimental use

Each extracted tooth was initially examined under $\times 10$ magnification (OMS2356, Zumax Medical Co., Ltd., Suzhou, Jiangsu, China), and specimens presenting carious lesions, cracks, restorations, or enamel defects were excluded. To obtain standardized samples, the teeth were sectioned horizontally 2 mm coronally to the cemento-enamel junction using a diamond disc with continuous water irrigation to prevent overheating and structural alterations. The crowns were subsequently divided longitudinally along the cervico-occlusal axis, producing two symmetrical halves. A unique identification code was assigned to each pair, with one half designated for bleaching treatment and the other retained as an untreated control. This allocation resulted in two experimental groups, each exposed to a different carbamide peroxide-based bleaching agent, and allowed for direct intra-tooth comparison of enamel morphological changes according to the composition and concentration of the whitening products. All fragments were embedded in autopolymerizing acrylic resin (UNIFAST Trad, GC America), and the exposed enamel surfaces were sequentially polished for two minutes per step with Soft-lex abrasive discs (3M ESPE, USA) of decreasing grit sizes: 42 μm , 30 μm , and 15 μm . After each polishing stage, specimens were rinsed thoroughly with distilled water to remove residual abrasives.

To simulate intraoral conditions and maintain enamel hydration throughout the entire experimental process, all samples were stored in artificial saliva at 37 °C in a biological incubator. This protocol was initiated immediately after extraction and maintained continuously during specimen preparation, bleaching treatment, and all subsequent analyses, including spectrophotometric evaluation and laser scanning confocal microscopy (LSCM). To ensure chemical stability and prevent microbial contamination, the artificial saliva solution was renewed every 48 hours. The composition of the solution included sodium bicarbonate

(2190 mg), potassium phosphate (1270 mg), magnesium chloride (125 mg), calcium chloride (441 mg), potassium chloride (820 mg), sodium fluoride (4.5 mg), nipasol (100 mg), nipagin (10 mg), sorbitol (24 mg), carboxymethylcellulose (8 mg), and distilled water (1000 mL), with the pH adjusted to a physiological value of 7.0. The solution was prepared in a closed-circuit pharmacy following the standardized protocol described by Vilhena et al. [7].

Bleaching Procedure

The experimental group included specimens treated with two professional carbamide peroxide-based whitening gels commonly used in dental practice:

- Opalescence Quick (Ultradent Products Inc., USA) – 45% Carbamide Peroxide

Opalescence Quick (Figure 2) is a chemically activated professional whitening gel containing 45% carbamide peroxide, with a neutral to slightly alkaline pH (5.6–7.2). Unlike light-activated systems, this formulation does not require external illumination. Carbamide peroxide gradually decomposes to release hydrogen peroxide, enabling a controlled oxidative reaction on the enamel structure. For each specimen, the gel was applied in a uniform layer approximately 1 mm thick over the exposed enamel surface. The treatment protocol consisted of three consecutive applications, each lasting 15 minutes, resulting in a total exposure time of 45 minutes per sample. After every application, the gel was completely removed, and the enamel surface was thoroughly rinsed with distilled water to eliminate any residual reactive components.



Figure 2. Presentation of the Opalescence Quick 45% bleaching gel – carbamide peroxide-based, chemically activated, light-free system.

- Pola Office+ (SDI, Australia) – 37.5% Carbamide Peroxide

Pola Office+ (Figure 3) is another in-office bleaching agent containing 37.5% carbamide peroxide, formulated with a neutral pH and desensitizing components such as fluoride and potassium nitrate to minimize post-treatment sensitivity. Similar to Opalescence Quick, it is chemically activated and does not require photoactivation. The gel was applied in a thin layer over the enamel surface, with the treatment protocol consisting of two applications of 20 minutes each. Between applications, the enamel was thoroughly rinsed with distilled water to remove any residual material. Pola Office+ is characterized by its slow release of hydrogen peroxide, providing a gradual and controlled oxidative effect on intrinsic pigments.



Figure 3. Presentation of the Pola Office+ 37.5% bleaching gel – carbamide peroxide-based, chemically activated, light-free system

All treated specimens were stored in saline solution throughout the experiment in order to prevent dehydration and structural alterations that may occur in a dry environment. Prior to the application of bleaching gels, dental surfaces were mechanically cleaned to remove organic residues and microbial biofilm, followed by thorough rinsing with distilled water. After treatment, representative enamel fragments were sectioned and mounted on glass slides for detailed microscopic evaluation using laser scanning confocal microscopy (LSCM). The exact composition of the two bleaching products employed in this study is presented in the table below (Table 1).

Table 1. Composition of the bleaching agents used in the study

Product name	Composition	Manufacturer	Lot number
Opalescence Quick	45% carbamide peroxid, pH = 5.6–7.2	Ultradent Products Inc., South Jordan, SUA	BWNV9
Pola Office+	37.5% carbamide peroxide, pH = 6.5–7	SDI Limited, Victoria, Australia	PO+2375-01

Confocal Microscopy Evaluation

Specimen analysis was performed using a laser scanning confocal microscope Olympus Fluoview FV1000 (Figure 4), equipped with UPLSAPO objectives of 10× (NA 0.40) and 20× (NA 0.75), allowing for detailed assessment of morphological changes induced by bleaching treatments. Excitation was carried out at wavelengths of 405 nm and 635 nm, depending on the objective used, with corresponding emission detection at 461 nm and 647 nm. With the 10× objective, images were acquired as high-resolution Z-stacks (1024 × 1024 pixels) at an optical sectioning step size of 1.5 μm, enabling three-dimensional reconstruction of the enamel surface using Bitplane Imaris v7.4 software. For the 20× objective, analysis was conducted using the same excitation (635 nm) and emission (647 nm) parameters, but at a higher resolution (1600 × 1600 pixels), with an optical sectioning interval of 1.2 μm. The scanning speed was set at 2 μs/pixel, laser transmissivity at 65%, and the photomultiplier tube (PMT) voltage adjusted to 629 V to optimize image contrast and clarity. This configuration provided high-precision three-dimensional reconstructions, highlighting structural alterations in enamel. To quantify these morphological variations, fluorescence intensity was measured in four distinct regions of each specimen – mesial, distal, buccal, and lingual/palatal – and the mean value was used for comparison between treated and control groups. Additional examinations at higher magnification (×40) were performed in reflection mode, using immersion oil and a He-Ne laser with a wavelength of 633 nm. Initially, low-magnification images were acquired to provide general orientation of the specimen, followed by a 3× digital zoom to allow detailed observation of prismatic microstructure. CLSM images revealed distinct variations in enamel surface morphology depending on the bleaching protocol applied. Areas with well-organized prisms appeared more translucent under laser illumination, reflecting uniform mineralization, whereas poorly organized inter-prismatic regions produced characteristic reflective patterns, resulting in a honeycomb-like appearance.



Figure 4. Laser scanning confocal microscope Olympus Fluoview FV1000 used for morphological evaluation of enamel specimens

Data Processing

As the methodology applied in this study was designed exclusively for the qualitative assessment of structural alterations in dental enamel using laser scanning confocal microscopy (LSCM), no conventional quantitative statistical analysis was performed. The interpretation of results was based on direct visual comparisons between bleaching-treated specimens and their corresponding control halves from the same tooth. Through this comparative approach, significant morphological changes were identified, including prism disorganization within the enamel structure, increased porosity, and widening of dentinal tubules. This non-invasive and descriptive evaluation method enabled a detailed observation of the structural effects induced by bleaching treatments, without the need for numerical measurements or statistical inference tools.

RESULTS

In Figure 5, the images on the left represent control specimens, showing a well-organized enamel prism structure and a uniform reflection of laser light. In contrast, the images on the right correspond to treated fragments, where evident morphological changes can be observed, including increased porosity, disorganization of the prismatic architecture, and areas of variable fluorescence intensity. These alterations qualitatively illustrate the oxidative impact of carbamide peroxide on the dental surface, as revealed by confocal microscopy.

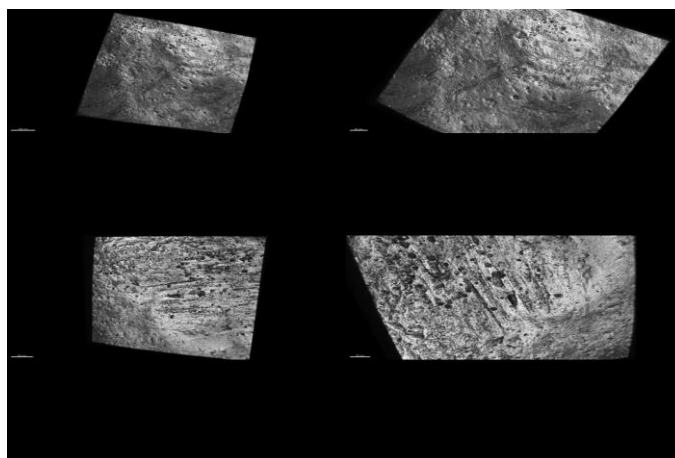


Figure 5. CLSM images illustrating enamel structure before and after bleaching treatment with carbamide peroxide (Opalescence Quick 45%)

Figure 6 presents four micrographs obtained through laser scanning confocal microscopy (CLSM), illustrating the morphological changes in dental enamel before and after treatment with the Pola Office+ 37.5% carbamide peroxide bleaching gel. The first two images (left) correspond to control specimens, which display a regular prismatic architecture and uniform reflectivity. In contrast, the treated samples (right) reveal partial prism disorganization, uneven fluorescence patterns, and a tendency toward widening of interprismatic spaces—features suggestive of early oxidative alterations induced by the bleaching agent. These observations support the moderate structural impact of Pola Office+, confirming a controlled interaction with enamel under simulated clinical application conditions.

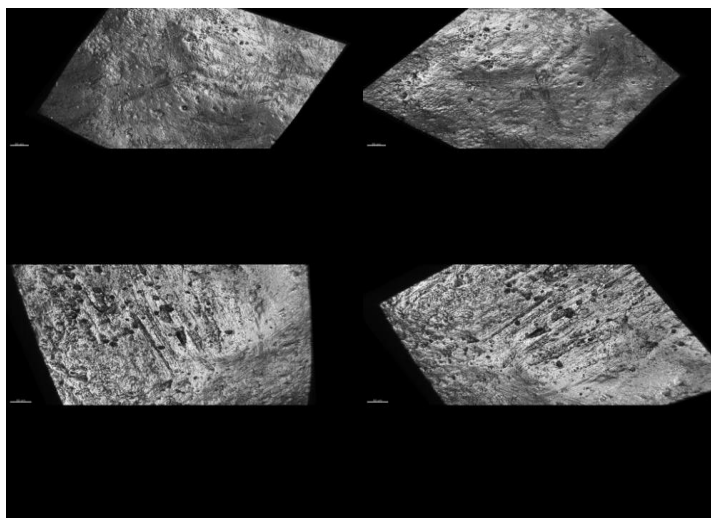


Figure 6. Structural appearance of dental enamel treated with Pola Office+ 37.5% – comparative CLSM images (control vs. treated specimen)

DISCUSSIONS

In today's culture, strongly influenced by social media, the appearance of a bright white smile is increasingly valued and often associated with health, self-confidence, and attractiveness. Dental aesthetics, closely linked to perceptions of beauty and overall well-being, has become an essential component of modern restorative practice [8]. A well-maintained, luminous smile contributes to positive social impressions, psychological well-being, and satisfaction in personal interactions [9]. Tooth whitening is a widely used clinical procedure aimed at improving tooth color and, consequently, the aesthetic outcome and patient satisfaction [10]. The effectiveness of bleaching treatments is influenced by several factors, including the type of whitening agent used, the pH of the oral environment, the application protocol, and the nature of the dental discolorations.

In this study, laser scanning confocal microscopy (CLSM) was employed to evaluate structural changes of enamel surfaces following exposure to peroxide-based bleaching agents. CLSM combines optical imaging with computational processing to generate high-resolution, real-time three-dimensional reconstructions of enamel surfaces. By using a laser beam to excite fluorophores, this technique enables visualization of peroxide diffusion through enamel, dentin, and biofilms, providing detailed two-dimensional images that highlight structural alterations. Compared with scanning electron microscopy (SEM) and other histological methods, CLSM offers significant advantages, particularly in assessing penetration depth and the distribution of bleaching agents within dental tissues. Moreover, CLSM is widely used in microbiological research for quantifying bacterial presence within

dentinal tubules, further demonstrating its versatility as a non-destructive and highly precise analytical method [11].

An important finding of this study was the difference in enamel response depending on the bleaching product used. Opalescence Quick 45%, due to its higher carbamide peroxide concentration, produced more pronounced alterations in enamel prism structure compared with Pola Office+ 37.5%. This observation is consistent with reports in the literature, which indicate that the efficacy of bleaching agents is directly proportional to the concentration of the active substance; however, higher concentrations are also associated with an increased risk of adverse effects, such as heightened sensitivity, morphological changes, and mineral loss [3,4].

With regard to patient perception and expectations of tooth whitening, studies show that there is increasing social pressure for an aesthetic smile, which often leads to frequent and sometimes excessive use of bleaching products. Therefore, understanding the long-term effects on enamel is essential. In a systematic review conducted by Rodríguez-Martínez et al. (2019), the need for balance between rapid aesthetic results and the preservation of oral health was emphasized [12]. Recent literature also highlights the importance of post-treatment remineralization strategies. Studies employing fluoride toothpastes or calcium-phosphate solutions after bleaching have reported favorable effects on restoring enamel hardness and reducing porosity induced by whitening procedures [13,14]. Consequently, the inclusion of remineralizing products in post-treatment protocols is considered a justified measure and is recommended by specialists in modern dental aesthetics [15]. Although the *in vitro* design of the present study did not allow for a direct evaluation of dentin hypersensitivity, it is important to note that high-concentration hydrogen peroxide-based agents are frequently associated with transient sensitivity in clinical practice. To mitigate this adverse effect, dentists commonly adopt preventive strategies such as limiting exposure time, using neutral pH formulations, spacing treatment sessions, and applying desensitizing agents before or after bleaching procedures.

Among the most effective desensitizing substances are 5% potassium nitrate, sodium fluoride varnishes, and casein phosphopeptide–amorphous calcium phosphate complexes (CPP-ACP). A recent meta-analysis confirmed that both potassium nitrate and CPP-ACP significantly reduce post-bleaching sensitivity compared with untreated control groups [16]. Other clinical studies have demonstrated that topical application of CPP-ACP (e.g., MI Paste®) or fluoride reduces thermal and tactile sensitivity following professional bleaching, without compromising treatment efficacy [16,17]. These findings underline the importance of incorporating desensitizing protocols as a standard practice in bleaching treatments, particularly when high-concentration agents are used.

With regard to the durability of whitening outcomes, numerous studies confirm that color stability is strongly influenced by diet, oral hygiene, and patient habits. Highly pigmented foods and beverages (such as coffee, red wine, and tea) may accelerate re-staining, particularly during the first two weeks post-treatment—a period in which adherence to the so-called “white diet” is recommended [14]. In addition, periodic use of maintenance products containing mild bleaching agents may help to prolong the initial whitening effect. The present study aligns with the conclusions of a recent meta-analysis evaluating the impact of different bleaching modalities on enamel microstructure, which reported that in-office treatments with professional products are generally safer than those carried out without clinical supervision [18]. Thus, in-office bleaching with carbamide peroxide, when performed according to recommended protocols, provides an effective balance between efficacy and preservation of dental structure.

An essential aspect to be further emphasized concerns the technology used to evaluate enamel structural changes—laser scanning confocal microscopy (LSCM). This non-invasive

method offers excellent resolution for the visualization of dental microstructures and enables three-dimensional observation of alterations produced by bleaching treatments. Compared with scanning electron microscopy (SEM), LSCM provides the advantage of preserving specimen integrity while highlighting subtle variations in reflectivity and fluorescence, which are indicative of prismatic microstructure disorganization [19,20]. In our study, qualitative analysis using LSCM revealed that, regardless of the bleaching agent applied, an increased exposure of dentinal tubules was observed following treatment compared with baseline conditions. These microstructural changes were consistently identified across all treatment groups, reflecting accentuated surface irregularities and visible exposure of dentinal tubules after bleaching. While this investigation focused on surface morphology and qualitative enamel changes visualized with LSCM, we recognize the importance of assessing peroxide penetration depth and associated demineralization. Naim et al. [21] conducted an in vitro quantitative evaluation of enamel demineralization depth after bleaching, using fluorescence to measure lesion penetration. Their results demonstrated that structural alterations may extend to subsurface enamel layers and vary according to peroxide concentration and application time. Although our methodology did not include depth measurements, future research should integrate such analyses to better characterize enamel responses to bleaching agents and to more accurately assess long-term risks to structural integrity [21].

From a clinical perspective, the microstructural changes observed in enamel may have significant implications for patient care. Increased surface roughness and porosity favor the retention of acids and dental biofilm, thereby enhancing the risk of erosion and caries, especially in acidic oral environments [21–23]. These structural alterations may also facilitate fluid migration through enamel, intensifying post-bleaching sensitivity [24]. Moreover, compromised enamel surfaces can negatively affect the adhesion of restorative materials: several studies have shown a significant reduction in bond strength—up to 60%—when restorations are placed immediately after bleaching, with substantial improvement only after a waiting period of 7–21 days [21,24]. These findings highlight the need for increased clinical caution: dentists should delay bonding procedures, recommend careful post-whitening care, and, where appropriate, apply remineralizing agents to restore enamel integrity and reduce sensitivity.

Differences between in-office and at-home whitening procedures represent another relevant topic of discussion. High-concentration carbamide peroxide (CP) gels (above 30%), such as those used in this study, offer the advantage of rapid and controlled action but carry a higher risk of structural alterations if not properly administered. By contrast, at-home whitening with gels containing 10–16% CP requires longer treatment times but is generally associated with milder effects on enamel [10]. Thus, the choice of whitening method should take into account both the patient's aesthetic expectations and the baseline condition of the dental structures.

Another key element is the pH of bleaching products. Studies have shown that neutral or slightly alkaline formulations are associated with a significantly lower risk of demineralization and better preservation of enamel hardness [23,25]. The products analyzed in the present study comply with these characteristics, supporting the notion that adverse effects can be minimized through the use of chemically optimized formulations. It is also important to note that pH influences the release kinetics of active oxidizing agents from CP, which directly affects both the efficacy and safety of the whitening procedure. An emerging factor in assessing the safety of bleaching treatments is their interaction with pre-existing restorative materials in the oral cavity. Carbamide peroxide has been shown to compromise the surface integrity of composite resins—particularly microhybrid and nanocomposites—by increasing surface roughness and reducing gloss [26]. Therefore, prior to bleaching, it is essential to identify and protect existing restorations, especially in the anterior region.

Furthermore, for patients with extensive restorations, replacement may be required post-treatment to achieve a uniform color match between natural teeth and restorative materials.

Recent literature has also introduced the concept of “biomimetic whitening,” referring to formulations enriched with remineralizing agents such as nanostructured hydroxyapatite or active fluorides. These additives are designed to compensate for oxidative effects and to support enamel repair in parallel with stain removal [27]. This research direction is promising, as it may significantly reduce adverse effects associated with conventional bleaching and pave the way for gentler and more personalized protocols. Finally, individual biological variability of enamel must be considered. Differences in thickness, degree of mineralization, porosity, and wear influence how each tooth responds to whitening. The present study attempted to control this variability by using paired samples (control and treated) from the same tooth; however, in clinical practice, individual responses may vary significantly. This reality underscores the importance of personalized evaluation and careful clinical monitoring during and after whitening procedures [28,29].

This study presents several limitations that should be considered when interpreting the results. The *in vitro* design restricts direct clinical extrapolation, as artificial saliva cannot fully replicate the complex enzymatic, immunological, and buffering properties of natural saliva, nor the dynamic oral environment influenced by salivary flow, temperature changes, mastication, and pH fluctuations. The analysis was limited to a small number of commercial bleaching products, reducing the generalizability of the findings across all formulations. Biological variability among extracted teeth, despite careful selection, also represents a potential confounding factor. Furthermore, assessments were performed immediately after bleaching, without long-term follow-up to evaluate potential remineralization or recovery processes. CLSM provided high-resolution qualitative insights but lacks quantitative surface roughness parameters; integrating profilometry, SEM, Raman spectroscopy, or micro-CT could strengthen future analyses. Finally, complementary mechanical tests such as microhardness or bond strength evaluations would provide clinically relevant data. Despite these limitations, the study contributes valuable information on the structural effects of carbamide peroxide bleaching and emphasizes the need for safe and evidence-based clinical protocols.

CONCLUSIONS

Carbamide peroxide-based tooth whitening remains an effective and widely accessible aesthetic procedure, with a measurable impact on patients' quality of life. Using laser scanning confocal microscopy, this study qualitatively demonstrated moderate morphological changes in enamel, such as partial prism disorganization and increased porosity, without severe compromise of tissue integrity. Differences between Opalescence Quick 45% and Pola Office+ 37.5% highlighted the role of concentration and exposure time in shaping structural outcomes, underscoring the need for careful product selection and adherence to clinical protocols. Overall, carbamide peroxide whitening can be considered safe when performed under professional supervision, particularly when supported by remineralizing strategies and post-treatment care. Future research should focus on long-term *in vivo* effects, interactions with restorative materials, and the development of biomimetic formulations capable of balancing aesthetic efficacy with enamel preservation.

Conflicts of Interest

The authors declare no conflict of interest.

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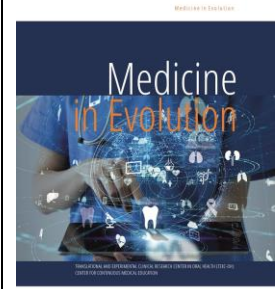
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The Influence of Oral Health on Quality of Life in Young Adults: A Public Health Perspective

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Abstract

1. Background/Objectives: Oral health plays an increasingly vital role in overall health and well-being. **2. Methods:** This cross-sectional design evaluated the impact of oral health on quality of life in adolescents and young adults. The study was conducted using a validated psychometric tool – the Oral Impact on Daily Performance (OIDP) questionnaire – which captures the self-perceived effect of oral conditions on various daily functions. Participants were recruited from a general adolescent and young adult population via online distribution channels. The target demographic included individuals aged approximately 14–25 years, either attending secondary school or university. The survey was administered using Google Forms platform, ensuring accessibility across various devices. Participants were informed about the purpose of the study and anonymity was guaranteed. Submission of the completed form was considered an expression of informed consent, in accordance with ethical research standards. Collected data were processed using Microsoft Excel. Descriptive statistics determined central tendency (mean, median), dispersion (standard deviation, range), and item-specific frequency distributions. **3. Results:** The study included a total of 76 participants, aged between 14 and 25 years. All participants completed the Oral Impact on Daily Performance (OIDP) questionnaire. Each respondent rated the frequency of 15 oral health-related impacts on a 0–4 scale. The mean OIDP Score among the group was 11.37, with scores ranging from 1 to 30, indicating a moderate burden of oral health on daily life activities across the sample. The difference between the psychosocial and appearance domains was the only one reaching statistical significance, suggesting that aesthetic and emotional concerns may affect participants differently and deserve further study in a larger sample. **4. Conclusion:** the findings from this study emphasize the importance of viewing oral health through a biopsychosocial lens, especially in adolescent and young adult populations.

Keywords: oral health, quality of life, adolescents, young adults, self-perceived health

INTRODUCTION

Oral health plays an increasingly vital role in overall health and well-being. While previously considered separately, the oral cavity is now recognized as interconnected with systemic health through intricate biological and behavioral pathways. A well-maintained mouth is not only crucial for basic functions like chewing, speaking, and social interactions but also for ensuring overall physiological equilibrium. Research conducted over the last twenty years has highlighted strong links between oral health and various systemic conditions. For instance, studies have shown associations between poor oral health and conditions such as cardiovascular disease, diabetes mellitus, respiratory infections, and specific neurodegenerative disorders. These findings underscore the importance of oral hygiene in maintaining overall health and preventing potential complications. [1-3]

The pathophysiological mechanisms connecting oral diseases to systemic illnesses are deeply rooted in the body's inflammatory response. For instance, when individuals suffer from periodontal disease, a condition characterized by inflammation and infection of the gums and surrounding tissues, it can lead to chronic low-grade systemic inflammation. This persistent inflammation not only affects the oral cavity but also has far-reaching consequences for overall health.

One significant impact is the potential contribution of periodontal disease to insulin resistance, a condition where cells fail to respond effectively to insulin, leading to elevated blood sugar levels. Moreover, the inflammatory processes associated with periodontal disease have been linked to the development of atherosclerosis, a condition characterized by the buildup of plaque in the arteries, increasing the risk of heart disease and stroke.

It is crucial to recognize that oral pathogens and their by-products have the ability to enter the bloodstream, acting as triggers for immune reactions that can influence distant organs. This phenomenon highlights the interconnected nature of the body's systems, where oral health can serve as a reflection of systemic well-being. Consequently, a paradigm shift has occurred in the understanding of oral health, moving beyond its traditional association with dental care to being recognized as a vital indicator of systemic risk. This shift underscores the importance of holistic health approaches that consider the impact of oral health on overall well-being.[4].

Adolescents and young adults are a demographic group that faces heightened risks attributed to various behavioral, environmental, and social factors. This population undergoes a significant transition from adolescence to adulthood, characterized by a decrease in parental supervision, an increase in autonomy, and a propensity to adopt lifestyle choices that can compromise oral health. For instance, the prevalence of tobacco use, alcohol consumption, body piercings, and the consumption of sugary foods is notably high among this age cohort. These behaviors directly impact the oral environment, leading to detrimental consequences.

When these detrimental habits are coupled with inconsistent oral hygiene practices, they create an environment conducive to the onset of dental issues such as cavities, gum disease, and inflammation of the soft tissues. The lack of regular brushing and flossing, combined with the consumption of cavity-causing sugary snacks and beverages, can result in the erosion of tooth enamel and the formation of cavities. Additionally, the use of tobacco products and excessive alcohol intake can exacerbate gum disease, leading to bleeding gums, bad breath, and potential tooth loss. [5]

The ramifications of inadequate oral health during adolescence extend far beyond mere clinical manifestations. In addition to dental caries and periodontal disease, young individuals may grapple with halitosis, heightened tooth sensitivity, enamel degradation, or

aesthetic concerns – all of which can profoundly affect their psychosocial development, academic achievements, and self-worth. These oral health issues, often dismissed as trivial, may harbor more profound social and emotional ramifications, particularly in environments sensitive to peer perception. Consequently, the impact of oral health transcends biomedical outcomes, permeating the domain of overall quality of life. [6-8]

Among the most common conditions associated with poor oral hygiene are:

- Dental caries, which arise from bacterial plaque accumulation and may result in pain, functional impairment, or tooth loss if untreated.
- Gingivitis and periodontitis, both of which may remain undiagnosed in early stages but can progress to irreversible bone loss and systemic effects.
- Halitosis, often underestimated, which can undermine confidence and social functioning.
- Tooth sensitivity, enamel wear, and mucosal irritation – discomforts that are frequently ignored but significantly affect daily life.

Within this broader construct, the term health-related quality of life (HRQoL) refers specifically to the impact of health conditions and their treatment on daily functioning and life satisfaction. In dentistry, a related construct—oral health-related quality of life (OHRQoL), has emerged to capture the subjective burden of oral conditions on an individual's day-to-day life. OHRQoL includes aspects such as pain, discomfort, ability to eat and speak, self-confidence, social interaction, and emotional well-being. It reflects both the functional and psychosocial consequences of oral diseases, which may not always be visible or measurable through clinical examination alone. [9,10]

The integration of OHRQoL into clinical and public health contexts recognizes that two individuals with similar oral pathologies may report very different impacts on their lives, depending on their psychological resilience, social support, aesthetic expectations, or occupational needs. In this way, OHRQoL serves as a bridge between clinical outcomes and patient experience, guiding treatment planning, preventive strategies, and healthcare policy. [11-13]

These issues highlight the need for early prevention, public awareness, and targeted interventions for young adults, particularly in societies where routine dental care is inconsistently accessed.

Aim and objectives

The purpose of this study is to investigate the multifaceted relationship between oral health and quality of life in young adults. By examining both clinical indicators and behavioral factors, this research aims to assess how oral hygiene habits, dietary choices, and harmful practices contribute to oral diseases, and in turn, how these diseases affect individuals' physical, emotional, and social well-being. The study further explores how oral health may act as a predictor or modifier of systemic risk, particularly in relation to chronic diseases. Ultimately, the findings aim to support public health initiatives, improve preventive strategies, and underscore the role of oral health as a cornerstone of general health and quality of life.

MATERIAL AND METHODS

This research employed a cross-sectional observational design aimed at evaluating the impact of oral health on quality of life in adolescents and young adults. The study was conducted using a validated psychometric tool – the Oral Impact on Daily Performance (OIDP) questionnaire – which captures the self-perceived effect of oral conditions on various daily functions.

Participants were recruited from a general adolescent and young adult population via online distribution channels. The target demographic included individuals aged approximately 14–25 years, either attending secondary school or university. The survey was administered using Google Forms platform, ensuring accessibility across various devices. Participants were informed about the purpose of the study and anonymity was guaranteed. Submission of the completed form was considered an expression of informed consent, in accordance with ethical research standards.

A total of 19 participants were included in the final analysis. Inclusion criteria were individuals aged between 14 and 25 years; Ability to read and understand the questionnaire independently; Voluntary participation with consent (parental consent assumed if under 18) and history of at least one oral health symptom or dental concern in the previous 6 months. Exclusion Criteria were represented by the presence of systemic conditions with known impact on oral function (e.g., autoimmune diseases, congenital syndromes). or recent major dental interventions (oral surgery or orthodontic appliance placement) within the last 3 months

The main data collection tool was the OIDP questionnaire, consisting of 15 items reflecting the frequency of difficulties encountered in specific daily activities due to oral problems. These activities include eating, speaking, sleeping, emotional expression, social interaction, academic performance, and personal comfort. Responses were captured on a 5-point Likert scale: 0 = Never, 1 = Rarely, 2 = Sometimes, 3 = Often, 4 = Always. For analytical purposes, responses were numerically coded from 0 to 4, corresponding directly to the original Likert scale. Higher scores indicated greater frequency of interference in daily performance due to oral health problems. Individual scores were summed to calculate a total OIDP score per participant, with a maximum possible value of 60. The questionnaire covered a six-month recall period and was fully anonymous. Responses were automatically exported from the Google Forms platform into Excel format for analysis.

Collected data were processed using Microsoft Excel. For each participant, individual item scores were analyzed and the total OIDP score was calculated. Descriptive statistics were applied to determine central tendency (mean, median), dispersion (standard deviation, range), and item-specific frequency distributions.

RESULTS

The study included a total of 76 adolescents and young adults, aged between 14 and 25 years. All participants completed the Oral Impact on Daily Performance (OIDP) questionnaire. Each respondent rated the frequency of 15 oral health-related impacts on a 0–4 scale, where 0 represented “never” and 4 represented “always.” The mean OIDP Total Score among the group was 11.37, with scores ranging from 1 to 30, indicating a moderate burden of oral health on daily life activities across the sample.

Figure 1 presents the complete list of items included in the OIDP questionnaire, labeled with corresponding codes (Q1–Q15). Each question targets a specific aspect of how oral health may interfere with daily functioning, ranging from eating and speaking to self esteem and participation in social or physical activities. These items collectively assess the multidimensional impact of oral health status on the quality of life of adolescents and young adults.

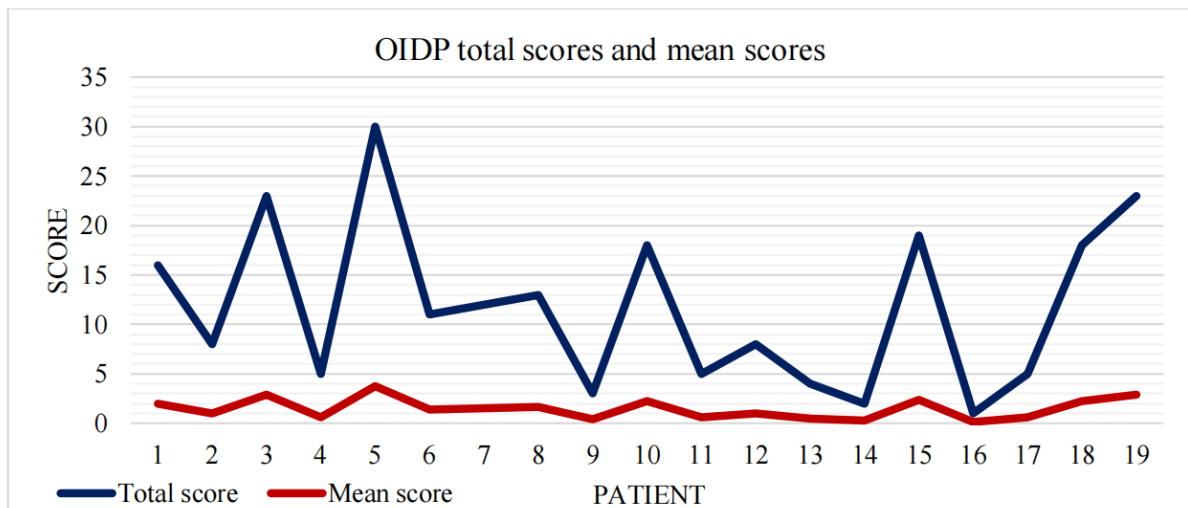


Figure 1. Distribution of OIDP total and mean scores across the sample

The chart shows that there is high inter-individual variability in perceived oral health impact. The highest burden, showed a total score of 30 and mean of 2.00. The lowest score was 1, mean 0.07, indicating minimal interference from oral symptoms. Several other patients reported substantial impacts, while others reported very limited disturbance. This variability indicates heterogeneous perceptions of oral health-related quality of life, even within a demographically narrow population.

To enhance interpretability, the 15 OIDP items were grouped into three conceptual domains: Functional Limitations (Q1, Q2, Q6, Q7, Q8, Q9, Q10), Psychosocial Impact (Q3, Q4, Q12, Q15), and Aesthetic and Somatic Concerns (Q5, Q11, Q13, Q14).

The items in the first domain assess the extent to which oral health affects essential daily functions such as eating, speaking, concentration, hygiene maintenance, and participation in activities. The third domain focuses on self-perception, emotional well-being, and social confidence in relation to oral health and the third evaluates the presence of pain, discomfort, and esthetic concerns that may interfere with taste, appearance, and physical activities.

Table 1. Mean scores for all domains

Functional Limitations							Psychosocial Impact				Aesthetic and Somatic Concerns			
Q1	Q2	Q6	Q7	Q8	Q9	Q10	Q3	Q4	Q12	Q15	Q5	Q11	Q13	Q14
0.89	0.52	0.31	0.52	0.73	0.42	0.84	0.21	1.26	1.31	0.84	1	1.42	0.84	0.63

For each participant, individual domain scores were calculated by summing the responses to grouped items corresponding to functional limitations, psychosocial impact, and aesthetic or somatic concerns, as seen in Table 1. This approach allowed a more nuanced analysis of the areas most affected by oral health issues. The functional domain yielded the lowest average score (0.56 ± 0.25), suggesting that basic oral functions such as eating, chewing, or maintaining oral hygiene were relatively less impaired among the study population. The appearance domain, encompassing concerns related to dental aesthetics and physical discomfort, showed a moderately higher impact, with a mean score of 0.82 ± 0.18 . The psychosocial domain recorded the highest average score (1.21 ± 0.25), indicating that emotional well-being and social interaction were the most adversely affected by oral health

problems. This distribution highlights that, for adolescents and young adults, the psychosocial consequences of oral disease may outweigh purely functional limitations.

To assess whether the differences between domain scores were statistically significant, Mann-Whitney U tests were applied between all domain pairs. This non-parametric test was selected due to the small sample size and non-normal distribution of scores (Table 2).

Table 1. Mann-Whitney U test

	Functional	Psychosocial	Appearance
Functional	-	159.5	218
Psychosocial	159.5	-	237.5*
Appearance	218	237.5*	-

* $p < 0.05$

The difference between the psychosocial and appearance domains was the only one reaching statistical significance, suggesting that aesthetic and emotional concerns may affect participants differently and deserve further study in a larger sample.

DISCUSSIONS

The present study aimed to explore the self-perceived impact of oral health on quality of life in a sample of adolescents and young adults using the validated Oral Impact on Daily Performance (OIDP) questionnaire. The findings contribute to a growing body of evidence demonstrating that oral health extends far beyond clinical symptomatology, encompassing psychosocial dimensions that deeply affect daily functioning, emotional well-being, and social confidence, particularly in younger populations.

The results of this study provide valuable insight into how oral health conditions influence the day-to-day lives of adolescents and young adults. The statistical analysis, supported by graphical representation, reveals meaningful trends in how participants perceive and report the burden of oral health-related issues on their quality of life. The findings are best understood not solely in terms of numerical differences between domains, but through the lens of lived experiences, where psychological and social dimensions often outweigh physical symptoms in perceived severity.

A prominent result of this study is the marked impact of psychosocial consequences, as reflected in the highest average scores within this domain (mean: 1.21 ± 0.25). Items such as self-consciousness about smiling (Q3), diminished self-esteem (Q4), and concern about dental appearance (Q12) were consistently scored higher than functional items. This trend suggests that for young individuals, the visibility of oral issues and their perceived esthetic implications may carry more emotional weight than pain or functional limitations. Adolescence and early adulthood are formative stages where peer perception and self-image are particularly salient, which may explain the elevated sensitivity to esthetic and emotional disturbances.

Consistent with prior research, the psychosocial domain emerged as the most affected area, with high average scores in questions related to self-esteem, appearance, and confidence. These results align with findings from studies conducted in similar age cohorts, such as the work by Masood et al. [12], who reported that adolescents place a high value on smile esthetics and social acceptance, often rating these concerns above pain or functional limitations. In another study conducted in Romania by Chisnoiu et al. (2022) [13], dental appearance and peer perception were found to be major drivers of dental anxiety and avoidance behavior, reinforcing the relevance of psychosocial burdens in youth oral health [12,13].

In contrast, functional limitations, such as difficulty chewing (Q9), speaking clearly (Q2), or maintaining oral hygiene (Q10), were perceived as relatively minor in this sample, as indicated by the lowest mean domain score (0.56 ± 0.25). While these functions are undeniably essential for physical well-being, their lower scores may reflect a combination of adaptive behaviors (e.g., avoiding problematic foods) and underreporting due to the absence of acute symptoms. Importantly, even when oral disease exists clinically, its interference with function may not yet have reached a threshold that prompts subjective concern in younger populations. This discrepancy supports the notion that subjective burden may not always reflect clinical severity, a phenomenon previously described in oral health-related quality of life (OHRQoL) literature [13]. For example, an individual with mild gingivitis may report high emotional distress if esthetic areas are affected, while another with advanced caries may underreport their burden due to low perceived impact on daily activities.

Another observation is the substantial inter-individual variability in both total and mean OIDP scores, visualized effectively in Figure 1. This graph juxtaposes each participant's total OIDP score (blue line) with their mean per-question score (red line), revealing striking differences among individuals. Such variability underscores the personalized nature of oral health burden. It supports the notion that two individuals with similar clinical findings may experience and report vastly different impacts on their emotional and social functioning, depending on factors such as personality, coping strategies, and social context. These differences could stem from a range of contextual variables including socioeconomic background, dental care access, health literacy, cultural norms, and individual resilience or coping mechanisms. The OIDP tool, while limited to standardized items, successfully captures this variability and highlights the need for individualized assessment in dental care planning.

From a statistical perspective, the analysis of domain differences through Mann-Whitney U testing further clarifies the relationships between the three grouped domains. Although not all comparisons reached statistical significance at the conventional $p < 0.05$ level, the significant result between the psychosocial and appearance domains ($U = 237.5$, $p = 0.02$) points toward a meaningful trend. This suggests that while esthetic/somatic concerns, such as pain (Q5), discomfort (Q15), or dry mouth (Q13), are notable, their emotional repercussions may weigh even more heavily on perceived quality of life. The lack of significance in comparison with the functional domain ($p = 0.54$ and $p = 0.27$, respectively) indicates that, in this sample, pure physical interference was less dominant in shaping daily experience.

Taken together, these results highlight the multifactorial nature of oral health's impact on young individuals. Beyond biological symptoms, it is the subjective experience of appearance, confidence, and social comfort that most often drives perceived burden. This finding is clinically important, as it signals that early-stage conditions—such as mild malocclusions, plaque, or gingivitis—may have disproportionate effects on well-being, even when they are not functionally debilitating. Furthermore, these insights argue strongly for the inclusion of patient-reported outcome measures (PROMs) like the OIDP in both routine dental care and public health monitoring, particularly in age groups undergoing major social and psychological development.

Several methodological limitations should be acknowledged. First, the cross-sectional design precludes conclusions about causality or temporal relationships. Second, data collection via self-administered online questionnaires may introduce reporting bias—participants may underestimate or overstate their symptoms depending on social desirability, recall accuracy, or comprehension of the items. Additionally, while the OIDP questionnaire has been validated internationally, cultural nuances may influence how individuals interpret and respond to subjective impact questions. This may affect score comparability across

populations. Another limitation is the lack of objective clinical data. Although the OIDP captures perceived burden effectively, correlating it with actual dental status (e.g., DMFT scores, periodontal indices) would provide a more holistic view of the oral health landscape. Moreover, demographic variables such as gender, income level, and prior dental history were not captured in this study, limiting our ability to stratify risk or identify vulnerable subgroups.

Despite these limitations, this study offers meaningful insights. It confirms that young people's oral health concerns are not merely clinical; they are deeply personal, social, and psychological. It also validates the OIDP as a feasible and informative instrument for public health screening in adolescent and student populations. By identifying which domains of life are most impacted, dental practitioners and policymakers can design more targeted preventive, esthetic, and psychosocial interventions.

Looking ahead, the integration of oral health-related quality of life (OHRQoL) measures into clinical practice could become a cornerstone of patient-centered dentistry. In educational settings, oral health promotion strategies should not only stress brushing technique or sugar intake but should also emphasize the psychological and social importance of a healthy, esthetic, and functional dentition. Universities and health services should collaborate to implement screening and counseling programs that are sensitive to the emotional dimensions of oral health.

Finally, this study adds to the growing consensus that dental care must be reframed – not just as a reactive intervention to disease, but as an essential part of holistic well-being that spans appearance, emotion, confidence, and functionality.

CONCLUSIONS

In conclusion, the findings from this study emphasize the importance of viewing oral health through a biopsychosocial lens, especially in adolescent and young adult populations. Traditional clinical indices alone may fail to capture the breadth of oral health's impact on life quality. Integrating subjective assessments like OIDP provides a more holistic understanding and supports the development of tailored interventions that address both clinical and psychosocial needs.

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Association of Oral Hygiene Products and Their Impact on Oral Health

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Abstract

1. Background/Objectives: Oral hygiene plays a crucial role in maintaining ecological balance within the oral cavity. Salivary pH is a key functional marker, closely linked to enamel demineralization–remineralization dynamics and microbial homeostasis. The study aimed to compare the influence of two oral hygiene regimens—using Elmex Anti-Caries Professional toothpaste alone versus its combination with alcohol-free Listerine Coolmint mouthwash—on salivary pH values in young adults. **2. Methods:** A controlled crossover design was applied to 30 participants aged 20–30 years. Saliva samples were collected under standardized conditions at 30 minutes and 2 hours after toothbrushing with Elmex toothpaste (Day 1) and after toothbrushing followed by mouthrinse with Listerine Coolmint (Day 2). Salivary pH was assessed using the Saliva Check Buffer kit, which provides semi-quantitative values in 0.5-unit intervals. Data were analyzed with descriptive statistics and the Wilcoxon signed-rank test. **3. Results:** Toothbrushing alone maintained salivary pH mostly in the physiological range of 6.5–7.0, while the combined regimen produced a significant alkalinizing effect, with values shifting towards 7.0–7.5 at both time points. Statistical analysis confirmed significant differences between the two protocols ($p < 0.001$). **4. Conclusion:** The combined use of fluoridated toothpaste and alcohol-free antiseptic mouthwash enhances salivary pH stability compared with toothbrushing alone, supporting remineralization and reducing acidogenic bacterial activity. These findings provide evidence-based support for integrating complementary oral hygiene products into daily preventive protocols, especially in young adults at increased risk for caries.

Keywords: Salivary pH, Oral hygiene, Fluoride toothpaste, Mouthwash

INTRODUCTION

Oral hygiene is an essential component of individual health, defined as the set of systematic, self-applied and/or professionally supported measures aimed at maintaining the health of the oral cavity, preventing the onset and progression of dento-periodontal diseases and preserving the functionality of the dento-maxillary apparatus [1]. The modern approach integrates daily practices – toothbrushing, use of floss and interdental brushes, oral irrigators and mouthwashes – with periodic prophylactic interventions in the office (professional scaling, topical fluoridation, sealing and advice on the correct techniques) [1]. The central purpose of these measures is to maintain an ecological balance at the level of the oral ecosystem, by controlling bacterial biofilm and preventing dysbiosis associated with gingival inflammation, caries and other pathologies [1,2].

In the absence of rigorous hygiene, the composition and metabolism of plaque become more adherent and pathogenic; the metabolism of fermentable carbohydrates by acidogenic microorganisms lowers the pH below the critical threshold of 5.5, favoring the demineralization of the enamel and the initiation of carious processes [1,2]. Well-structured hygiene routines – adapted to age, oral health status and individual particularities – directly influence the quality of the oral environment. Clinical evidence indicates that a constant sanitation regimen supports the maintenance of salivary pH in the physiological range 6.2-7.6, a favorable condition for the remineralization and inhibition of acidophilic bacteria [2]. The educational dimension is also crucial: early formation of correct habits and compliance with periodic check-ups correlate with lower prevalences of caries and periodontal diseases in adolescents and young adults [1].

Oral hygiene products have evolved to respond to the multifactorial etiology of oral diseases. Toothpastes, mouthwashes and auxiliary means (floss, interdental brushes, irrigators) are the core of home interventions, complemented by topical fluoride gels, concentrated antiseptic solutions and formulas for dentine sensitivity or incipient periodontal disease [3]. Paste formulas include abrasives (hydrated silica, calcium carbonate), surfactants (e.g. sodium lauryl sulfate), as well as fluorides (NaF, SnF₂, sodium monofluorophosphate) for remineralizing, antacid and antibacterial effects; incorporation of fluoride into the enamel crystal in the form of fluorhydroxyapatite increases resistance to acid attack and inhibits bacterial enzymes involved in carbohydrate metabolism [4,5]. Mouthwashes, selected according to clinical objective, may contain antimicrobials (chlorhexidine, essential oils, cetylpyridine), anti-inflammatory, hemostatic or remineralizing agents (fluorides) and, in some cases, desensitizers (potassium nitrate, arginine) [4-6].

Fluoride remains the cornerstone of caries prophylaxis, acting in the demineralization-remineralization dynamics and exerting antibacterial effects by reducing the acidogenicity of the biofilm (e.g. inhibition of enolase and glucose transport in cariogenic bacteria) [4,7]. Clinical efficacy depends on local bioavailability, modeled by pharmaceutical form, concentration, contact time and post-application behaviors; Immediate loosening after brushing increases salivary retention of fluorine ions and enamel exposure [8]. The different chemical forms – NaF, SnF₂, amine fluorides – exhibit distinct stability, release, and adhesion profiles, with SnF₂ additionally providing antimicrobial benefits when properly stabilized [9].

Salivary pH is a functional marker of oral homeostasis. Neutral or slightly alkaline values support remineralization and inhibit acidophilic bacteria, while dropping below pH 5.5 triggers demineralization [10]. Diet, hydration, salivary flow, medication, smoking and hygiene quality modulate this parameter; a correct regimen, especially with fluorinated products (NaF, SnF₂), contributes to acid neutralization and pH stabilization, synergistic with the buffering capacity of saliva (bicarbonate, phosphates, proteins) [7,9,10]. Clinical studies

show that consistent hygiene practices reduce the acidogenic microbial load and increase buffer capacity, accelerating the return of pH to physiological values after acid challenges [9,10].

The association of toothpaste with mouthwash is supported by the literature for biofilm control, reducing gingival inflammation, and maintaining acid-base balance [4,5]. However, synergies depend on ingredient compatibility and timing of use. Certain compounds (e.g., chlorhexidine or surfactant anions such as sodium lauryl sulfate) may reduce local fluoride retention if mouthwash is used immediately after brushing; Therefore, it is recommended to postpone the administration by 15-60 minutes or to use it at times of the day without brushing, respecting the chemical compatibility of the products [6,11]. Recent meta-analyses and reviews support the implementation of combined strategies especially in patients with increased carious/periodontal risk, xerostomia, halitosis or orthodontic treatments, in accordance with the current direction of personalized prophylaxis [3,12-15].

In this context, there is a growing interest in how concrete combinations of products influence salivary pH, a clinical indicator that is easy to measure and relevant for the demineralization-remineralization balance. In view of the possible interactions between fluorides, surfactants and other active agents, it becomes necessary to assess the effects on pH when toothpaste and mouthwash are used in combination [4-6,9,11]. The present paper aims to investigate, in an experimental setting, the impact of the association of Elmex Anti-Caries Professional toothpaste (GABA International AG, Therwil, Switzerland) with alcohol-free Listerine Coolmint mouthwash (Johnson & Johnson Consumer Inc., Skillman, NJ, USA) on salivary pH. The results may have direct clinical implications for optimizing hygiene recommendations in the young population at high risk of carious lesions and for refining evidence-based prophylactic protocols [3-6,9-15].

Aim and objectives

The overall aim of the study is to comparatively evaluate the influence of two oral hygiene regimens on salivary pH in young adults: the exclusive use of Elmex Anti-Caries Professional toothpaste and the association of the same paste with alcohol-free Listerine Coolmint mouthwash, under controlled conditions and at defined time intervals of 30 minutes and 2 hours.

The specific objectives aim to determine the salivary pH values 30 minutes and 2 hours after the exclusive use of Elmex Anti-Caries Professional paste and after the use of Elmex paste followed by alcohol-free Listerine Coolmint mouthwash, respectively. The research also aims to compare the values obtained in the two regimes at each measurement moment and over the overall temporal evolution, as well as to estimate the clinical relevance of the observed differences, through indicators such as the average pH difference and the proportion of participants who maintain protective values above the thresholds of 6.2 and 7.0.

Another objective is to explore the mechanistic explanations of the observed pH variations, related to the composition of the tested products, namely fluorides, surfactants or antimicrobials. The paper also aims to formulate evidence-based practical recommendations on the sequence and timing of the use of toothpaste and mouthwash in oral prophylaxis in the young population.

As complementary directions, the study can analyze individual pH variations from baseline and time to return to neutrality, the influence of control factors such as resting salivary flow, interval from last food intake or initial caryoperiodontal status, as well as participants' perception of tolerability and compliance with the two oral hygiene regimens.

MATERIAL AND METHODS

The study included 30 volunteers, young adults aged between 20 and 30, Romanian citizens, with medium or higher level of education. All participants have signed the informed consent and agreement for the processing of personal data before the start of the procedures. The recruitment was carried out anonymously, voluntarily and non-discriminatory, with the subjects being fully informed about the purpose, methodology, benefits and potential risks of the research, as well as the data protection measures. After consent, subjects were clinically evaluated for eligibility.

Inclusion and exclusion criteria

Participants aged 20–30 years, who report daily use of oral hygiene products, with a history of mild oral conditions (caries, dental hypersensitivity, gingivitis), without active carious lesions at initial examination, and without incorrectly adapted dental/prosthetic restorations were included. Subjects with active oral conditions (ulcerative lesions, abscesses, untreated infections), recent drug treatments with an impact on saliva (antibiotics, antidepressants, anticholinergics), active smokers, people with dental interventions in the last 4 weeks or with systemic diagnoses that may alter salivary composition/flow (diabetes, Sjögren's syndrome, autoimmune diseases) were excluded. The establishment of these criteria aimed at clinical homogeneity and the reduction of confounding factors; All determinations were performed by a single examiner to limit inter-observer variability.

Material

Toothpaste: Elmex Anti-Caries Professional (GABA International AG, Therwil, Switzerland), aminofluoride-based formula (olaflur), with increased fluoride adhesion to enamel and prolonged release (Figure 1).



Figure 1. Characteristics of Elmex Anti-Caries Professional toothpaste (GABA International AG, Therwil, Switzerland)

Mouthwash: Alcohol-free Coolmint Listerine (Johnson & Johnson Consumer Inc., Skillman, New Jersey, USA), with antimicrobial essential oils (eucalyptol, thymol, menthol, methyl salicylate), chosen for antiseptic efficacy and oral tolerability (Figure 2).

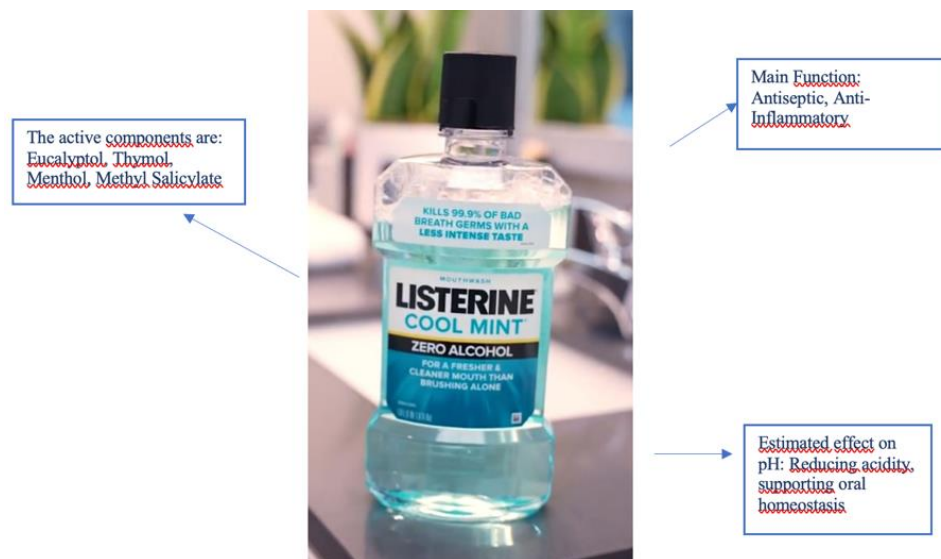


Figure 2. Features of Alcohol-Free Listerine Coolmint Mouthwash (Johnson & Johnson Consumer Inc., Skillman, New Jersey, USA)

Saliva testing: Saliva Check Buffer kit (GC Corporation, Tokyo, Japan), including measuring cups, colorimetric pH/buffer capacity strips, pipettes and wax strips for flow stimulation, used according to the manufacturer's instructions (Figure 3).

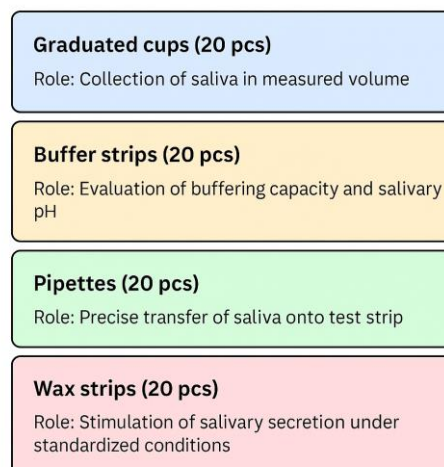


Figure 3. The components of the Saliva Check Buffer kit and their role in the experimental protocol

Study design

A crossover design was used, with each participant serving as their own control. The interventions were carried out on two consecutive mornings, at a comparable time interval (08:00–10:00), prior to the ingestion of food/beverages and any other oral hygiene procedure, to control circadian saliva variations.

Experimental protocol

Day 1: brush your teeth for 2 minutes with Elmex Anti-Caries Professional, using a brush with medium hardness and modified horizontal technique on all dials. After brushing, without ingestion of food/liquids, samples of non-induced saliva were collected every 30 minutes and every 2 hours.

Day 2: identical brushing protocol, followed immediately by rinsing for 30 seconds with 20 ml of alcohol-free Listerine Coolmint. Harvests every 30 minutes and 2 hours after rinsing. Each participant provided four samples in total (two for the paste alone regime and

two for the paste + mouthwash regimen). All harvests were carried out in the sitting position, at rest, without mechanical or gustatory stimulation.

pH collection and measurement

The samples were collected by expectoration in graded sterile containers, at the defined times. The pH measurement was carried out with colorimetric strips of the Saliva Check Buffer kit, by immersion according to the instructions and visual reading after about 10 seconds, based on the standardized color scale. The method is semi-quantitative, reporting ranges (e.g., 6.0–6.5; 6.5–7.0), with potential variability minimized by using a single trained operator. The readings were taken in natural light, on a white background.

Control of disruptive factors

Participants were instructed to avoid acidic foods/drinks, alcohol, smoking and the use of oral hygiene products other than those indicated by the protocol in the 24 hours prior to each visit. The collections were carried out at similar times on the two days, before any food or liquid intake.

Data logging

For each participant, an individual form with an anonymous code was filled in, noting the basic demographics, the regime applied and the pH values at each time, as well as observations on the volume, color or viscosity of saliva. The data were centralized in a password-protected electronic database (Excel format), with predefined fields for the analyzed variables.

Statistical analysis plan

The descriptive analysis included the mean, median, standard deviation and coefficient of variation for pH at 30 minutes and 2 hours, in each regimen. The distributions were verified with the Shapiro–Wilk test. Given that most of the sets did not follow normal ($p < 0.05$), even comparisons between regimes and time moments were performed with the nonparametric Wilcoxon test for paired samples. Comparative charts and summary tables were generated in Microsoft Excel; The accuracy of the calculations was verified by independent recalculations.

Ethics and privacy

The study complied with the principles of biomedical research ethics and data protection regulations (GDPR). Participant identities were kept confidential through anonymous coding, and access to the database was restricted to the principal investigator. The crossover design, standardized procedures, and the use of a single examiner were chosen to enhance the internal validity and reproducibility of the results.

RESULTS

The results obtained in this study were structured on the stages of the experimental protocol, following the evolution of salivary pH after using the two oral hygiene regimens tested: simple brushing and combined brushing with mouthwash.

In the first stage, which focused exclusively on the effect of brushing with Elmex Anti-Caries Professional toothpaste (GABA International AG, Therwil, Switzerland), salivary pH values were predominantly in the physiological range of 6.5–7.0, both at 30 minutes and at 2 hours post-brushing. The slightly upward trend observed in a small number of participants, including reaching the 7.0 threshold at two hours, reflects the cumulative effect of slow-release fluoride and brushing-induced salivary stimulation. These data confirm that the exclusive use of fluoridated toothpaste maintains a stable salivary pH close to neutral, an optimal condition for the prevention of demineralization and inhibition of cariogenic microorganisms (Table 1).

At 30 minutes, all 30 participants (100%) had salivary pH values within the range of 6.5–7.0. At 2 hours, 29 participants (96.7%) remained within the range of 6.5–7.0, while 1 participant (3.3%) recorded a salivary pH value of 7.0.

Table 1. Salivary pH values after simple brushing, at 30 minutes and 2 hours

Participant	Ph in 30 min	Ph in 2 h
P01	6.5–7.0	6.5–7.0
P02	6.5–7.0	6.5–7.0
P03	6.5–7.0	6.5–7.0
P04	6.5–7.0	6.5–7.0
P05	6.5–7.0	7.00
P06	6.5–7.0	6.5–7.0
P07	6.5–7.0	6.5–7.0
P08	6.5–7.0	6.5–7.0
P09	6.5–7.0	6.5–7.0
P10	6.5–7.0	6.5–7.0
P11	6.5–7.0	6.5–7.0
P12	6.5–7.0	6.5–7.0
P13	6.5–7.0	6.5–7.0
P14	6.5–7.0	6.5–7.0
P15	6.5–7.0	6.5–7.0
P16	6.5–7.0	6.5–7.0
P17	6.5–7.0	6.5–7.0
P18	6.5–7.0	6.5–7.0
P19	6.5–7.0	6.5–7.0
P20	6.5–7.0	6.5–7.0
P21	6.5–7.0	6.5–7.0
P22	6.5–7.0	6.5–7.0
P23	6.5–7.0	6.5–7.0
P24	6.5–7.0	6.5–7.0
P25	6.5–7.0	6.5–7.0
P26	6.5–7.0	6.5–7.0
P27	6.5–7.0	6.5–7.0
P28	6.5–7.0	6.5–7.0
P29	6.5–7.0	6.5–7.0
P30	6.5–7.0	6.5–7.0

In the second step, by combining brushing with the use of alcohol-free Listerine Coolmint mouthwash (Johnson & Johnson Consumer Inc., Skillman, New Jersey, USA), an obvious increase in salivary pH values was observed. At 30 minutes after the application of the combined regimen, most participants had pHs in the range of 7.0–7.5, and this trend was maintained or accentuated at 2 hours, with several cases reaching the upper threshold of the reference range. These findings suggest an additional alkalizing effect, attributed to antimicrobial essential oils in mouthwash, which reduce bacterial acidogenic activity and reflexively stimulate salivary secretion (Table 2).

At 30 minutes: 27 participants (90%) had salivary pH values in the range of 7.0–7.5, while 3 participants (10%) remained in the range of 6.5–7.0.

At 2 hours: all 30 participants (100%) had salivary pH values in the range of 7.0–7.5.

Table 2. Salivary pH values after brushing and rinsing with mouthwash, at 30 minutes and 2 hours

Participant	pH in 30 min	pH in 2 h
P01	7	7.0–7.5
P02	7.0–7.5	7.0–7.5
P03	6.5–7	7.0–7.5
P04	7.0–7.5	7.0–7.5
P05	7.0–7.5	7.0–7.5
P06	7.0–7.5	7.0–7.5
P07	6.5–7	7
P08	7	7.0–7.5
P09	7.0–7.5	7.0–7.5
P10	7.0–7.5	7.0–7.5
P11	7.0–7.5	7.0–7.5
P12	7	7.0–7.5
P13	7.0–7.5	7.0–7.5
P14	7.0–7.5	7.0–7.5
P15	7.0–7.5	7.0–7.5
P16	7.0–7.5	7.0–7.5
P17	6.5–7	7.0–7.5
P18	7.0–7.5	7.0–7.5
P19	7	7.0–7.5
P20	7.0–7.5	7.0–7.5
P21	7.0–7.5	7.0–7.5
P22	7.0–7.5	7.0–7.5
P23	6.5–7	7.0–7.5
P24	7.0–7.5	7.0–7.5
P25	7.0–7.5	7.0–7.5
P26	7.0–7.5	7.0–7.5
P27	7.0–7.5	7.0–7.5
P28	7.0–7.5	7.0–7.5
P29	7	7.0–7.5
P30	6.5–7	7.0–7.5

The comparative analysis between the two protocols revealed clear differences. Simple brushing kept the pH in the range of 6.5–7.0, while brushing followed by mouthwash determined higher values, frequently in the range of 7.0–7.5, especially at 2 hours post-intervention. This synergistic effect between the amine fluoride in the paste and the antiseptic essential oils in the mouthwash indicates a superior impact on the oral acid-base balance, with potential benefits in remineralization and in reducing the activity of acidophilic microorganisms (Table 3).

Table 3. The results of the Wilcoxon test

Compare	W Statistic	p-value	Interpret
T30 – Day 1 vs Day 2	0.0	0.0000	statistically significant difference
T120 – Day 1 vs Day 2	0.0	0.0000	statistically significant difference

Statistical interpretation by the Wilcoxon test for paired samples confirmed significant differences between the two protocols. Both at 30 minutes and 2 hours post-intervention, salivary pH values were significantly higher in the case of the combined regimen ($p = 0.0000$ for both intervals). This statistical significance reinforces the clinical relevance of the results, supporting the superiority of the protocol that integrates brushing and rinsing with alcohol-free antiseptic mouthwash.

Overall, the data obtained confirm the initial hypothesis that the combination of Elmex Anti-Caries Professional toothpaste with alcohol-free Listerine Coolmint mouthwash results in a more pronounced and stable increase in salivary pH compared to simple brushing. These results validate the importance of hybrid oral hygiene strategies, with direct implications in the prevention of tooth decay and in the maintenance of oral homeostasis (Figure 4).

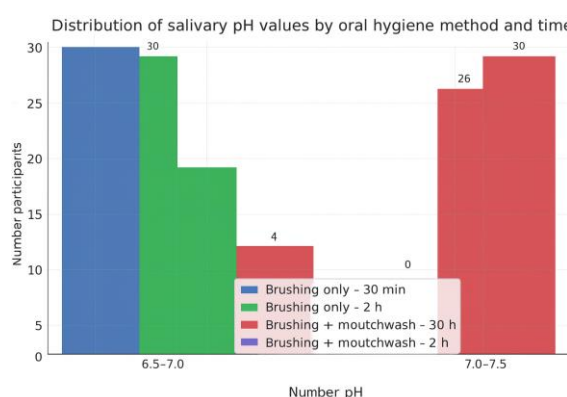


Figure 4. Distribution of salivary pH values at 30 minutes and 2 hours after simple brushing and mouthwash brushing

DISCUSSIONS

The results obtained in this study are congruent with recent data from the literature, confirming the significant impact of oral hygiene products on the acid-base balance of saliva and, implicitly, on general oral health. The increase in pH values observed after both protocols tested, with a more pronounced trend in the case of the combined use of toothpaste and mouthwash, demonstrates the effectiveness of this strategy in maintaining an alkaline oral environment, conducive to enamel remineralization and inhibition of acidophilic microorganisms.

Fluoride, a central element in caries prophylaxis, has once again demonstrated its role in stabilizing salivary pH. According to the study by Albahrani et al. (2022), increased fluoride retention in the salivary by avoiding rinsing with water immediately after brushing prolongs the protective effect and promotes enamel remineralization [7]. The results of the present research, where pH values remained in the physiological range of 6.5-7.0 after simple brushing, correlate with these conclusions, suggesting that fluoride potentiates the natural buffer response of saliva.

The addition of antiseptic mouthwash had an additional effect of increasing salivary pH, which corresponds to the observations of Jiemkim et al. (2023) and Memarpour et al. (2024), who showed that the association of fluoridated paste with mouthwash reduces tooth erosion and enhances enamel protection by enhancing buffering capacity and reducing microbial levels [4,6]. Also, Raj et al. (2021) highlighted that this combination lowers postprandial salivary acidity and protects more effectively against incipient carious lesions [5].

These findings are also supported by recent guidelines in the field, which recommend the integration of complementary products to optimize oral hygiene. Gallione et al. (2025) and

Ingleshwar et al. (2024) emphasize the importance of including the combination of fluorinated toothpaste and antiseptic mouthwash in standard protocols, especially among young people, a category at high risk for cavities and with often poor hygiene habits [14,3]. Thus, the results of the current study align with these recommendations, demonstrating the synergistic role of fluoride and antiseptic essential oils in maintaining a favorable salivary pH and reducing the risk of cariogenic.

However, the interpretation of the results must be carried out taking into account certain limitations. The small sample size ($n=30$), made up of young adults, limits the generalization of the conclusions to the general population. Also, pH monitoring was performed only at two points (30 minutes and 2 hours), without an assessment of the long-term effect persistence. Another important limitation is the semi-quantitative measurement method used, based on the Saliva Check Buffer kit, which estimates the pH in ranges of 0.5 units and does not provide exact values.

To deepen these results, future research should adopt a longitudinal design, which tracks changes in pH and other biochemical (fluorine, calcium) and microbiological parameters over longer periods. The diversification of the investigated population, by including children, the elderly, people with active oral diseases or with varied eating and hygiene habits, would provide a more complete picture of the effects of oral hygiene products. In addition, testing of other combinations of pastes and mouthwashes, with different types of fluoride and bioactive compounds, could highlight relevant variations in the ability to maintain oral homeostasis.

This study confirms the effectiveness of the combined fluoridated toothpaste-antiseptic mouthwash regimen in maintaining a more stable and alkaline salivary pH, with direct clinical implications in preventing cavities and protecting oral health. At the same time, the data obtained offer an additional argument for integrating this type of routine into personalized oral hygiene recommendations, thus contributing to reducing the incidence of oral pathologies at the population level.

CONCLUSIONS

The results of the study clearly demonstrate that the combined use of a fluoridated toothpaste and an antiseptic mouthwash leads to a significant and stable increase in salivary pH values compared to simple brushing. This evolution highlights the effectiveness of the combined diet in maintaining a more alkaline oral environment, favorable to enamel remineralization and reducing the activity of acidophilic bacteria involved in the etiology of dental caries.

Maintaining the salivary pH in a neutral or slightly alkaline range, both 30 minutes and 2 hours post-intervention, confirms the protective role of fluoride and essential oils in the composition of mouthwash on the oral acid-base balance. These results align with the data in the literature, which emphasize the importance of an optimal salivary pH for the prevention of microbiological imbalances and inflammatory gum-periodontal diseases.

Beyond its experimental value, the study also makes an educational contribution, supporting the need to promote a complete oral hygiene routine, which includes both daily brushing and the use of an effective mouthwash, with a prophylactic role. The consistent implementation of these measures can contribute to reducing the prevalence of caries and other untreated oral pathologies, having a positive impact on oral health at the population level.

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