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CITRULLUS COLOCYNTHIS (L.) SCHRAD (BITTER APPLE): AN OVERVIEW OF ITS TRADITIONAL USES, PHYTOCHEMISTRY AND PHARMACOLOGICAL POTENTIAL

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ABSTRACT

Citrullus colocynthis (L.) Schrad is an important cucurbit plant, commonly distributed in the sandy areas of the world. In the Indian continent it is mainly found in the North West, the Punjab and in Central and southern India. The Citrullus colocynthis plant is usually known for its traditional uses as a remedy in the treatment of asthma, diabetes, common cold, leprosy, cough, bronchitis, joint pain, jaundice, cancer, toothache, mastitis, and in gastrointestinal disorders such as gastroenteritis, indigestion, dysentery, constipation, colic pain and other microbial infections. Phytochemicaly the compounds like glycosides, alkaloids, flavonoids, phenolic acids fatty acids, carbohydrates and essential oils were reported from the plant and the main components isolated from the Citrullus colocynthis plant are Cucurbitacins. The plant has been studied extensively for its wide range of pharmacological activities, which include anticancer, antidiabetic, antioxidant, cytotoxic, antimicrobial, anti-inflammatory antilipidemic, and insecticide but the therapeutic potential for cardiovascular, gut, airways and many other diseases remain to be explored.

Keywords: *Citrullus colocynthis, Traditional uses, Cucurbitacins.*

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INTRODUCTION

The survey from The World Health Organization indicated that about 70-80% of the world's population relies on nonconventional medicine, mainly of herbal sources, in their primary healthcare. This is especially the case in developing countries where the cost of consulting a western style doctor and the price of medication are beyond the means of most people (1, 2). There are numbers of significant drugs and biologically active compounds developed from the traditional medicinal plants.

Plants showed a wide range of pharmacological activities including antioxidant, antimicrobial, anti-inflammatory, anticancer, hypolipidemic, cardio-vascular, analgesic, antipyretic, central nervous stimulants, immunological, respiratory, and many other pharmacological effects (3).

The Cucurbitaceae family is one of the most genetically diverse groups of food plants (4). Some well-known members of this family are bitte rapple, gourd, cucumber, melon, and pumpkin (5). Due to consumer awareness on the health benefits of cucurbit plants and fruits, their production seems to have increased over the time. Over the last two decades, India has been the largest producer of cucurbit followed by Egypt, United States of America, Russia and Republic of Iran (4).

Citrullus colocynthis (L.) Schrad (family Cucurbitaceae) is widely available in India and the Southern Islands (6). The fruit of Citrullus colocynthis is commonly called Colocynth/ Bitter Apple in English, Indrayan in Hindi, Hanjal in Urdu, Kattu Kattuvellari in Malayalam, Anedri in Sanskrit, Rakhal in Bengali, and Pcitummatti in Tamil (7, 8).

Phytochemicaly Citrullus colocynthis have many bioactive compounds like glycosides, alkaloids, flavonoids, fatty acids, carbohydrates and essential oils (9-11). The main components isolated from the Citrullus colocynthis plant are Cucurbitacins. Several chemical compounds isolated from the Citrullus colocynthis fruits, pulps and seeds are listed in table 1.

This review presents an overview on traditional uses along with recent studies covering the pharmacological and toxicology of the Citrullus colocynthis plant.

Table 1. List of chemical constituents isolated from the Citrullus Colosynthis plant.

Chemical Class	Isolated Compounds	Plant Part	Reference
Glycosides, flavonoids and phenolic acids	2-O-β-D-glucopyranosyl-Cucurbitacin I	Fruit	12
	2-O-β-D-glucopyranosyl-CucurbitacinL	Fruit	12
	Isosaponarin	Fruit, seeds	12, 13
	Isoorientin 3-o-methylether	Fruit, seeds	12, 13
	Isovitexin	Fruit, seeds	12, 13
	Quercetin	Fruits	14, 15
	Catechin	Fruit pulp	15
	Myricetin	Fruit pulp	15
	Gallic acid	Fruit pulp	15
	Kaempferol	Fruit pulp	15
	p-Hydroxybenzoicacid	Fruit pulp	15
	Caffeic acid	Fruit pulp	15
	Chlorogenic acid	Fruit pulp	15
	Vanillic acid	Fruit pulp	15
	Ferulic acid	Fruit pulp	15
	Sinapic acid	Fruit pulp	15
	p-Coumeric acid	Fruit pulp	15
Alkaloids	Choline	Whole fruit, pulp	10, 16, 17
	Choline	Fruit pulp	18
Cucurbitacins	Curcubitacin A	Fruit	19, 20
	Curcubitacin B	Whole fruit	17, 21
	Curcubitacin C	Fruit	19
	Curcubitacin D	Fruit	19
	Curcubitacin E	Whole fruit	17, 21, 22
	Curcubitacin I	Whole fruit	17, 20
	Curcubitacin J	Fruit	20

Chemical Class	Isolated Compounds	Plant Part	Reference
	Curcubitacin K	Fruit	20
	Curcubitacin L	Fruit	20
	Colocynthosides A	Fruit	20
	Colocynthosides B	Fruit	20
Fatty acids	Palmitic acid, stearic acid, linoleic acid, oleic acids	Seed oils	23 -25
	Myristic, palmitic, stearic, oleic, linoleic and linolenic acids	Seeds	13
Tocopherols and Carotenes	α-tocopherol, γ-tocopherol, β-carotene	Seed oil	26

TRADITIONAL USES

Colocynthis is a very old remedy in Indian medicine. The fruit has been described as cathartic and useful in biliousness, fever, constipation and intestinal parasites. The root is used in janndice, ascites, rheumatism and urinary diseases. The physicians use this drug extensively in their practice as a drastic purgative in ascites and jaundice and in various uterine conditions, especially in amenorrhea. (27).

Worldwide Citrullus colocynthis is widely used in different parts of the world for the treatment of a number of diseases including leprosy, jaundice, constipation, diabetes, asthma, cancer, bronchitis, joint pain and mastitis (6, 28 -30).

In India and Pakistan, the fruits are used for the treatment of bacterial infections, intestinal disorders, diabetes and cancer in humans as well as animals (6, 30 - 33).

Traditionally Citrullus colocynthis is the communally used plant for the treatment of diabetes, in tropical and subtropical countries, (34 - 37).

Citrullus colocynthis is used for the treatment of hypertension and diabetes in Morocco (38 - 40).

In the United Arab Emirates Citrullus colocynthis is one of the most popular folk medicines because of its anti-inflammatory property (41). The fruit has therapeutical application in the stimulation of intestinal peristalsis and soften bowel contents by an irritant action on the enteric mucosa (42 - 44)

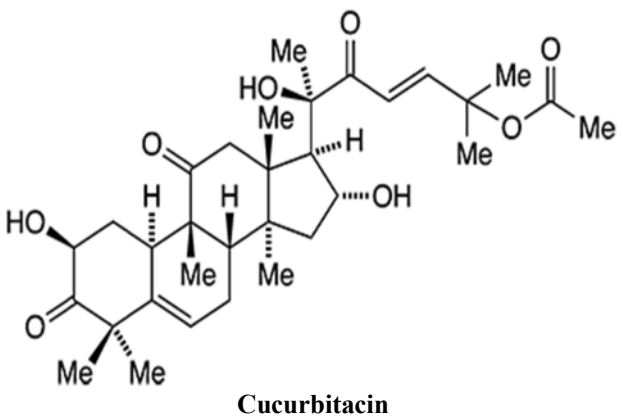
A decoction of the different parts of this plant is used to treat cancer, rheumatic pain and as a hepato protective agent (30, 44, 45).

Fruits and seeds, are often used to treat urinary infections (46), and different parts of the plant are also used to treat many other illness such as rheumatism, hypertension, dermatological problems, gynecological and pulmonary infections, in Tunisia, and other Mediterranean countries (47, 46).

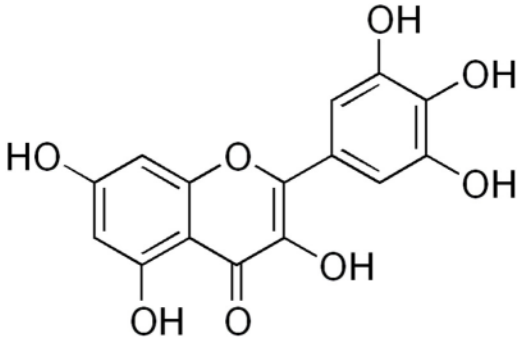
In Saudi Arabia, fruits of Citrullus colocynthis are used as antirheumatic, purgative, anthelmintic, carminative and as a remedy for skin and sore throat infections (19). The fruit is also a blood purifier and remedy enlargement of spleen and tumors.

The seeds of Citrullus colocynthis are used for the treatment of diabetes, while the leaves are used for the treatment of jaundice and asthma (48 - 50). In Israel Citrullus colocynthis is also well-known as a source of seed oil and its fruit has been used as a laxative (51).

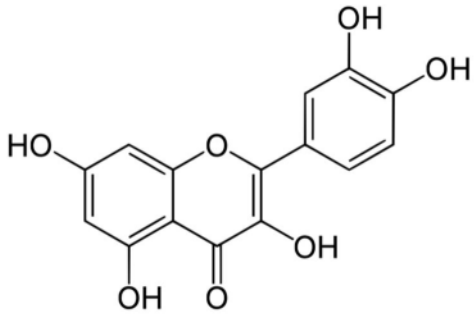
Table 2. Chemical Structures of some chemical Compounds present in Citruluss Colosynthis.



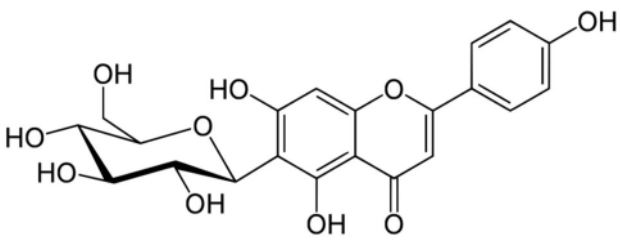
Cucurbitacin



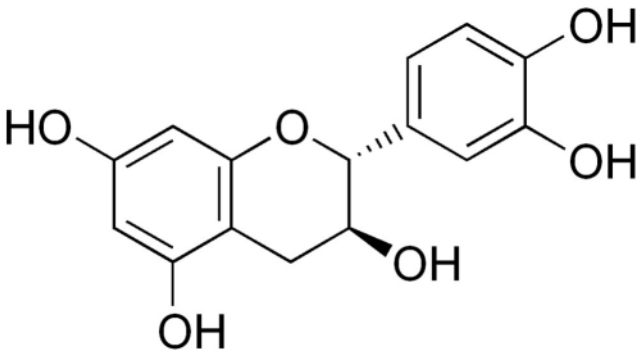
Myricetin



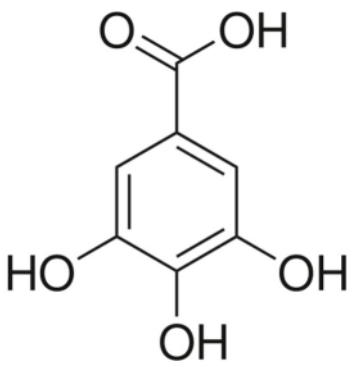
Quercetin



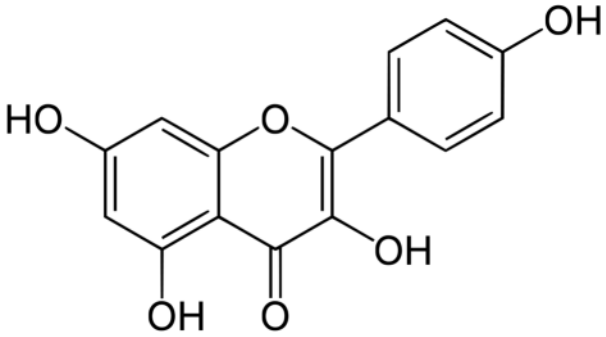
Isovitexin



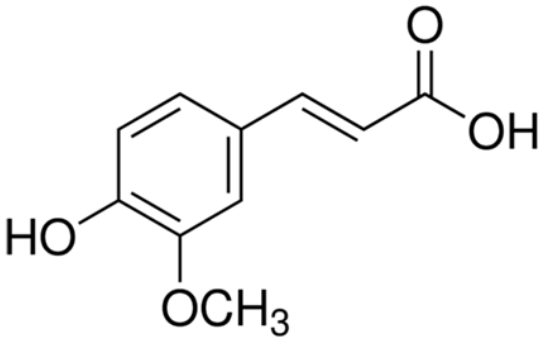
Catechin



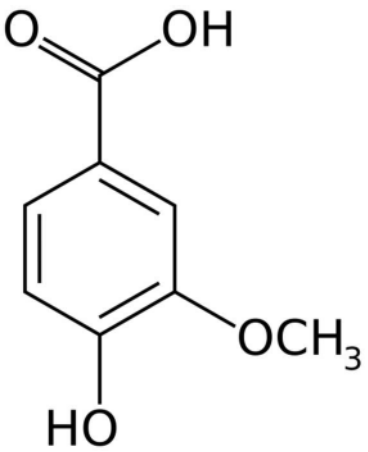
Gallic acid



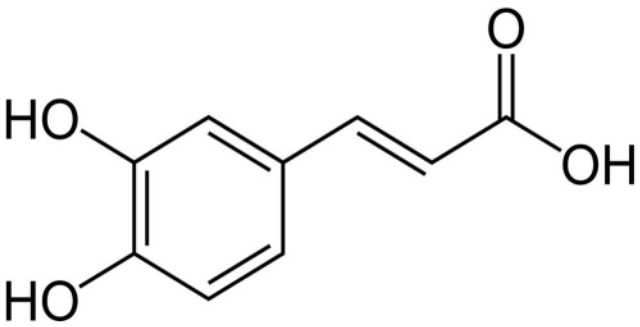
Kaempferol



Ferulic acid



Vanillic acid



Caffeic acid

Table 3 . Pharmacological Activities of different parts of Citruluss Colocynthis plant

Pharmacological activity	Part/ Activity	Extract/ compound	Dose/conc.	Results	References
Antimicrobial activity	Fruit/ in vitro	Water extract	5 mg/mL	inhibition against Staphylococcus aureus	10
	Fruit/ in vitro	Ethanol extract	500 mg/mL	Inhibition against Staphylococcus aureus,	17
	Fruit/ in vitro	Ethanol extract	500 mg/mL	Inhibition against Bacillus cereus	17
	Fruit/ in vitro	Ethanol extract	500 mg/mL	Inhibition against Klebsiella pneumonia	17
	Fruit/ in vitro	Cucurbitacin B	500 mg/mL	Inhibition against Staphylococcus aureus	17
	Fruit/ in vitro	Cucurbitacin B	500 mg/mL	Inhibition against Bacillus cereus	17
	Fruit/ in vitro	Cucurbitacin B	500 mg/mL	Inhibition against Klebsiella pneumonia	17
	Fruit/ in vitro	Cucurbitacin E	500 mg/mL	Inhibition against Staphylococcus aureus	17
	Fruit/ in vitro	Cucurbitacin E	500 mg/mL	Inhibition against Bacillus cereus	17
	Fruit/ in vitro	Cucurbitacin E	500 mg/mL	Inhibition against Klebsiella pneumonia	17
	Fruit/ in vitro	Cucurbitacin I	500 mg/mL	Inhibition against Staphylococcus aureus	17
	Fruit/ in vitro	Cucurbitacin I	500 mg/mL	Inhibition against Bacillus cereus	17
	Fruit/ in vitro	Cucurbitacin I	500 mg/mL	Inhibition against Klebsiella pneumonia	17
Anti-inflammatory Activity	Fruit/ in vivo	Water extract	4 mg/kg	97.29% reduction in edema after 6 hour in Carrageenan-induced paw edema model in rats	46
	Fruit/ in vitro	Aqueous ethanol extract gel	2 g of gel/3%	45% reduction in edema in Carrageenan-induced paw edema model in rats	52
Hypolipidemic Activity	Seed/in vivo	Powder	300 mg/Day	Decrease in the level of triglyceride and cholesterol concentration in non-diabetic hyperlipidemic patients	37
	Fruit/ in vivo	Aqueous ethanol extract	1.2 g/kg/day	Reduction in Serum cholesterol levels in Hyperlipidaemic Rabbits	45
Antidiabetic Activity	Fruit/ in vivo	Petroleum ether extract	300 mg/kg	reduction in blood glucose levels in STZ induced diabetic rats	9
	Fruit/ in vivo	Hydro-ethanol extract	300 mg/kg	Reduction in total cholesterol, triglycerides, phospholipids and free fatty acids levels in diabetic rats	53
	Fruit/ in vivo	Aqueous extract	300mg/kg	Decrease of blood glucose in normoglycaemic rabbits	54
	Seed/ in vivo	Aqueous extract	300mg/kg	Reduction in the plasma level of AST and LDH in	55

Pharmacological activity	Part/ Activity	Extract/ compound	Dose/conc.	Results	References
				Streptozotocin induced diabetic rats	
	Fruit/ in vivo	Alkaloid rich fraction	50 mg/kg	Decrease of blood glucose from 132 to 120 mg/100 mL after 6 h of normoglycaemic rabbits	54
	Fruit/ in vivo	Glycoside rich fraction	50 mg/kg	Decrease of blood glucose from 132 to 89 mg/100 mL after 6 h of normoglycaemic rabbits	54
	Fruit/ in vivo	Saponin rich fraction	50 mg/kg	Decrease of blood glucose from 132 to 84mg/100 mL after 6 h of normoglycaemic rabbits	54
Antioxidant Activity	Fruit/ in vivo	Hydro-ethanol extract	300 g/kg	Increase in the enzymatic and non-enzymatic components of the oxidative system in the liver of Alloxan induced diabetic rats	53
	Seed/in vitro	Aqueous extract	IC5=0.021mg/mL	DPPH radical scavenging activity in term of IC50 of aqueous extract of Citrullus colocynthis seeds was tested	46
	Fruit/ in vitro	Methanol extract	2500 µg/mL	88% DPPH radical scavenging activity was found against ascorbic acid	56
	Fruit/ in vitro	Methanol extract	2500 µg/mL	61.4% Nitric oxide scavenging activity was found against ascorbic acid	56
	Fruit/ in vitro	Cucurbitacin glycosides	IC 50 145 µM	ABTS radical scavenging properties	57
Antiallergic Activity	Fruit/ in vivo	Methanol extract	200 mg/kg	72.5% inhibition in ear passive cutaneous anaphylaxis (PCA) reaction in mice	20
	Fruit/ in vivo	Cucurbitacin E2-O- β-D-glucopyranoside	200 mg/kg	49.7% inhibition in ear passive cutaneous anaphylaxis (PCA) reaction in mice	20
Anticancer Activity	Fruit/ in vitro	Cucurbitacin B, Cucurbitacin E (1:1)	20 µM	inhibited the growth of ER+ MCF-7 and ER-MDA-MB-231 breast cancer cell lines	21
	Fruit/ in vitro	Alkaloid rich fraction	IC50 3.30 µg/mL	Alkaloid rich fraction demonstrated strong cytotoxicity towards Artemia salina naupli	16
	Fruit/ in vitro	Cucurbitacin B	1–100 µM	Inhibited cellular proliferation of human laryngeal cancer cell line (Hep-2)	58
	Fruit/ in vitro	Cucurbitacin B	LD50 = 0.1 µM	Cucurbitacin B inhibited 50% growth at 0.1 µM of human GBM celllines in liquid culture	59
Insecticidal activity	Fruit/ in vitro	Ethanol extract	LC50 11,003ppm	Against Aphis craccivora	60
	Fruit/ in vitro	Chloroform extract	LC50 17,328ppm	Against Aphis craccivora	60

Pharmacological activity	Part/ Activity	Extract/ compound	Dose/conc.	Results	References
	Fruit/ in vitro	Methylenechloride extract	LC50 19,497ppm	Against Aphis craccivora	60
	Fruit/ in vitro	n-hexane extract	LC50 23,065ppm	Against Aphis craccivora	60

PHARMACOLOGICAL ACTIVITY

The traditional medicinal applications of Citrullus colocynthis have inspired many pharmacological investigations. Several extracts and isolated compounds have been evaluated for their biological activities, especially anticancer and antidiabetic activities. There seems to be an interest in developing new anticancer/ antitumor drugs from Citrullus colocynthis due to its high contents of cucurbitacins.

1. Antimicrobial Activity

Results from different studies demonstrated antimicrobial and anticandidal activities of Citrullus colocynthis extracts and the antimicrobial effect varied from population to population.

Aqueous and methanolic extracts were tested for their antimicrobial activities. Antibiotic sensitivity test strain was carried out by using the standard Disc diffusion method including two antifungal drugs. The aqueous extract showed high antibacterial activity against Staphylococcus aureus and E. coli with considerable lower antibacterial against bacillus subtillus and Klebseilla pneumoniae and the methanolic extract showed high antibacterial activities against *B. subtilis, S. pyogenes, S. typhi* with very less activitiy against S. faecalis and inactive against P. mirabilis V. cholera and P. vulgaris (61)

Antibacterial Activity

Ethanolic extracts from stem, root, leaves and fruit of Citrullus colocynthis were tested for their broad spectrum of antibacterial effects against selected positive and negative bacterial strains. The extracts were found to be active against Gram positive bacteria (B. subtilis, B. pumilis and S. auerus) but the extract have poor response against Gram-negative bacteria (E. coli and P. aeruginosa). (62)

The Citrullus colocynthis extracts have a broad spectrum of antibacterial effects in both strains i.e. in Gram-positive as well as in Gram-negative bacterial strains (46,47).

Different solvents extracts of Citrullus colocynthis were studied for their antibacterial activity against some pathogenic bacteria i.e. B. cents, E. coli, S. typhimurium, S. aureus, S. epidermidis M. smegmatis P. aeruginosa, and P. vulgaris (33). Most of the extracts exhibited moderate MIC within the range of 20–100 µg/mL against all the bacterial pathogens.

3. Antifungal Activity

Methanolic and aqueous extracts were screened for their antifungal and antimycotoxigenic activity against *Aspergillus ochraceus* and *Aspergillus flavus* and the extracts showed a very good antifungal activity against *A. ochraceus*, but not against *A. flavus*. The extracts have good antiochratoxigenic power in liquid medium (63)

In another study methanolic extracts were screened for their antifungal activity against six different species of fungi, for which stock culture was maintained in GPYS (Glucose peptone yeast and sucrose) medium. The extracts exhibited high anti-fungal activity against A. flavus, A. fumigatus and Mucor sp. but Penicillium sp., C. albicans and Rhizopus sp. did not show any antifungal activity (13)

4. Anti-inflammatory activity

In modern therapeutical practice, non-steroidal anti-inflammatory drugs (NSAIDs) are known to cause gastrointestinal tract ulceration (50), but NSAIDs with selective cox-2 inhibitory action have been reported to cause fetal cardiac toxicity with less ulcerogenic effects, based on which some of medicine with these effects have been withdrawn from the market. Therefore, there is an increased need for safer anti-inflammatory drugs with very less or no side effects.

Traditionaly, Citrullus colocynthis is used in folk medicines because of its anti-inflammatory activities (41, 46)

Citrullus colocynthis extracts with different solvents were reported for their in vivo anti-inflammatory activity using the carrageenan-induced paw edema model in albino rats (52).

In another study the fruit aqueous extract was studied for its anti- inflammatory effect at 4mg/kg in carrageenan-induced paw edema assay in rats (46) thus above study validated the medicinal use of Citrullus collocynthis as an anti-inflammatory and analgesic agent as well as in rheumatoid arthritis.

In two different animal models, the methanolic extract from the leaf of Citrullus colosynthis shows decrease in the level of carrageenan, serotonin and prostaglandin E1-induced paw edema with about 48% inhibition in prostaglandin E1-induced paw edema model and 35% inhibition in carrageenan

air-pouch model with decrease in the volume of exudate and migration of monocytes and neutrophils (64).

The anti-inflammatory activity found may be due to the presence of therapeutically active flavonoids such as quercetin, apigenin, lutteolin and naringein (65). Flavonoids have their therapeutic use as an anti-inflammatory agent as they are known to prevent the synthesis of prostaglandins (66)

In another study, fruit extract cream was studied for its topical anti-inflammatory effect in carrageenan-induced edema in rats. In this experiment, the commercial ELISA kit was used to estimate the tissue levels of TNF-α and IL-6. The results showed that the fruit extract cream (2–8%) dose-dependently reduced the carrageenan-induced paw edema and reversed the changes in the level of TNF-α and IL-6. (67).

5. Antidiabetic activity

Diabetes mellitus is fairly well known and well-conceived as an entity in the world. It is the fastest growing metabolic disorder with symptomatic treatment and required lifelong use of chemical agents, which produced many side effects with addition to high cost. Hence there is need of a much safer and more effective antidiabetic agent.

Traditionaly, Citrullus colocynthis is used as an antidiabetic agent in different countries. (55).

Citrullus colosynthes fruit extract stimulates the production and activity of insulin. (34, 68).

There is significant reduction in glycaemia and in the level of thiobarbituric acid reactive substances (TBARS) when petroleum ether fruit extract was screened for its antidiabetic effect in Streptozotocin induced diabetic albino rats (9)

Tertiary and quaternary alkaloids, glycosides and saponins, isolated from the Citrullus colocynthis fruits administration oral (50mg/Kg) in normoglycaemic rabbits. No change in elevated glucose level shown by alkaloids. Whereas the glycosidic component significantly reduced the blood glucose level. The saponin component show hypoglycemic effect at much lower doses at 10–20 mg/kg in alloxan-induced diabetic rabbits indicating that the glycosides and saponin components are responsible for the hypoglycemic effect of the fruit of Citrullus colocynthis (68).

When the plant extract is administered orally at a dose of 300mg/kg daily for 2 weeks, it significantly decreases the plasma level of AST and LDH, while it failed to decrease the increased blood level of ALP and GGT in STZ induced diabetic rats (55).

Different extracts from the Citrullus colocynthis root were investigated for biochemical parameters in normal and alloxan-induced diabetic rats. Root significant reduce the blood glucose level about 58.70% when compared to ethanol extract about 36.60% and chloroform extract about 34.72%.

There is also significant improvement in parameters like body weight, serum protein, serum creatinine, and serum urea as well as lipid profile with aqueous extract and it also restored the serum level of total and conjugated bilirubin, SGOT, SGPT and alkaline phosphatase. (69).

All these studies support the use of Citrullus colocynthis as a safer and effective antidiabetic agent.

6. Antilipidemic activity

Citrullus colocynthis was studied for its lipid lowering effect, both in animal as well as in human subjects.

When ethanol extract of the plant was studied for its anti lipidemic effect, it was found that the level of serum cholesterol was reduced to normal level at a dose of 1.2g/kg/day in hyperlipidemic rabbits. (45).

In a clinical study, Citrullus colocynthis seeds powder at a dose of 300 mg significantly reduced the triglyceride and cholesterol level in hyperlipidemic non-diabetic patients. (37).

7. Gastrointestinal effect

Methanolic seed extract was investigated for anti-ulcerogenic activity and it was significant reduced in the gastric volume 1.68±0.18, free acid 39.86±3.86 and total acidity 61.23±1.87 at dose of 200 mg/kg, with maximum inhibition of ulcerogenicity of 71.57% in pyloric ligation induced ulcers model in Wistar albino rats (70)

8. Anticancer Activity

There are many research studies available in the literature for the anticancer activity of the Citrullus colocynthis extract and its isolated compounds (22, 57-59, 71).

The cucurbitacin glycoside extract from Citrullus colocynthis leaves was studied in human breast cnacer cell growth. The cucurbitacin glucosides combination (1:1) at a dose of 20 μM inhibited the growth of selected human breast cancer celllines (21).

Cucurbitacin B (1–100 μM) when studied on human laryngeal cancer cellline (Hep-2) inhibited cellular proliferation and the flow cytometry analysis showed that the treatment with cucurbitacin B resulted in accumulation of cells at the G2/M phase of the cell cycle and cell apoptosis in a dose and time dependent manner (58).

When cucurbitacin B was tested on human Glioblastoma Multiforme (GBM) celllines in liquid culture at 0.1 μ M (ED50), it significantly inhibited 50% growth of GBM celllins. In Soft-gel assays cucurbitacin B inhibited nearly all of the GBM clonogenic cells at 10^{-8} M, indicating that this drug might be a good candidate for clinical trial (59).

Cucurbitacin E has also cytotoxic and anti-cancer effects. Cucurbitacin L also show a cytotoxic effect when studied

against KB and HeLa celllines, but was less potent than cucurbitacin I, which was isolated from Citrullus colocynthis (72, 73, 22).

9. Antioxidant activity

Citrullus colocynthis extracts are a rich source of poly phenol and plant sterol so they can be used as antioxidants (25).

The methanolic fruit extract was evaluated for its free-radical scavenging effect and the highest antioxidant and free radical scavenging ability was observed at a concentration of 2500 mg/ml(56).

Cucurbitacin glycoside isolated from Citrullus colocynthis exhibited ABTS radical scavenging properties with IC50 at 145 μM, probably through the involvement of a direct scavenging effect on several free-radicals (57).

The aqueous extract of Citrullus colocynthis seeds show very potent DPPH radical scavenging activity with IC50 of 0.021mg/mL. (46).

The various study strongly supported the use of Citrullus colocynthis as a source of natural antioxidant agents.

10. Effect on hair growth

Petroleum ether extract from *Citrullus colocynthis* was evaluated to study the effect on hair growth and the extract in oleaginous ointment base was applied topically on shaved denuded skin albino rats.

The hair growth initiation time as well as the cycle for hair growth completion was recorded. In standard animals minoxidil 2% solution was applied topically. The time required for hair growth initiation was reduced to half on treatment with the petroleum ether extracts compared with untreated control animals as well as the time required for complete hair growth was also decreased. The treatment was effective in bringing a more prominent number of hair follicles (>70%) to the anagenic stage than standard minoxidil (67%). The result of treatment with 2% and 5% petroleum ether extracts were comparable with the standard minoxidil (74).

TOXICITY

The fruit pulp extract of Citrullus colocynthis was studied for teratogenicity during the early stage of pregnancy in rats at a dose of 40.6 mg/kg body weight, equivalent to one fourth of the LD50 of the extract. The study displayed treatogenic effects, when gross anatomical observation is done on the 20th day of gestation it revealed a high percentage of resorbed fetuses, smaller size and weight fetuses as well as absence of coccygeal vertebrae, metacarpal and metatarsal bones, and carpal and tarsal bones. (75).

Pulp and seed extracts of Citrullus colocynthis at a dose of 100 or 200 mg/kg/day were tested on rabbits. After one month the surviving animals were sacrificed and specimens from the liver, small intestine and kidney were prepared for morphological evaluation. The survival rate for the animals treated with 200 mg/kg/day of pulp extract was zero and the animals treated with 100 mg/kg/day of pulp extract showed sever lesions in the liver, kidney and small intestine. On the other hand, animals treated with seed extract at a dose of 100 or 200 mg/kg/day displayed only minor intestinal insult (76).

The study showed that the drug is severely poisonous. Due to the presence of cucurbitacin glycoside content it has a strongly irritating and painful effect on mucous membranes. Overdose usually associated with vomiting, colic irritation, bloody diarrhea and kidney irritation, follows the intake of toxic dosages (0.6 to 1 g), and then increased diuresis which progressed to anuria. Lethal dosages, starting at 2 g lead to convulsions, paralysis and, if untreated, to death through circulatory collapse. The treatment for poisonings should proceed symptomatically following gastric lavage (77).

CONCLUSION

In this review, we have documented the existing traditional uses and summarized the recent research to the pharmacology of the Citrullus colocynthis plant.

Many traditional uses have been validated on the bases of phytochemical and modern pharmacological studies but still many need to be validated.

Different extracts and isolated components from the Citrullus colosynthis plant have been found to possess various biological and pharmacological activities, especially in the area of anti-inflammatory, antidiabetic, antioxidant, anticancer, insecticidal, antilipidemic and antimicrobial.

It is evident that the different parts of this plant possessed a huge potential for further studies and could be utilized in several medical applications.

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CONFLICT OF INTEREST

Authors do not have any conflict of interest.

REFERENCES

1. Dyson A. (1998). Discovering indigenous healing plants of the herb and fragrance gardens at Kirstenbosch National Botanical Garden. Cape Town.
2. Chan K. (2003). Some aspects of toxic contaminants in herbal medicines. *Chemosphere*. *52*, 1361-1371.
3. Al-Snafi AE. (2016). Chemical constituents and pharmacological effects of Citrullus colocynthis-A review. *IOSR Journal of Pharmacy*. *6*, 57-67.

4. Zaini NAM, Anwar F, Hamid AA, et al. (2011). Kundur [Benincasa hispida (Thunb.) Cogn.]: A potential source for valuable nutrients and functional foods. *Food Research International. 44*, 2368-2376.
5. Robinson RW, Decker-Walters DS. (1997). Crop production science in horticulture. Cucurbit, 6, CAB International. Wallingford, Oxon, U.K., New York, N.Y.
6. Upadhyay B, Roy S, Kumar A. (2007). Traditional uses of medicinal plants among the rural communities of Churu district in the Thar Desert, India. *Journal of ethnopharmacology*. *113*, 387-399.
7. Kumar D, Kashyap SK, Maherchandani S. (2009). Antibacterial activity of some plant extracts. Veterinary Practitioner. 10, 148–151.
8. Amamou F, Bouafia M, Chabane-Sari D, et al. (2011). Citrullus colocynthis: a desert plant native in Algeria, effects of fixed oil on blood homeostasis in Wistar rat. *Journal of Natural Product and Plant Resources*. *1*, 1-7.
9. Jayaraman R, Shivakumar A, Anitha T, et al. (2009). Antidiabetic effect of petroleum ether extract of Citrullus colocynthis fruits against streptozotocin-induced hyperglycemic rats. *Rom J Biol Plant Biol. 4*, 127-34.
10. Najafi S, Sanadgol N, Nejad BS, et al. (2010). Phytochemical screening and antibacterial activity of Citrullus colocynthis (Linn.) Schrad against Staphylococcus aureus. *Journal of Medicinal Plants Research. 4*, 2321-2325.
11. Salama HM. (2012). Alkaloids and flavonoids from the air dried aerial parts of Citrullus colocynthis. *Journal of Medicinal Plants Research*. *6*, 5150-5155.
12. Delazar A, Gibbons S, Kosari AR, et al. (2006). Flavone C-glycosides and cucurbitacin glycosides from Citrullus colocynthis. *DARU Journal of Pharmaceutical Sciences*. *14*, 109-114.
13. Gurudeeban S, Satyavani K, Ramanathan T. (2010). Bitter apple (Citrullus colocynthis): An overview of chemical composition and biomedical potentials. *Asian Journal of Plant Sciences*. *9*, 394.
14. Meena MC, Patni V. (2008). Isolation and identification of flavonoid "quercetin" from Citrullus colocynthis (Linn.) Schrad. *Asian J Exp Sci. 22*, 137-142.
15. Hussain AI, Rathore HA, Sattar MZ, et al. (2013). Phenolic profile and antioxidant activity of various extracts from Citrullus colocynthis (L.) from the Pakistani flora. *Industrial crops and products. 45*, 416-422.
16. Mukherjee A, Patil SD. (2012). Effects of alkaloid rich extract of Citrullus colocynthis fruit on Artemia salina and human cancerous (MCF-7 and HEPG-2) cells. *J. PharmaSciTech. 1*, 15-19.
17. Ali AA, Alian MA, Elmahi HA. (2013). Phytochemical analysis of some chemical metabolites of Colocynth plant [Citrullus colocynthis L.] and its activities as antimicrobial and antiplasmidial. *J Basic Appl Sci Res*. *3*, 228-36.
18. Sayed MD, Balbaa SI, Afifi MSA. (1973). Nitrogenous bases of the different organs of Citrullus colocynthis. *Planta medica. 24*, 260-265.
19. Adam SEI, Al-Yahya MA, Al-Farhan AH. (2001). Response of Najdi sheep to oral administration of Citrullus colocynthis fruits, Nerium oleander leaves or their mixture. *Small Ruminant Research*. *40*, 239-244.
20. Yoshikawa M, Morikawa T, Kobayashi H, et al. (2007). Bioactive saponins and glycosides. XXVII. Structures of new cucurbitane-type triterpene glycosides and antiallergic constituents from Citrullus colocynthis. *Chemical and pharmaceutical bulletin*. *55*, 428-434.
21. Tannin-Spitz T, Grossman S, Dovrat S, et al. (2007). Growth inhibitory activity of cucurbitacin glucosides isolated from Citrullus colocynthis on human breast cancer cells. *Biochemical pharmacology. 73*, 56-67.
22. Chen JC, Chiu MH, Nie RL, et al. (2005). Cucurbitacins and cucurbitane glycosides: structures and biological activities. *Natural product reports*. *22*, 386-399.
23. Sawaya WN, Daghir NJ, Khan P. (1983). Chemical characterization and edibility of the oil extracted from Citrullus colocynthis seeds. *Journal of Food Science. 48*, 104-106.
24. Sadou H, Sabo H, Alma MM, et al. (2007). Chemical content of the seeds and physico-chemical characteristic of the seed oils from Citrullus colocynthis, Coccinia grandis, Cucumis metuliferus and Cucumis prophetarum of Niger. *Bulletin of the Chemical Society of Ethiopia. 21*, 323-330.
25. Sebbagh N, Cruciani-Guglielmacci C, Ouali F, et al. (2009). Comparative effects of Citrullus colocynthis, sunflower and olive oil-enriched diet in streptozotocin-induced diabetes in rats. *Diabetes & metabolism. 35*, 178-184.
26. Kalhoro MA, Afza N, Saleem M, et al. (2002). Pharmacochemical studies of the oil, aerial parts, pulp and peel of Citrullus colocynthis. *Journal of the Chemical Society of Pakistan. 24*, 274-276.
27. Chopra RN, Chopra IC. (1994). *Indigenous drugs of India*. Academic publishers.
28. Chopra RN. (1958). Chopra's Indigenous drug of India, UN Dhar & Sons Pvt. *Ltd., Calcutta*. *12*, 495.
29. Abo KA, Fred-Jaiyesimi AA, Jaiyesimi AEA. (2008). Ethnobotanical studies of medicinal plants used in the management of diabetes mellitus in South Western Nigeria. *Journal of ethnopharmacology*. *115*, 67-71.
30. Sultan A, Khan FU, Iqbal H, et al. (2010). Evaluation of chemical analysis profile of Citrullus colocynthis growing in southern areas of Khyber Pukhtunkhwa Pakistan. *World Applied Sciences Journal. 10*, 402-405.
31. Sharma SK, Medicinal Plants Used in Ayurveda 1998. *National Academy of Ayurveda, Ministry of Health and Family Welfare, Govt. of India, New Delhi, India*.
32. Katewa SS, Galav PK. (2005). Traditional herbal medicines from Shekhawati region of Rajasthan.
33. Sharma A, Singh S, Nag TN. (2010). Antibacterial activity of Citrullus colocynthhis and Tribulus terrestris against some pathogenic bacteria. *Asian Journal of Microbiology and Biotechnology and Environmental Sciences. 12*, 633-637.

34. Nmila R, Gross R, Rchid H, et al. (2000). Insulinotropic effect of Citrullus colocynthis fruit extracts. *Planta Medica. 66*, 418-423.
35. Aburjai T, Hudaib M, Tayyem R, et al. (2007). Ethnopharmacological survey of medicinal herbs in Jordan, the Ajloun Heights region. *Journal of Ethnopharmacology. 110* 294-304.
36. Huseini HF, Darvishzadeh F, Heshmat R, et al. (2009). The clinical investigation of Citrullus colocynthis (L.) schrad fruit in treatment of Type II diabetic patients: a randomized, double blind, placebo-controlled clinical trial. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives. 23*, 1186-1189.
37. Rahbar AR, Nabipour I. (2010). The hypolipidemic effect of Citrullus colocynthis on patients with hyperlipidemia. *Pakistan journal of biological sciences: PJBS. 13*, 1202-1207.
38. Ziyyat A, Legssyer A, Mekhfi H, et al. (1997). Phytotherapy of hypertension and diabetes in oriental Morocco. *Journal of ethnopharmacology. 58*, 45-54.
39. Eddouks M, Maghrani M, Lemhadri A, et al. (2002). Ethnopharmacological survey of medicinal plants used for the treatment of diabetes mellitus, hypertension and cardiac diseases in the south-east region of Morocco (Tafilalet). *Journal of ethnopharmacology. 82*, 97-103.
40. Tahraoui A, El-Hilaly J, Israili ZH, et al. (2007). Ethnopharmacological survey of plants used in the traditional treatment of hypertension and diabetes in south-eastern Morocco (Errachidia province). *Journal of ethnopharmacology*. *110*, 105-117.
41. Wasfi IA, Bashir AK, Abdalla AA, et al. (1995). Anti-inflammatory activity of some medicinal plants of the United Arab Emirates. *International journal of pharmacognosy. 33*, 124-128.
42. Goldfain D, Lavergne A, Galian A, et al. (1989). Peculiar acute toxic colitis after ingestion of colocynth: a clinicopathological study of three cases. *Gut. 30*, 1412-1418.
43. AL RARAJ S. (1995). Haemorrhagic colitis induced by Citrullus colocynthis. *Annals of tropical medicine and parasitology*. *89*, 695-696.
44. Barth A, Müller D, Dürrling K. (2002). In vitro investigation of a standardized dried extract of Citrullus colocynthis on liver toxicity in adult rats. *Experimental and Toxicologic Pathology. 54*, 223-230.
45. Daradka H, Almasad MM, Qazan WSH, et al. (2007). Hypolipidaemic effects of Citrullus colocynthis L. in rabbits. *Pakistan journal of biological sciences: PJBS. 10*, 2768.
46. Marzouk B, Marzouk Z, Haloui E, et al. (2010). Screening of analgesic and anti-inflammatory activities of Citrullus colocynthis from southern Tunisia. *Journal of ethnopharmacology. 128*, 15-19.
47. Marzouk B, Marzouk Z, Décor R, et al. (2009). Antibacterial and anticandidal screening of Tunisian Citrullus colocynthis Schrad. from Medenine. *Journal of ethnopharmacology. 125*, 344-349.
48. Baquar SR, Tasnif M. (1967). *Medicinal plants of southern west Pakistan* (No. 3). Botany Section, Central Laboratories, Pakistan Council of Scientific and Industrial Research.
49. Kirtikar KR, Basu BD, Blatter E, et al. (1984). Indian Medicinal Plants. Lalit Mohan Basu, Allahabad, India.
50. Qureshi R, Bhatti GR, Memon RA. (2010). Ethnomedicinal uses of herbs from northern part of Nara desert, Pakistan. *Pak J Bot. 42*, 839-851.
51. Schafferman D, Beharav A, Shabelsky E, et al. (1998). Evaluation ofCitrullus colocynthis, a desert plant native in Israel, as a potential source of edible oil. *Journal of Arid Environments. 40*, 431-439.
52. Aly AM, Naddaf A. (2006). Anti-inflammatory activities of Colocynth topical gel. *J Med Sci. 6*, 216-21.
53. Dallak M. (2011). In vivo, hypolipidemic and antioxidant effects of Citrullus colocynthis pulp extract in alloxan-induced diabetic rats. *African journal of biotechnology. 10*, 9898-9903.
54. Abdel-Hassan IA, Abdel-Barry JA, Mohammeda ST. (2000). The hypoglycaemic and antihyperglycaemic effect of Citrullus colocynthis fruit aqueous extract in normal and alloxan diabetic rabbits. *Journal of ethnopharmacology*. *71*, 325-330.
55. Al-Ghaithi F, El-Ridi MR, Adeghate E, et al. (2004). Biochemical effects of Citrullus colocynthis in normal and diabetic rats. *Molecular and cellular biochemistry. 261*, 143-149.
56. Kumar S, Kumar D, Manjusha, et al. (2008). Antioxidant and free radical scavenging potential of Citrullus colocynthis (L.) schrad. Methanolic fruit extract. *Acta Pharm. 58*, 215-220.
57. Tannin-Spitz T, Bergman M, Grossman S. (2007). Cucurbitacin glucosides: Antioxidant and free-radical scavenging activities. *Biochemical and Biophysical Research Communications. 364*, 181-186.
58. Liu T, Zhang M, Deng Y, et al. (2008). Effects of cucurbitacin B on cell proliferation and apoptosis in Hep-2 cells. *Lin Chuang er bi yan hou tou Jing wai ke za zhi Journal of Clinical Otorhinolaryngology, Head, and Neck Surgery*. *22*, 403-407.
59. Yin D, Wakimoto N, Xing H, et al. (2008). Cucurbitacin B markedly inhibits growth and rapidly affects the cytoskeleton in glioblastoma multiforme. *International Journal of Cancer. 123*, 1364-1375.
60. HM T, HM AY, AZ AA, et al. (2009). Insecticidal effect of cucurbitacin E glycoside isolated from Citrullus colocynthis against Aphis craccivora.
61. Bauer RW, Deutsch M, Mutchler GS, et al. (1960). Nuclear Orientation of Mn 54 and Mn 52 m. *Physical Review. 120*, 946.
62. Memon U, Brohi AH, Ahmed SW, et al. (2003). Antibacterial screening of Citrullus colocynthis. *Pakistan journal of pharmaceutical sciences. 16*, 1-6.
63. Gacem MA, Khelil AOEH, Gacemi B, et al. (2013). Antimycotoxigenic and antifungal activities of Citrullus colocynthis seeds against Aspergillus flavus and Aspergillus ochraceus contaminating wheat stored. *African journal of biotechnology*. *12*, 6222-6231.

64. Rajamanickam E, Gurudeeban S, Ramanathan T, et al. (2010). Evaluation of anti-inflammatory activity of Citrullus colocynthis. *International Journal of Current Research. 2*, 67-69.
65. Khare CP. (2008). *Indian medicinal plants: an illustrated dictionary*. Springer Science & Business Media.
66. Havsteen BH. (2002). The biochemistry and medical significance of the flavonoids. *Pharmacology & therapeutics. 96*, 67-202.
67. Pashmforosh M, Rajabi Vardanjani H, Khodayar MJ. (2018). Topical anti-inflammatory and analgesic activities of Citrullus colocynthis extract cream in rats. *Medicina. 54*, 51.
68. Bnouham M, Ziyyat A, Mekhfi H, et al. (2006). Medicinal plants with potential antidiabetic activity-A review of ten years of herbal medicine research (1990-2000). *International Journal of Diabetes and Metabolism. 14*, 1.
69. Agarwal V, Sharma KA, Upadhyay A. (2012). Hypoglycemic effects of Citrullus colocynthis roots. *Acta Pol Pharm.* 69, 75-9.
70. Gill NS, Kaur S, Arora R, et al. (2011). Screening of antioxidant and antiulcer potential of Citrullus colocynthis methanolic seed extract. *Res J Phytochem. 5*, 98-106.
71. Liu T, Zhang M, Zhang H, et al. (2008). Combined antitumor activity of cucurbitacin B and docetaxel in laryngeal cancer. *European journal of pharmacology. 587*, 78-84.
72. Lavie D, Willner D, Merenlender Z. (1964). Constituents of Citrullus colocynthis (L.) Schrad. *Phytochemistry. 3*, 51-56.
73. Cassady JM, Suffness M. (1980). Terpenoid antitumor agents. *Anticancer agents based on natural product models. 201*.
74. Roy RK, Thakur M, Dixit VK. (2007). Effect of citrullus colocynthis. On hair growth in albino rats. *Pharmaceutical biology*. *45*, 739-744.
75. Elgerwi AA, Benzekri Z, El-Magdoub A, et al. (2013). Qualitative identification of the active principles in Citrullus colocynthis and evaluation of its teratogenic effects in albino rats. *International Journal of Basic & Clinical Pharmacology. 2*, 438-445.
76. Shafaei H, Esmaeili A, Rad JS, et al. (2012). Citrullus colocynthis as a medicinal or poisonous plant: a revised fact. *Journal of Medicinal Plants Research*. *6*, 4922-4927.
77. Fleming T. (2000). PDR for herbal medicines: From Medical Economics Company. *New Jersy: USA*, *253*.

ANTITUMOR EFFECT OF THE SYNTHESIZED CHALCONE ANALOGUES ON HeLa CELL LINE

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ABSTRACT

Chalcones represent a type of flavonoids which are located at vegetative and reproductive organs of plants and they can be metabolic progenitor molecules for several flavonoids and isoflavonoids. Many studies indicated that molecular structure of chalcone accountable for their anti-tumor, anti-inflammatory and anti-oxidant effects. The aim of our research was to investigate anti-tumor effect and mechanism of action of three synthesized chalcone analogues on HeLa cells. The anti-tumor effectiveness of chalcone analogues was compared to effects of the dehydrozingerone and cisplatin that were used as referent substances.
The viability of the treated cells was evaluated using MTT assay. Evaluation of cell death was determined by flow cytometry and cells were stained with Annexin V-FITC/7-AAD. The result of our research indicated that used chalcones have stronger antitumor effect relative to the dehydrozingerone and cisplatin.
The IC_{50} values of the chalcones ranged between 1.69-6.18 μM, with CH1 being more cytotoxic after 24 h of treatment, while CH3 being more cytotoxic after 48 h of treatment on HeLa cells. All investigated chalcones induced apoptosis in HeLa cells via mitochondrial pathway, which was detected expression Bax and Bcl-2 proteins.
Our results provided evidence that chalcones induced apoptosis in HeLa cervical carcinoma through the intrinsic apoptotic pathway. These findings provide insights into the molecular mechanism of chalcones-induced cell death.

Keywords: *Apoptosis, cervical cancer, chalcones, cytotoxicity, HeLa.*

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INTRODUCTION

Cervical cancer (CC) represents the third most common malignancy in women worldwide. In many countries, this type of cancer is the leading cause of death for women. High mortality rate from CC comes as a result of late diagnosis and limitations of therapy (1). The limitations in therapy approaches are defined with: drug resistance of tumor cells, the non-selectivity of anti-cancer drugs towards the healthy cells and the abundance of unwanted side effects (2). Due to the existence of the above mentioned drawbacks, it is necessary to discover and synthesize new anti-cancer agents that would have more efficient antitumor effect with less unwanted effects on healthy cells. In order to eliminate tumor cells and its expansion, antitumor drug effect directly provokes different types of cell death mechanisms and pathways in cancer cells (2). However, the failure for cancer eradication lays in the fact that cancer cells overcome goals of antitumor therapy acquiring apoptosis-resistance during treatment (3). As a final result, cancer cell survival is activated and propagation of the tumor continues. To date, scientific research indicates that there are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (4). The intrinsic pathway involves distortion of the outer mitochondrial membrane potential while the extrinsic pathway involves binding of cells death receptors. Like apoptosis, autophagy represents highly regulated process that eliminates and recycles degenerative cells in order to enable cell survival. Protective role of autophagy in cancer development is achieved with the elimination of certain proteins or damaged organelles (5). Due to this fact, manipulation with autophagy in cancer cells may result in accelerated tumor cell death (6). During the past 80 years chloroquine has been widely used as a potent antimalarial drug, however due to its numerous biological effects, including inhibition of cell growth and induction of apoptosis in cancer cells, chloroquine as a potent inhibitor of autophagy is used as anti-cancer drug (7). Chalcone represent a class of flavonoids that occur naturally in fruits and vegetables, and metabolic precursors of some flavonoids and isoflavonoids (8). Various studies have shown that the chemical structure of chalcone is responsible for their anti-tumor, anti-inflammatory and anti-oxidant effects (8) (9). Chalcone have certain advantages compared to the current cancer drugs such as less toxicity towards healthy cells, lower prices, higher availability etc.

We herein report for the first time, antitumor effect of previously synthesized chalcones analogue on HeLa cell line.

METHODS

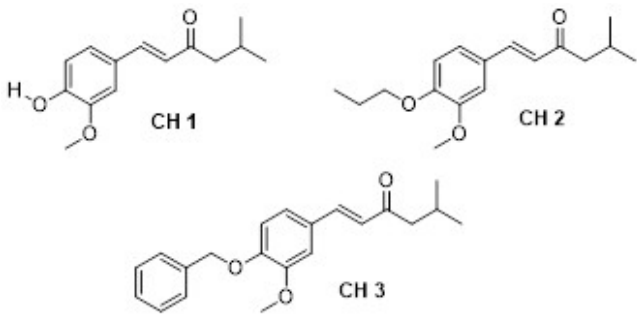
Chemistry

All starting chemicals were commercially available and used as received, except that the solvents were purified by distillation. Chromatographic separations were carried out using silica gel 60 (Merck, 230-400 mesh ASTM) whereas silica gel on Al plates, layer thickness 0.2 mm (Merck), was used for TLC. IR spectra were recorded on a Perkin-Elmer One FT-IR spectrometer with a KBr disc, ν in cm^{-1}; NMR spectra were recorded on a Varian Gemini 200 MHz spectrometer (200 MHz for ^{1}H and 50 MHz for ^{13}C), using $CDCl_3$ as solvent and TMS as the internal standard. ^{1}H and ^{13}C NMR chemical shifts were reported in parts per million (ppm) and were referenced to the solvent peak; $CDCl_3$ (7.26 ppm for ^{1}H and 76.90 ppm for ^{13}C).

Reagents and compounds

Chloroquine was obtained from Sigma Aldrich (C6628) and dissolved in ultrapure water at final concentration of 20 μM. Three newly synthesized chalcone analogues (E)-1-(4-hydroxy-3-methoxyphenyl)-5-methylhex-1-en-3-one, (CH1) (E)-1-(3-methoxy-4-propoxyphenyl)-5-methylhex-1-en-3-one, (CH2) (E)-1-(4-(benzyloxy)-3-methoxyphenyl)-5-methylhex-1-en-3-one) (CH3), (Figure 1) were tested for antitumor activity on HeLa cell line and results are presented in this article.

Figure 1. Structures of tested chalcone analogues.



The referent substances (dehydrozingerone (DHZ) was a gift from our colleagues, Faculty of Chemistry; University of Kragujevac; cisplatin (cisplatin) CAS 15663-27-1 - Calbiochem) were dissolved in DMSO (at final concentration of DMSO less than 0.5%). Investigated concentrations of CH 1, CH 2, and CH 3 were 0.3, 1, 3, 10, 30, 100, and 300 μM and for referent substances (DHZ and cisplatin) were 3, 10, 30, and 100 μM. - Annexin V-FITC and 7-AAD (7-amino-actinomycin-D) - were purchased from Abnova (KA3806).

Cell lines and culturing method

Human epithelial cervical carcinomas cells (HeLa) and human fibroblast lung cell line (MRC-5) were purchased from the American Type Culture Collection (ATCC® CCL-2™ and CCL-171™). Cells (passage 5 and 6) were cultured in complete growth DMEM medium (Dulbecco's Modified Eagle's medium, Sigma Aldrich D5671). *Cells were maintained* in culture at 37°C in atmosphere containing 5% CO_2 and 95% air. The cells were classified in three large groups – first group- control cells (HeLa and MRC-5 cells) cultured in complete medium only; second - experimental groups, cells treated with different chalcones and third group cells treated with different concentrations of referent substances during 24 and 48 h period.

MTT Cell Viability Assay

The viability of the three chalcones on HeLa cancer cells and noncancerous MRC-5 cells during 24 and 48 h period was determined using MTT assay (10). The absorbance was measured at 595 nm using a micro-plate reader (Zenyth 3100, Anthos Labtec Instruments).

Determination of IC_{50} values for investigated substances

The percentage of cytotoxic cells was calculated using the formula: Cytotoxicity (%) = (1 - (exp. group (ABS)) / (control group (ABS)) x 100). The IC_{50} values of investigated substances were calculated using Microsoft Office Excel free add-in (ed50v10.xls.) for linear regression.

Effect of CH analogues on apoptosis and autophagy – flow cytometry

In order to evaluate type of the cell death induced with investigated substances, staining procedure with Annexin V-FITC/7-AAD (7-amino-actinomycin-D) was performed using flow cytometry analysis (11). For autophagy detection pre-treatment with CQ 20 μM was performed on experimental and control cell group. Cell samples were analyzed by flow cytometer Cytomics FC500 (Beckman Coulter, USA). Obtained data were analyzed using Flowing Software and presented by dot plots.

Morphological analysis

Both control and experimental HeLa cells were seeded in 24 well plates and allowed to attach for 24 h. After incubation period, cells were exposed to vehicle (VEH)-containing complete media and different concentrations (3, 10, 30 and 100 μM) of CH, DHZ and cisplatin during 48 h period. Cells were visualized with phase contrast microscopy under 100 X magnification on Olympus microscope (model BX51).

Flow cytometry analysis of apoptosis-related proteins

In order to explain the mechanism of apoptosis, we analyzed the presence of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2. HeLa cells were incubated for 24 h with IC_{50} values of CH1, CH2 and CH3 or culture medium alone (control) harvested, washed with ice cold PBS, resuspended, fixed and permeabilized (using Fixation and Permeabilization Kit, eBioscience). Two types of staining were separately performed. For Bcl-2 staining permeabilized cells were incubated with FITC-conjugated 1:500 anti-Bcl-2 monoclonal antibody (mhbcl01, Life technologies) for 20 min at room temperature. For Bax staining cells were incubated with primary 1:500 anti-Bax antibody (N20, sc-493, Santa Cruz Biotech. Inc) for 30 min at room temperature. These cells had been washed, incubated with appropriate secondary antibody ((1:1000 Alexa 488-conjugated antibody (11001, Invitrogen, USA)) for 30 minutes, washed with PBS and analyzed by flow cytometry. The Bcl-2 and Bax expression levels were expressed as mean fluorescence index (MFI).

Statistical analysis

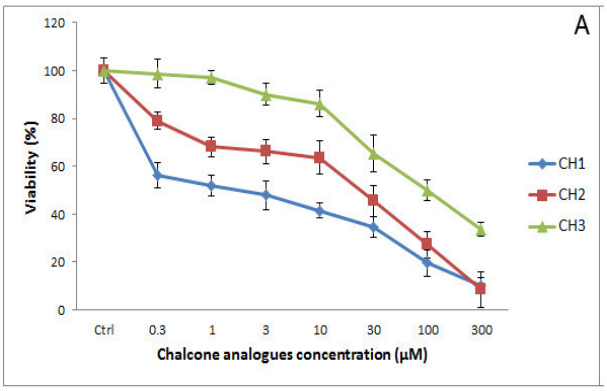
The data are presented as the mean ± standard deviation for at least three repeated individual experiments for each group. In order to study significant differences between two groups, the Student's *t*-test was used. All data are represented as means ± SEM.

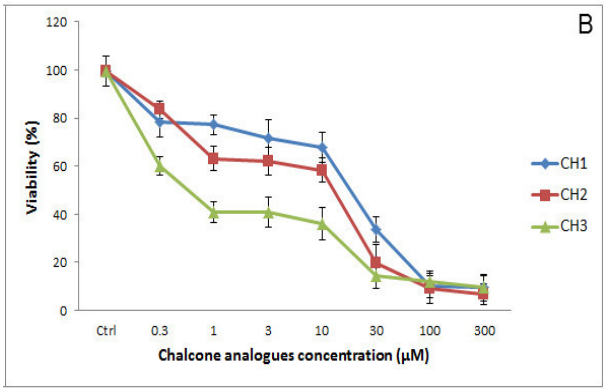
RESULTS

According to the previously described processes, new series of compounds were synthesized in very good yields (12). Starting compound (CH1) was prepared by Claisen-Schmidt condensation of natural product vanillin and 4-methylpentan-2-one. Alkylation of free phenolic group in CH1, set of corresponding *O*-alkyl derivatives was obtained.

After 24 and 48 h of treatment with chalcones, the percentage of viable HeLa cells decreased significantly (Figure 2). The results obtained by MTT test showed that CH1 was most efficient and decreased viability of HeLa cells relative to both of CH2 and CH3 after 24 h treatment. In the case of the lowest applied dose (0.3 μM), CH1 decreased viability of HeLa cells for 56.21%, while CH2 and CH3 had 79.00% and 98.68% after 24 h treatment. The highest applied dose (300 μM) of chalcones decreased viability of HeLa cells for 10.36 (CH1), 8.48% (CH2) and 33.72% (CH3) after 24 h treatment. After 48 h treatment lowest dose of chalcone analogues decreased viability of HeLa cells for 78.34% (CH1), 83.53% (CH2) and 60.09% (CH3 while the highest applied dose resulted in 9.68%, 6.84% and 9.58%, respectively.

Figure 2. The effects of the various concentrations of new chalcone analogues on viability of HeLa cancer cells after 24 h treatment (A) and 48 h (B). Viability was quantified by MTT assay. Results are mean ± SD of three experiments ($p<0.05$)



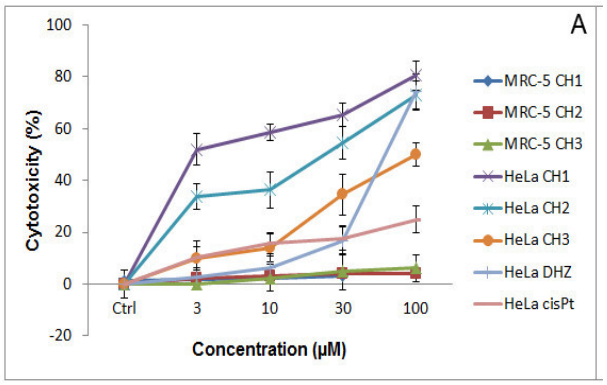


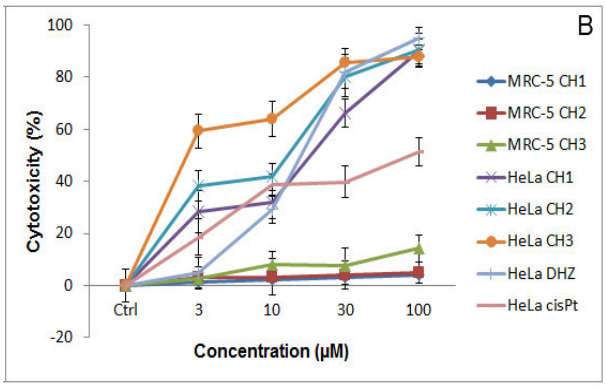
Based on the obtained values of the IC_{50} test, we compared the cytotoxic effect on HeLa and MRC-5 cells between chalcones and reference substances during 24 and 48 h. Interestingly, CH1 resulted in various cytotoxic effects after 24 h of treatment in comparison to the cytotoxic effects chalcones after 48 h of treatment. The chalcone CH1 exhibited significant cytotoxic effect on the HeLa cells after 24 h of treatment with the IC_{50} value of 3.97 μM compared to CH2 and CH3 (IC_{50} 4.11 and 6.18 μM), respectively (Table 1). However, CH3 chalcone analogue exhibited more significant cytotoxic effect on HeLa cells after 48 h of treatment (IC_{50} 1.69 μM) compared to both of CH 1 and CH2 (IC_{50} 3.67 and 3.5 μM) (Table 1). After 48 h CH3 chalcone analogue exhibited the most efficient cytotoxic effect on HeLa cells compared to all tested agents (IC_{50} 1.69 μM) (Table 1). After 24 and 48 h treatment with all investigated chalcone analogues, IC_{50} values for MRC-5 cells were > 200 μM and IC_{50} value for DHZ was > 300 μM. However, *cisplatin* exhibited a stronger cytotoxic effect on noncancerous MRC-5 cells (IC_{50} values for 24 and 48 h were > 150 and > 40 μM, respectively) compared to chalcones and DHZ (Table 1). Our results also showed that chalcone analogues increased cytotoxicity of the cancer cells in comparison to DHZ and *cisplatin* (Figure 3). All tested chalcone analogues had stronger cytotoxic effect on HeLa cells after 24 h (IC_{50} 3.97, 4.11 and 6.18 μM) compared to *cisplatin* (IC_{50} of 9.70 μM), and less significant cytotoxic effect compared to DHZ (IC_{50} 3.61 μM) after 24 h of treatment (Table 1). After 48 h, all chalcone analogues exhibited more efficient cytotoxic effect (IC_{50} 3.67, 3.5 and 1.69 μM) compared to *cisplatin* (IC_{50} 3.8 μM). However, only CH3 chalcone analogue had more efficient cytotoxic effect on HeLa cells after 48 h compared to DHZ (IC_{50} 2.41 μM) (Table 1).

Table 1. IC50 values (μM) of chalcone analogues (CH), cisplatin and dehydrozingerone (DHZ) after 24 and 48 h treatment of HeLa cancer cells and noncancerous MRC-5 cell.

HeLa IC_{50}	**CH1**	**CH2**	**CH3**	**cisPt**	**DHZ**
24 h	3.97 ± 1.45	4.11 ± 1.46	6.18 ± 1.72	9.70 ± 1.22	3.61 ± 2.16
48 h	3.67 ± 1.17	3.5 ±1.1	1.69 ± 0.68	3.8 ± 0.4	2.41 ± 0.57
MRC-5 IC_{50}	**CH1**	**CH2**	**CH3**	**cisPt**	**DHZ**
24 h	> 200	> 200	> 200	< 150	< 300
48 h	> 200	> 200	> 200	< 40	< 300

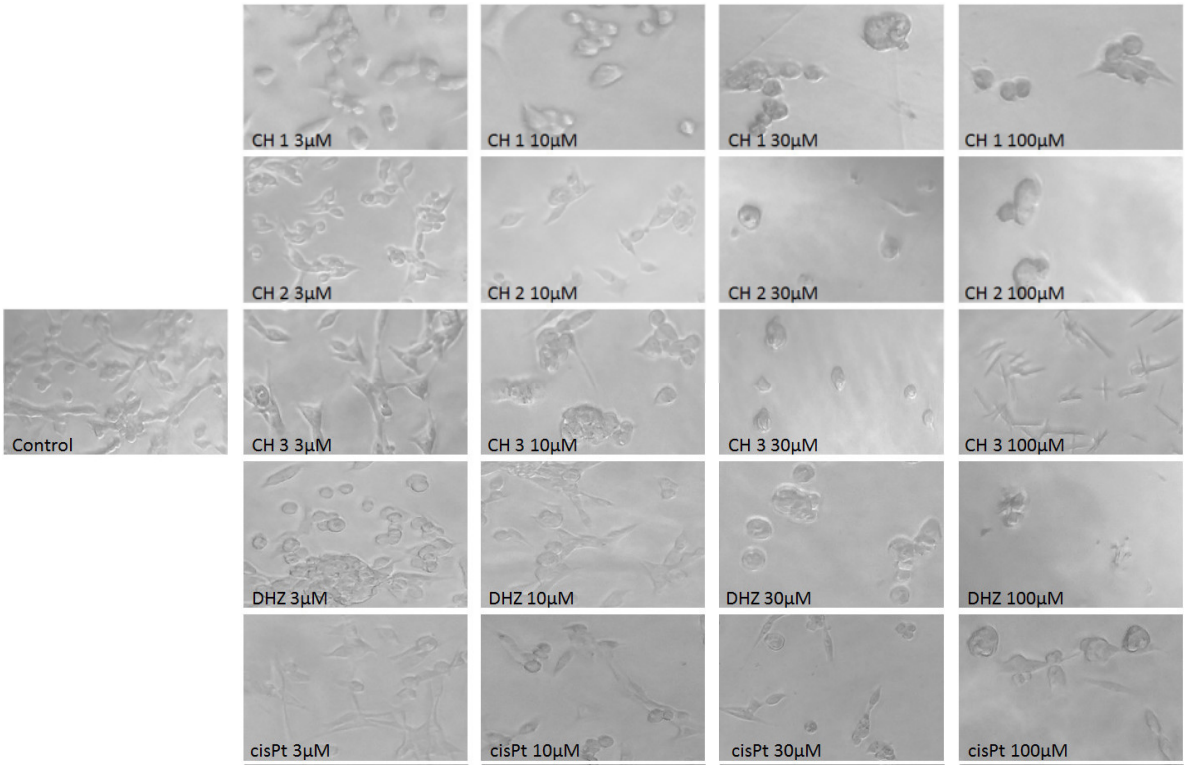
Figure 3. The effects of new synthesized chalcone analogues on cytotoxicity of HeLa and MRC-5 cells after 24 h (A) and 48 h (B) in comparison to DHZ and cisplatin treatment. Results are mean ± SD of three experiments ($p<0.05$).





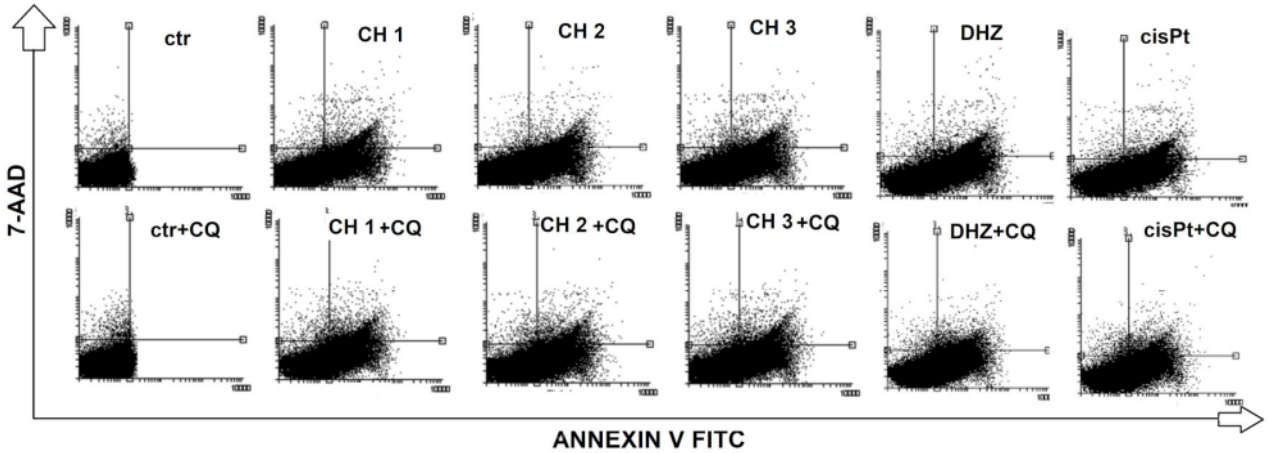
In our next experiment using phase contrast microscopy, we have determined changes in HeLa cells viability, cell and nuclear shape after 48 h exposure to chalcone analogues and referent substances. The morphological changes of treated HeLa cells were compared to the morphology of the control cells (Figure 4). Our results indicated a significant reduction in the number of treated HeLa cells compared to the number of control cells. Morphological changes of the treated cells were manifested as: cell rounding, loss of normal cell shape and cell attachment. Complete loss of the cell morphological characteristics were statistically significant in the case of all investigated CH analogues starting from 3 μM. Intensity of morphological changes of the treated cells was directly dose dependent.

Figure 4. Morphology of the HeLa cells after 48 h treatment with investigated substances. Equal number of HeLa cells were plated in 24 well plates and allowed to attach for 24 h. Cells were exposed to vehicle (VEH)-containing complete media and different concentrations of CH, DHZ and cisplatin during 48 h period and morphology of the cells was analysed on microscope.



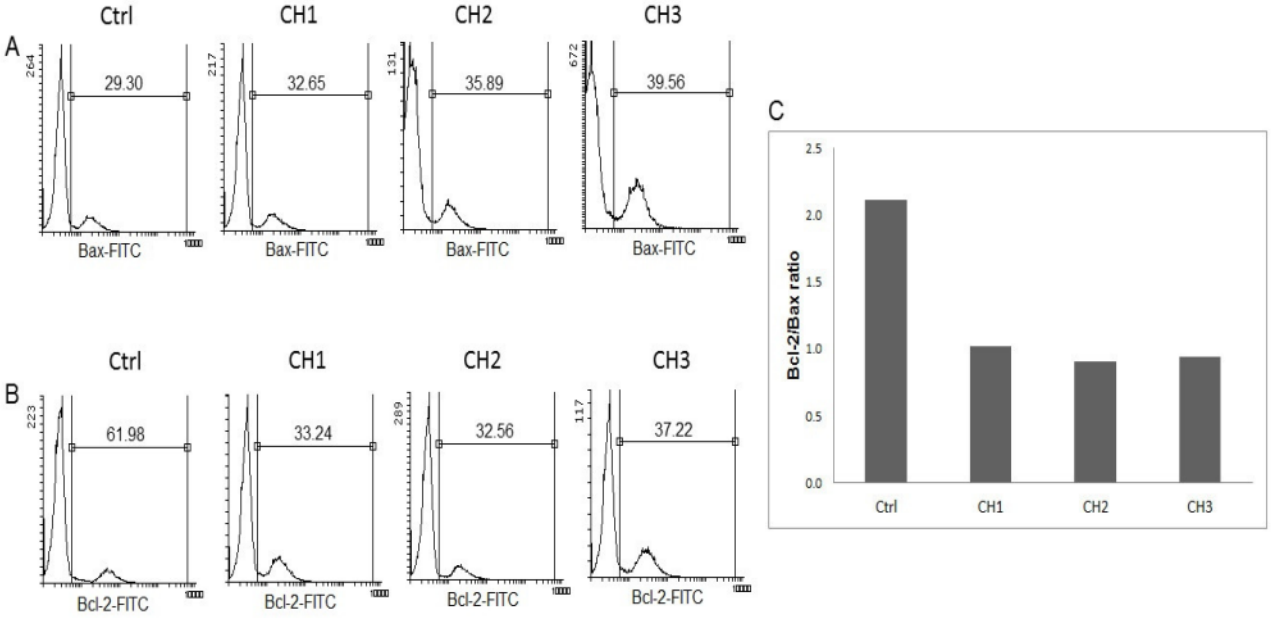
Next we have investigated the mechanism of their killing abilities by studying their apoptotic induction by applying the Annexin V-FITC/7-AAD staining. Our results clearly showed that apoptosis plays an important role in the death of HeLa cells induced with all chalcone analogues (Figure 5). Specifically, after 48 h treatment of HeLa cells with chalcone analogues (3.67, 3.5 and 1.69 μM), our results showed that 37.30, 40.48 and 39.17%, of cells were in early apoptosis; 3.55, 2.81 and 2.93%, of cells were in late apoptosis; 0.74, 0.69 and 0.75% of cells were in necrosis. After 48 h treatment with cisplatin and DHZ (3.8 and 1.76 μM), our results showed that 37.98 and 41.69% of cells were in early apoptosis; 3.92 and 4.1% of cells were in late apoptosis; 0.62 and 0.66% of cells in were necrosis (Figure 5). In our next experiment we evaluated if CH co-treatment with chloroquine (CQ - 20 μM) promoted apoptosis in HeLa through the inhibition of autophagy. Our results indicated that CQ did not influence apoptosis in control HeLa cells. The percentage of the control cells undergoing early apoptosis was 6.12%, while after CQ treatment this percentage was 5.16%. Similar effect of CQ on the percentage of the control cells in late apoptosis (ctrl - 0.07%; CQ - 0.08%) and necrotic cells (ctrl - 1.53 %; CQ - 1.77%) was observed (Figure 5). Our results were similar to experimental cells. Results indicated that co-treatment with CQ had no statistically significant effect regarding the distribution (percentage) of the apoptotic and necrotic HeLa cells (Figure 5). Our results presented on Figure 5, showed that co-treatment with CQ did not affect CH analogues induced apoptosis, indicating that autophagy was not involved in mechanism of cytotoxic action of tested substances. The apoptotic type of cell death was further confirmed by flow cytometric analysis of apoptosis-related protein expression in HeLa cells. The expression of anti-apoptotic Bcl-2 decreased in treated cells, while expression of pro-apoptotic active Bax increased (Figure 6 A and B). The Bcl-2/Bax ratio consequently declined (Figure 6 C).

Figure 5. Effects of CH analogues on apoptosis and autophagy in HeLa cells. Flow cytometry analysis of Annexin V-FITC/7-AAD stained HeLa cells after 48 h of the treatment with CH, DHZ, cisplatin and co treatment with CQ.



	ctrl	ctrl+CQ	CH 1	CH 1+CQ	CH 2	CH 2+CQ	CH 3	CH 3+CQ	DHZ	DHZ+CQ	cisPt	cisPt+CQ
EA	6.12	5.16	37.30	36.64	40.48	41.78	39.17	35.13	41.69	39.28	37.98	32.06
LA	0.07	0.08	3.55	3.67	2,81	3.82	2.93	3.78	4.10	3.39	3.92	3.97
N	1.53	1.77	0.74	0.87	0.69	0,99	0.75	1.06	0.66	0.69	0.62	0.92

Figure 6. Expression of apoptosis-related proteins in untreated (control) and treated HeLa cells presented by histograms (Fig. 6 A and B) and bar chart (Fig 6. C). The mean fluorescence intensity (MFI) of Bax (A) and Bcl-2 staining (B) are indicated on histograms. Bar chart (C) showing Bcl-2/Bax ratio in untreated and treated cells.



DISCUSSION

Numerous researches indicate that chalcones (benzylidene acetophenone's) and their analogues display cytotoxic, anticancer (8) (9) (13) antimalarial (7) activity etc. These abundance of biological activities develops as a result of modification of 1,3-diphenyl propenone template with different functional groups or atoms (14). Due to different biological effects, we have synthesized three new chalcone analogues, and for the first time we have investigated their cytotoxic and apoptotic effects on HeLa and MRC-5 cell line. The cytotoxic and apoptotic effects of different chalcone analogues on human carcinoma cell line HeLa, MCF-7, SKBR-3, A549, PC3 and human HT-29 are demonstrated in several studies (15) (16) (17) (18). Results of these studies showed that different types of chalcone analogues had high cytotoxic effect on human cancer cells and low cytotoxic effect on healthy cells (17). Our results correlated with the previous

studies and indicate that low IC_{50} values of our CH analogues induced strong cytotoxic effect on HeLa cells with low cytotoxic effect towards healthy cells. Dehydrozingerone is structurally related to chalcones (19). In investigation conducted by Jin et al., (19) authors indicate that newly synthesized chalcones showed significant and similar cytotoxic activity against both KB and KB-VCR. Ignacia et al., (20) and Burmudzija et al., (21) reported that Mannich base DHZ derivatives exhibited higher antioxidant and cytotoxic activity compared to DHZ. Similar to findings of Jin et al., (19) we have evaluated and compared cytotoxic effects of our chalcone analogues with DHZ. Results of our study showed that CH analogues had similar cytotoxic effect compared to DHZ. ElMoanem et al., (22) showed that their chalcone derivatives exerted superior activity against HCT-116 and PC-3 cell lines in comparison with cisplatin. Manik and Kuntal (13) showed promising activity of anthraquinone based chalcone compounds on proliferation rate of HeLa cells with similar IC_{50} values to cisplatin. Morphological changes of cytotoxic cells include cell rounding, loss of normal cell shape and cell attachment. Different literature sources indicated presence of morphological changes of chalcone treated cells (18) (23) (24). Similar to findings of Solano et al., (24), we have determined changes in HeLa cells viability, cell shape and nuclear shape compared to control (Figure 4). Our results indicate that cell number of treated HeLa cells was significantly lower compared to the control HeLa cells. Intensity of morphological changes of the treated cells directly correlated to the concentrations of investigated substances. Literature data that investigated the effect of different chalcone analogues on type of the cell death pointed out that chalcones induced apoptosis (3) (13). Our results showed that all investigated CH analogues induced apoptosis in HeLa cell line. In some literature data, it has been reported that application of chloroquine with chalcone analogues enhances apoptotic responses of treated cells (25) (26). However, our results presented on Figure 5, pointed out that co-treatment with CQ did not affect CH analogues induced apoptosis, indicating that autophagy was not involved in the mechanism of cytotoxic action of tested substances. Various sources of literature identify chalcones as powerful inducers of apoptosis in different tumor cells. However their apoptotic mechanisms differ. For instance, two chalcone derivates acted on human hepatoma cells line (HepG2 and Huh-7) through caspase dependent intrinsic pathways, increased expression of Bax and decreased the levels of Bcl-2 triggering the mitochondrial apoptotic pathway in T24 and HT-1376 cells (27). Also, in a study by Ignacio et al., (28) synthetic chalcone increased expression of Bax and decreased the levels of Bcl-2 in human leukemia U-937 cells. Flavokawain C induces apoptosis in HCT-116 cells by activating both the intrinsic and extrinsic apoptotic pathway (29). In our study, we provided evidence that chalcone analogue induces apoptosis HeLa cells that was accompanied by the activation of Bax and decrease of Bcl- 2 protein expression which further leads to the release of cytochrome c from mitochondria into cytosol and cleavage/activation of caspase-3. Also, our results showed that the resulting net effect led to a lower ratio of Bcl-2 / Bax which might be responsible for the chalcone analogues-induced apoptosis in HeLa cells.

In conclusion, for the first time we have determined mechanism of action for these chalcone analogues on treated HeLa cells. Our results provided evidence that chalcone analogues induced apoptosis in HeLa cervical carcinoma through the intrinsic apoptotic pathway. These findings provide insights into the molecular mechanism of chalcone analogues-induced cell death. These compounds shows good promise to be further evaluated as a potential therapeutic agents for the treatment of human carcinoma.

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REFERENCE

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018 CA: A Cancer J Clin 2018;68:7–30.
2. Galluzzi L, Vitale I, Aaronson SA et al. Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018 Cell Death {&} Differ 2018;25:486-541
3. Baig S, Seevasant I, Mohamad J, Mukheem A, Huri HZ, Kamarul T. Potential of apoptotic pathway-targeted cancer therapeutic research: Where do we stand Cell Death and Disease 2016;7:1-11.
4. Wong RS. Apoptosis in cancer: from pathogenesis to treatment Journal of Experimental & Clinical Cancer Research 2011;30:87.
5. Gewirtz DA. The Four Faces of Autophagy: Implications for Cancer Therapy. Cancer Research 2014;74:647-51.
6. Monma H, Iida Y, Moritani T et al. Chloroquine augments TRAIL-induced apoptosis and induces G2/M phase arrest in human pancreatic cancer cells. PLoS One 2018;13:1-16.
7. Al-Bari AA. Chloroquine analogues in drug discovery: New directions of uses, mechanisms of actions and toxic manifestations from malaria to multifarious diseases. J Antimicrob Chemother. 2014;70:1608–21.
8. Zhang EH, Wang RF, Guo SZ, Liu B. An update on antitumor activity of naturally occurring chalcones. Evidence-based Complement. Altern. Med 2013;13:1-22.
9. Vogel S, Barbic M, Jürgenliemk G, Heilmann J. Synthesis, cytotoxicity, anti-oxidative and anti-inflammatory activity of chalcones and influence of A-ring modifications on the pharmacological effect. Eur J Med Chem. 2010;45:2206–13.
10. Riss TL, Moravec RA, Niles AL et al. Cell Viability Assays. Assay Guid Man 2013;114(8):785–96.
11. Koopman G, Reutelingsperger CPM, Kuijten GAM, Keehnen RMJ, Pals ST, Van Oers MHJ. Annexin V for

Flow Cytometric Detection of Phosphatidylserine Expression on B Cells Undergoing Apoptosis 1994;84:1415-20.
12. Muškinja J, Ratkovi Z, Rankovi B, Kosani M. Synthesis of O -Alkyl Derivatives of Dehydrozingerone Analogues. Kragujev J Sci 2016;38:97–106.
13. Das M, Manna K. Chalcone Scaffold in Anticancer Armamentarium: A Molecular Insight. J Toxicol 2016;16:14.
14. Bukhari SNA, Jasamai M, Jantan I. Synthesis and biological evaluation of chalcone derivatives (mini review). Mini Rev Med Chem 2012;12:1394–403.
15. Kelter G, Eichhorn E, Beckers T, Fiebig HH, Sellmer A, Mahboobi S. Synthesis and cytotoxic activity of 2-acyl-1H-indole-4,7-diones on human cancer cell lines. Eur J Med Chem 2004;40:85–92.
16. Pilatova M, Varinska L, Perjesi P et al. In vitro antiproliferative and antiangiogenic effects of synthetic chalcone analogues. Toxicol Vitr 2010;24:1347–55.
17. Syam S, Abdelwahab S, Al-Mamary M, Mohan S. Synthesis of Chalcones with Anticancer Activities. Molecules (Basel, Switzerland) 2012;17:6179-95.
18. Luković J, Mitrović M, Zelen I, Čanović P, Zarić M, Nikolić I. Antitumor Effect of the Chalcone Analogue, (E) -1- (4-Ethoxy-3-Methoxyphenyl) -5- Methylhex-1-en-3-One on HeLa Cell Line. Serbian J Exp Clin Res. 2018;0(0):1–7.
19. Tatsuzaki J, Bastow KF, Nakagawa-Goto K, Nakamura S, Itokawa H, Lee K-H. Dehydrozingerone, Chalcone, and Isoeugenol Analogues as in Vitro Anticancer Agents [#]. J Nat Prod 2006;69:1445–9.
20. Ignacia C, Hayun H, Arrahman A et al. Synthesis, Anti-inflammatory, and Antioxidant Activity of Mannich Bases of Dehydrozingerone Derivatives. J Young Pharm. 2018;10:6-10.
21. Burmudzija AZ, Muskinja JM, Kosanic MM et al. Cytotoxic and Antimicrobial Activity of Dehydrozingerone based Cyclopropyl Derivatives. Chem Biodivers 2017 Aug;14:1-16.
22. ElMonaem HSA, Abdel-Aziz NI, Morsy MA et al. Synthesis, in vitro antiproliferative evaluation and molecular docking of new tetrazole-chalcone and tetrazole-pyrazoline hybrids. J Appl Pharm Sci 2018;8:75–87.
23. Rasul A, Yu B, Zhong L, Khan M, Yang H, Ma T. Cytotoxic effect of evodiamine in SGC-7901 human gastric adenocarcinoma cells via simultaneous induction of apoptosis and autophagy. Oncol Rep 2012;27:1481–7.
24. Solano JD, González-Sánchez I, Cerbón MA et al. The products of the reaction between 6-amine-1,3-dimethyl uracil and bis-chalcones induce cytotoxicity with massive vacuolation in HeLa cervical cancer cell line. Eur J Med Chem 2013;60:350–9.
25. Tang ZH, Chen X, Wang ZY et al. Induction of C/EBP homologous protein-mediated apoptosis and autophagy by licochalcone A in non-small cell lung cancer cells. Sci Rep 2016;6:1-10.
26. Zhao S, Ma C-M, Liu C-X et al. Autophagy inhibition enhances isobavachalcone-induced cell death in multiple myeloma cells. Int J Mol Med 2012;30:939–44.
27. Ramirez-Tagle R, Escobar CA, Romero V et al. Chalcone-induced apoptosis through caspase-dependent intrinsic pathways in human hepatocellular carcinoma cells. Int J Mol Sci. 2016;17:1-18.
28. Brouard I, Quintana J, Del Rosario H, Saavedra E, Estévez F. 6′-Benzyloxy-4-bromo-2′-hydroxychalcone is cytotoxic against human leukaemia cells and induces caspase-8- and reactive oxygen species-dependent apoptosis. Chem Biol Interact 2018;298:137–45.
29. Phang C-W, Karsani SA, Sethi G, Abd Malek SN. Flavokawain C Inhibits Cell Cycle and Promotes Apoptosis, Associated with Endoplasmic Reticulum Stress and Regulation of MAPKs and Akt Signaling Pathways in HCT 116 Human Colon Carcinoma Cells. PLoS One 2016;11:1-27.

ASSOCIATIONS BETWEEN METABOLIC SYNDROME, ULCERATIVE COLITIS, AND FECAL SST2 AND CXCL8 LEVELS: UNVEILING NEW INFLAMMATORY PATHWAYS

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ABSTRACT

Ulcerative Colitis (UC), a chronic inflammatory bowel disease, exhibits complex interactions with metabolic disorders such as Metabolic Syndrome (MetS), which can significantly impact disease progression and patient outcomes. Among the multitude of players in this intricate network, soluble ST2 (sST2) and Chemokine (C-X-C motif) ligand 8 (CXCL8) have emerged as critical mediators of immune responses, potentially modulating the disease course in UC patients with co-existing MetS. This study aimed to investigate the association between Metabolic Syndrome (MetS) and immune response modulation in patients with Ulcerative Colitis (UC). UC patients, stratified by the presence of MetS, underwent clinical, endoscopic, and histological evaluation, along with blood and fecal biochemical analyses. Serum and fecal concentrations of sST2 and CXCL8 were measured and compared between groups. UC patients with MetS exhibited lower white blood cell (WBC) count, higher levels of metabolic markers, and milder disease severity on clinical, endoscopic, and histological scales. Serum concentrations of sST2 and CXCL8 were similar between UC patients with and without MetS. However, fecal levels of these cytokines were significantly elevated in UC patients with MetS, suggesting a localized intensified immune response. Our findings indicate a potential dichotomy in the immune response of UC patients with MetS, characterized by a dampened systemic inflammation and heightened local immune response. The elevated fecal levels of sST2 and CXCL8 underscore a potentially unique immune modulation within the gut in the presence of MetS. These findings shed new light on the pathophysiological interplay between MetS and UC and may provide new avenues for targeted therapeutic strategies.

Keywords: *Ulcerative Colitis, Metabolic Syndrome, sST2, CXCL8, Immune Response.*

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INTRODUCTION

Inflammatory bowel diseases (IBD), such as ulcerative colitis (UC), are chronic, relapsing disorders that involve inflammation of the gastrointestinal tract (1,2). The etiology of IBD is complex and multifactorial, with genetic, immunologic, and environmental factors playing pivotal roles in disease onset and progression (3,4). UC, characterized by continuous inflammation and ulceration of the colonic mucosa, presents a significant clinical challenge due to its unpredictable course and the necessity for lifelong management (5). It has been consistently associated with various extraintestinal manifestations and comorbidities, which can substantially impact the patient's quality of life (6).

One such comorbidity is metabolic syndrome (MetS), a cluster of conditions that includes obesity, insulin resistance, hypertension, and dyslipidemia (7). The incidence of MetS is on the rise globally, and its association with IBD, particularly UC, has garnered increased attention (8). Interestingly, recent studies have unveiled a potential protective effect of MetS on the inflammatory and immunopathogenic processes underlying UC (9, 10). This is contrary to the traditional view that MetS tends to exacerbate inflammatory diseases (11).

The protective influence of MetS on UC has been reflected through several parameters, including clinical and endoscopic scores, histopathological changes, phenotype of inflammatory cells, and cytokine levels in the liquid fraction of feces (10). Despite the somewhat paradoxical nature of this relationship, it has provided novel insights into the complex interplay between metabolic disorders and immune-mediated inflammatory diseases.

In this context, the role of key inflammatory and immune biomarkers, such as soluble ST2 (sST2) and chemokine (C-X-C motif) ligand 8 (CXCL8, also known as interleukin-8), gains importance. sST2, a member of the interleukin-1 receptor family, is known to act as a decoy receptor for interleukin-33, thereby inhibiting its proinflammatory actions (12). Elevated sST2 levels have been associated with various inflammatory conditions (12). On the other hand, CXCL8 is a potent chemokine that attracts neutrophils to the site of inflammation, thus playing a crucial role in inflammatory responses (13).

In this study, we sought to compare the systemic and fecal levels of sST2 and CXCL8 in patients with UC with and without MetS. Our findings aimed to elucidate further the intricate relationship between UC and MetS, with a particular emphasis on the local intestinal environment, contributing to the understanding of the pathophysiology of UC and potentially informing the development of novel therapeutic strategies.

MATERIAL AND METHODS

Ethics Guidelines Compliance

This study was conducted under the joint initiative of the Center for Gastroenterology, Clinical Center of Kragujevac and the Center for Molecular Medicine and Stem Cell Research at the Faculty of Medical Sciences, University of Kragujevac, Serbia. The research was granted approval by the respective Ethics Committees of these institutions. In keeping with ethical research standards, the principles of Good Clinical Practice and the Declaration of Helsinki were strictly adhered to throughout the study. Prior to participating, all patients provided their written informed consent for the collection and analysis of blood and tissue samples. The Clinical Center Kragujevac's medical staff provided continuous supervision to all participants during the course of the study.

Study Population

In this cross-sectional, observational study, we included patients diagnosed with both Ulcerative Colitis (UC) and Metabolic Syndrome (MetS). Our cohort consisted of 70 patients, including 40 males and 30 females, ranging in age from 21 to 81 years. Each patient had a histologically confirmed diagnosis of UC and MetS at the inception of the study. We captured demographic and clinical data for all participants and processed this data using SPSS Statistical Analysis Software.

Patients were excluded if they had a previous diagnosis of colorectal cancer, Crohn's disease, or if their UC had been treated previously with antibiotics, aminosalicylates, corticosteroids, immunosuppressants, statins, or biological therapies. All patients underwent a comprehensive evaluation, including physical examination, routine laboratory testing, and diagnostic imaging such as chest X-rays, abdominal ultrasound and computed tomography scans, and endoscopy.

MetS Diagnosis

Patients diagnosed with UC were included in the study and divided into two groups based on the presence or absence of MetS. The diagnosis of MetS was established using the Adult Treatment Panel III (ATP III) criteria (14). According to the ATP III criteria, MetS is diagnosed when three or more of the following five criteria are present:

1. Abdominal obesity, as defined by a waist circumference >102 cm (40 inches) in men and >88 cm (35 inches) in women.
2. Serum triglycerides ≥150 mg/dL (1.7 mmol/L) or drug treatment for elevated triglycerides.
3. Serum high-density lipoprotein (HDL) cholesterol <40 mg/dL (1.03 mmol/L) in men and <50 mg/dL (1.29 mmol/L) in women or drug treatment for low HDL cholesterol.
4. Blood pressure ≥130/85 mmHg or drug treatment for elevated blood pressure.

5. Fasting plasma glucose level above 99 mg/dL (5.5 mmol/L) and/or a 2-hour post-load plasma glucose level exceeding 140 mg/dL (7.8 mmol/L), or the use of medication to treat elevated blood glucose.

For all patients included in the study, the relevant measurements were taken at the baseline. Waist circumference was measured midway between the lower rib margin and the iliac crest. Blood samples were taken after an overnight fast to determine serum triglyceride, HDL cholesterol, and fasting plasma glucose levels. Blood pressure was measured using a standard sphygmomanometer after the patient had been seated and resting for at least five minutes.

Patients meeting three or more of these ATP III criteria were classified as having MetS and included in the UC+MetS group, while those not fulfilling these criteria were included in the UC only group.

Assessment of Ulcerative Colitis Severity

The severity of UC was determined using a combination of endoscopic, clinical, and histological evaluations.

The Mayo endoscopic sub-score was used for grading the severity of endoscopic lesions in UC patients (15). This scoring system classifies the severity into four categories: 0 - normal or inactive disease; 1 - mild disease (erythema, decreased vascular pattern, mild friability); 2 - moderate disease (marked erythema, lack of vascular pattern, friability, erosions); 3 - severe disease (spontaneous bleeding, ulceration).

Clinical severity of UC was assessed using the Truelove and Witts clinical activity index and the Mayo clinical index (16). The Truelove and Witts index characterizes UC activity based on six parameters: number of bowel movements per day, presence of blood in stool, body temperature, pulse rate, hemoglobin level, and erythrocyte sedimentation rate. The Mayo clinical index, on the other hand, is a composite score assessing stool frequency, rectal bleeding, physician's global assessment, and endoscopic findings.

Histological assessment of UC severity was performed using the Geboes grading system (17). This scoring system provides a detailed evaluation of various histological features, including architectural changes, inflammatory infiltrate, neutrophils in the lamina propria, crypt destruction, erosion or ulceration, and granulation tissue or neovascularization.

All endoscopic, clinical, and histological evaluations were conducted by experienced gastroenterologists and pathologists blinded to the patients' metabolic status.

Quantification of Fecal and Serum Cytokine Levels

The process for the preparation of fecal samples adhered to previously outlined methodologies (18). In brief, 1g of fecal material was diluted and homogenized in 5mL of a protease inhibitor cocktail (P8340, Sigma Aldrich, St. Louis, MO, USA) through stirring. Concurrently, blood samples were obtained from all study participants at 8 am, and serum was separated, collected, and stored at -80 °C until subsequent analysis. Levels of sST2 and CXCL2 in the serum and fecal supernatants of UC patients were determined through the use of commercial ELISA kits, strictly adhering to the manufacturer's instructions.

Statistical Analysis

All statistical computations in the study were executed using SPSS software (version 22.0; IBM Corp., Armonk, NY, USA). Descriptive statistics were utilized to summarize the demographic and clinical features of the study participants. Continuous variables were presented as mean ± standard error of mean (SE), with their distribution being verified by the Shapiro-Wilk test. Categorical variables, on the other hand, were expressed as frequencies and percentages. The comparison of continuous variables between two groups was performed using Student's t-test (for normally distributed data) or the Mann-Whitney U test (for data that was not normally distributed). Comparisons of categorical variables were conducted.

RESULTS

Blood Parameter Findings in UC Patients with Metabolic Syndrome

A total of 70 patients participated in the study, with 58 of them presenting Ulcerative Colitis (UC) along with Metabolic Syndrome (MetS), while 12 patients had UC without MetS. In the context of blood parameters, patients with UC and MetS demonstrated a significantly lower white blood cell (WBC) count compared to patients with UC alone (8.6±1.0 x 10^9/L vs 10.3±1.4 x 10^9/L; $p<0.01$). Additionally, the levels of blood Potassium (4.6±0.3 mmol/L vs 4.0±0.2 mmol/L; $p<0.05$) and Sodium (140±2.5 mmol/L vs 136±2.0 mmol/L; $p<0.05$) were notably increased in the UC+MetS group.

Evaluation of lipid profile and liver function tests revealed significantly higher levels of cholesterol (5.8±0.4 mmol/L vs 3.6±0.3 mmol/L; $p<0.01$), triglycerides (2.1±0.2 mmol/L vs 1.6±0.2 mmol/L; $p<0.01$), and low-density lipoprotein (LDL) (3.5±0.3 mmol/L vs 1.7±0.2 mmol/L; $p<0.01$) in the UC+MetS group. Levels of aspartate aminotransferase (AST) (28±4.0 U/L vs 22±3.0 U/L; $p<0.05$) and alanine aminotransferase (ALT) (30±4.0 U/L vs 24±3.0 U/L; $p<0.05$) were also elevated in UC patients with MetS, indicating altered liver function.

Regarding renal function, both urea (5.5±0.5 mmol/L vs 3.2±0.4 mmol/L; $p<0.05$) and creatinine (90±7.0 µmol/L vs 75±6.0 µmol/L; $p<0.05$) were significantly higher in the UC+MetS group, suggestive of possible alterations in renal function.

However, there was no significant difference detected between the two groups in terms of erythrocyte count, platelet count, and concentrations of hemoglobin, albumin, and

globulin. Similarly, levels of free thyroxine (fT4), thyroid-stimulating hormone (TSH), chloride (Cl^-), phosphate (P^-), calcium (Ca^{2+}), high-density lipoprotein (HDL), gamma-glutamyl transferase (GGT), and lactate dehydrogenase (LDH) did not differ significantly between the groups with both ulcerative colitis and metabolic syndrome (UC+MetS) and the group with only ulcerative colitis (UC).

These findings indicate that patients with both UC and MetS demonstrate a distinct profile of blood parameters and biochemical markers, potentially reflective of the systemic implications of the co-existing conditions.

The Protective Influence of MetS: Endoscopic and Histopathological Evidence in UC Patients

In the evaluation of endoscopic and clinical severity, patients with UC and MetS consistently demonstrated lower scores compared to those with UC alone. The Mayo endoscopic subscore in the UC+MetS group was significantly lower, with patients predominantly falling in the category of score 1, indicating mild disease characterized by slight mucosal erythema, decreased vascular pattern, and mild friability (1.80±0.40 vs 2.60±0.30; p<0.001). This was in stark contrast to UC patients without MetS, who exhibited frank friability, marked erythema, an absent vascular pattern, and erosions, typically characteristic of moderate disease severity.

Similar findings were observed in the Mayo clinical subscore, with a lower average score in the UC+MetS group compared to the UC only group (1.75±0.20 vs 2.20±0.50; p<0.01), indicating milder clinical disease activity in the presence of MetS. The Truelove and Witts clinical activity index also showed a trend towards lower scores in the UC+MetS group, although this difference did not reach statistical significance.

Consistent with the endoscopic and clinical evaluations, histopathological assessment revealed milder inflammatory changes in the UC+MetS group. Chronic inflammatory infiltration was significantly less severe in UC patients with MetS compared to those without MetS (1.85±0.30 vs 2.40±0.40; p=0.034). Similarly, the extent of eosinophilic infiltration was also reduced in the UC+MetS group (1.60±0.35 vs 2.20±0.50; p=0.031).

These results suggest a potential protective effect of MetS on both the endoscopic appearance and the histopathological features of UC, which might be associated with altered local immune responses in the gut.

Differential Fecal Levels of sST2 and CXCL8 in Ulcerative Colitis Patients with Metabolic Syndrome: A New Perspective on Disease Pathogenesis

Our study aimed to evaluate the concentrations of sST2 and CXCL8 in both the serum and fecal liquid fractions across all UC patients, shedding light on their roles in UC progression in the context of MetS.

Focusing on the systemic concentration of sST2, as shown in Figure 1A, we found no statistically significant difference between UC patients with MetS and those without (713.71±49.99 pg/ml vs 686.35±82.34 pg/ml; p= 0.526). This suggests that the presence of MetS does not significantly impact the systemic levels of sST2 in patients suffering from UC.

Interestingly, a different scenario was observed when evaluating the concentration of sST2 in the fecal liquid fraction. UC patients with MetS had significantly higher fecal sST2 levels (997.69±108.79 pg/ml) when compared to those without MetS (721.14±246.37 pg/ml), reaching statistical significance (p= 0.026) (Figure 1B). This finding suggests a heightened local release or decreased clearance of sST2 in the intestinal lumen among UC patients with MetS.

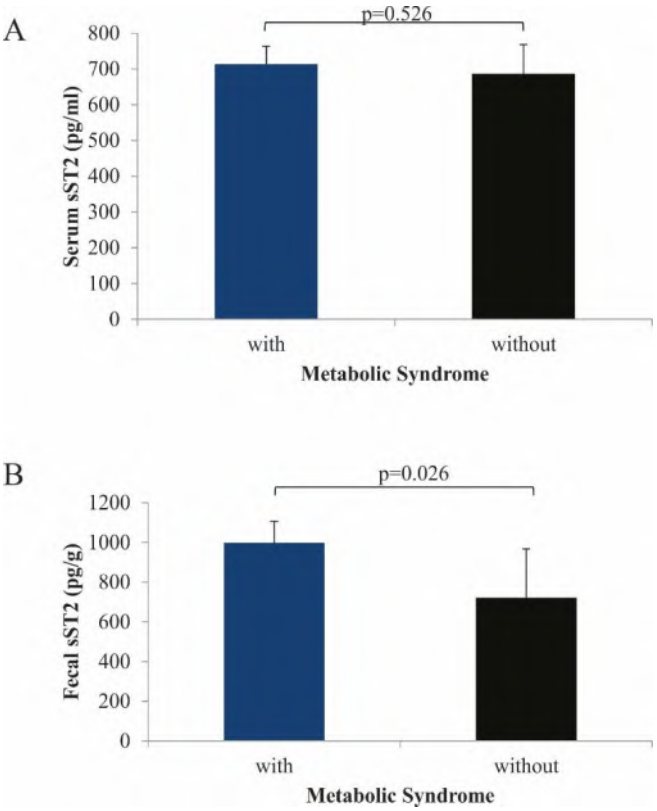

Figure 1. Levels of sST2 in ulcerative colitis (UC) patients, with and without co-existing metabolic syndrome (MetS). The graph illustrates both serum (A) and fecal (B) sST2 concentrations. Statistical significance between groups was determined using either the Student's t-test or the Mann-Whitney U test, as appropriate. Data are presented as mean ± standard error of the mean.

Regarding CXCL8, our findings mirrored those of sST2. The systemic concentration of CXCL8 did not differ significantly between the two groups of UC patients (121.88±25.26 pg/ml vs 90.40±31.73 pg/ml; p= 0.534) (Figure 2A). However, fecal concentrations of CXCL8 were significantly higher in UC patients with MetS (4882.73±631.40 pg/ml)

compared to those without MetS (2474.84±1081.97 pg/ml; p= 0.027) (Figure 2B).

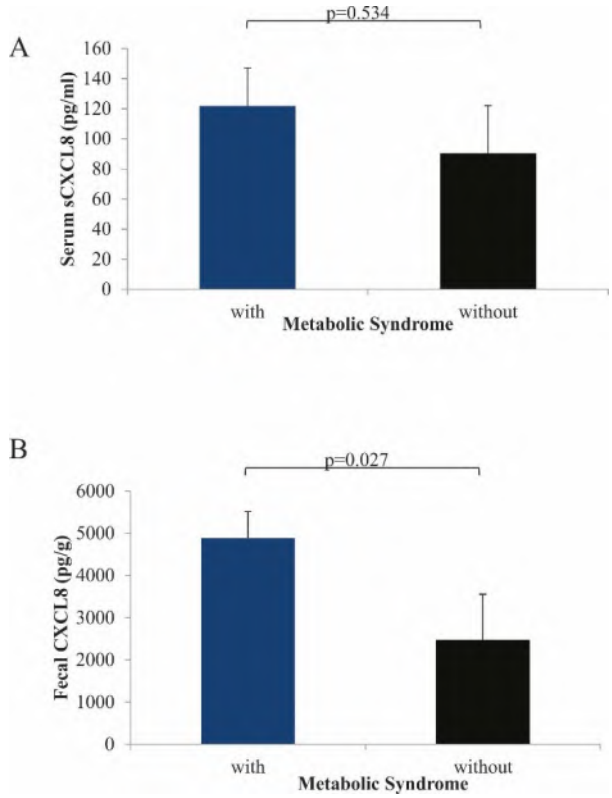

Figure 2. Depicts the levels of CXCL8 in patients diagnosed with ulcerative colitis (UC), with and without co-existing metabolic syndrome (MetS). The serum (A) and fecal (B) CXCL8 concentrations are represented in the figure. For the determination of statistically significant differences, we employed either the Student's t-test or the Mann-Whitney U test, as per the data distribution. Data are articulated as mean ± standard error of the mean.

These observations highlight the distinct influence of MetS on the local intestinal milieu in UC patients, potentially affecting the inflammatory response and disease progression.

DISCUSSION

In the present study, we investigated the serum and fecal levels of two key inflammatory markers, soluble ST2 (sST2) and chemokine (C-X-C motif) ligand 8 (CXCL8, or interleukin-8), in patients with ulcerative colitis (UC), with and without co-existing metabolic syndrome (MetS). The intricate relationship between these two conditions, in the context of the gut inflammatory status, is an emerging field of interest, given the potential protective influence of MetS on the immunopathogenic processes underlying UC.

Our study elucidates a complex interplay between Metabolic Syndrome (MetS) and Ulcerative Colitis (UC), with findings demonstrating distinctive clinical, endoscopic, histological, and biochemical signatures in UC patients with MetS. These results align with previous studies, suggesting a modulatory influence of MetS on UC progression (10). The blood analyses exhibited a significant reduction in white blood cell (WBC) count among UC patients with MetS, which could potentially be attributed to the systemic anti-inflammatory effects of MetS. Elevated levels of Potassium, Sodium, cholesterol, triglycerides, LDL, AST, ALT, urea, and creatinine were also detected in this cohort. These alterations are in line with the classic presentation of MetS and underscore the systemic metabolic alterations that might be influencing the inflammatory process in UC (19).

Additionally, Mayo endoscopic and clinical subscores were lower in this cohort, indicating milder disease presentation and potentially suggesting a protective effect of MetS in UC. Histopathological observations further corroborated this pattern, with less severe chronic inflammatory and eosinophilic infiltration noted in the intestinal mucosa of MetS patients. These insights substantiate the need for further research to unravel the mechanistic underpinnings of these findings, aiding in the refinement of therapeutic strategies for UC patients with MetS.

Our study has identified a fascinating pattern of sST2 distribution, a key molecule in immune response and inflammation, in patients with UC and MetS. We found that systemic concentrations of sST2 were similar in both groups of UC patients, but fecal sST2 levels were significantly higher in those with MetS. This notable disparity may suggest that MetS influences intestinal immune responses in UC, as indicated by the heightened fecal sST2 levels, potentially implying an intensified local immune response to control gut inflammation.

sST2, a receptor decoy for IL-33, has an intriguing dual role in inflammatory disorders (20). While various cells, including activated leukocytes, can produce and release IL-33, the role of IL-33 can be protective in conditions such as obesity, atherosclerosis, and experimental fulminant hepatitis, and proinflammatory in other contexts (21, 22). Prior research has shown that sST2 inhibits macrophage activation and the resultant production of pro-inflammatory cytokines like TNF-α, IL-6, and IL-12 (23). Meanwhile, it does not affect the production of anti-inflammatory mediators such as IL-10 and TGF-β (23).

As inflammation progresses, activated macrophages, fibroblasts, and other cell types produce sST2 (24). Once released, sST2 may further inhibit the pro-inflammatory response through a negative feedback mechanism, most likely by inactivating TLRs (25). Thus, sST2 functions as a crucial participant in negative feedback to prevent an uncontrolled inflammatory reaction (23). Bearing this in mind, the heightened release of sST2 in subjects with MetS may halt the progression of chronic inflammatory processes and limit local tissue damage. This observation provides further impetus to explore the role of MetS in modulating local immune processes in UC.

CXCL8, also known as Interleukin-8 (IL-8), is a chemoattractant cytokine produced by various tissue and blood cells, with a specific targeting of neutrophils and only weak effects on other blood cells (26). It plays a crucial role in attracting and activating neutrophils in areas of inflammation, exemplified by its significant role in conditions like UC where neutrophil functions are paramount (27). Additionally, CXCL8 is a member of the Interleukin-8 supergene family, sharing structural homologies with other small chemotactic peptides and common regulatory pathways with other cytokines (28). This highlights the complex and multifaceted functionality of CXCL8 in both physiological and pathological processes. The observation of elevated fecal levels of CXCL8, a neutrophil chemoattractant, in UC patients with MetS is another key finding from our study. Despite the systemic inflammation reportedly reduced in patients with both UC and MetS, as seen through our lower histological inflammation scores, the high fecal concentration of CXCL8 appears to be counterintuitive at first glance (10).

The contrasting local and systemic immune responses in UC patients with MetS can be reconciled when considering the specific role of CXCL8. This cytokine, known for its ability to attract neutrophils to the site of inflammation, may play a role in marshalling immune resources specifically to the gut (29). The high fecal concentration of CXCL8 could indicate a vigorous local immune response to contain the inflammation within the gut. This potentially explains the lower systemic inflammation markers and milder clinical and histological presentation observed in our MetS cohort.

On the other hand, the elevated fecal CXCL8 concentrations suggest a heightened local immune response. This seemingly contradictory observation underlines the complex interplay between MetS and UC and the possible compartmentalization of the immune response. The systemic immune response, as shown by general markers of inflammation, might be suppressed or dampened. Still, locally, within the gut, there seems to be an intensified immune response, as suggested by elevated levels of sST2 and CXCL8.

It's important to note that MetS, characterized by chronic low-grade systemic inflammation, is frequently associated with immune dysfunction (30). This could possibly alter the immune response in the gut in patients with UC, leading to an apparent decrease in systemic inflammation and a paradoxical increase in local gut inflammation. The exact mechanisms through which MetS influences the local gut inflammation, specifically in the context of UC, require further exploration. It may involve complex interplays among gut microbiota, intestinal epithelial barrier integrity, immune cell function, and metabolic regulation.

One potential limitation of our study is the cross-sectional design, which precludes conclusions about the causal relationship between elevated fecal sST2 and CXCL8 levels and the progression or severity of UC in the presence of MetS. Future longitudinal studies, ideally with larger patient cohorts and including other potential biomarkers, are warranted to validate and extend our findings.

CONCLUSION

This study reveals an intricate relationship between Metabolic Syndrome (MetS) and Ulcerative Colitis (UC), demonstrating that UC patients with MetS exhibit distinct clinical, endoscopic, histological, and biochemical profiles compared to those without MetS. Key findings include lower Mayo endoscopic and clinical subscores, milder chronic inflammatory and eosinophilic tissue infiltration, and significantly higher fecal concentrations of sST2 and CXCL8 in UC patients with MetS, underscoring the potential influence of MetS on UC progression. These observations prompt a reevaluation of the complexity of UC pathogenesis and the role of metabolic factors in its modulation, necessitating further research to validate these findings and refine therapeutic strategies.

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CONFLICT OF INTEREST

We extend our heartfelt gratitude to all individuals and institutions whose indispensable contributions and steadfast support made this research possible.

REFERENCES

1. Vuyyuru SK, Kedia S, Sahu P, Ahuja V. Immune-mediated inflammatory diseases of the gastrointestinal tract: Beyond Crohn's disease and ulcerative colitis. JGH Open. 2022;6(2):100-11.
2. Hendrickson BA, Gokhale R, Cho JH. Clinical aspects and pathophysiology of inflammatory bowel disease. Clin Microbiol Rev. 2002;15(1):79-94.
3. Kumar M, Garand M, Al Khodor S. Integrating omics for a better understanding of Inflammatory Bowel Disease: a step towards personalized medicine. Journal of Translational Medicine. 2019;17(1):419.
4. Kofla-Dlubacz A, Pytrus T, Akutko K, Sputa-Grzegrzolka P, Piotrowska A, Dziegiel P. Etiology of IBD-Is It Still a Mystery? Int J Mol Sci. 2022;23(20).
5. Gajendran M, Loganathan P, Jimenez G, Catinella AP, Ng N, Umapathy C, et al. A comprehensive review and update on ulcerative colitis(). Dis Mon. 2019;65(12):100851.
6. Lichtenstein GR, Shahabi A, Seabury SA, Lakdawalla DN, Espinosa OD, Green S, et al. Increased Lifetime Risk of Intestinal Complications and Extraintestinal Manifestations in Crohn's Disease and Ulcerative Colitis. Gastroenterol Hepatol (N Y). 2022;18(1):32-43.
7. Fahed G, Aoun L, Bou Zerdan M, Allam S, Bou Zerdan M, Bouferraa Y, et al. Metabolic Syndrome: Updates on

Pathophysiology and Management in 2021. International Journal of Molecular Sciences. 2022;23(2):786.
8. Li W, Qiu X, Ma H, Geng Q. Incidence and long-term specific mortality trends of metabolic syndrome in the United States. Front Endocrinol (Lausanne). 2022;13:1029736.
9. Verdugo-Meza A, Ye J, Dadlani H, Ghosh S, Gibson DL. Connecting the Dots Between Inflammatory Bowel Disease and Metabolic Syndrome: A Focus on Gut-Derived Metabolites. Nutrients. 2020;12(5).
10. Jovanovic M, Simovic Markovic B, Gajovic N, Jurisevic M, Djukic A, Jovanovic I, et al. Metabolic syndrome attenuates ulcerative colitis: Correlation with interleukin-10 and galectin-3 expression. World J Gastroenterol. 2019;25(43):6465-82.
11. Fahed G, Aoun L, Bou Zerdan M, Allam S, Bou Zerdan M, Bouferraa Y, et al. Metabolic Syndrome: Updates on Pathophysiology and Management in 2021. Int J Mol Sci. 2022;23(2).
12. Griesenauer B, Paczesny S. The ST2/IL-33 Axis in Immune Cells during Inflammatory Diseases. Frontiers in Immunology. 2017;8.
13. Cambier S, Gouwy M, Proost P. The chemokines CXCL8 and CXCL12: molecular and functional properties, role in disease and efforts towards pharmacological intervention. Cellular & Molecular Immunology. 2023;20(3):217-51.
14. Rezaianzadeh A, Namayandeh SM, Sadr SM. National Cholesterol Education Program Adult Treatment Panel III Versus International Diabetic Federation Definition of Metabolic Syndrome, Which One is Associated with Diabetes Mellitus and Coronary Artery Disease? Int J Prev Med. 2012;3(8):552-8.
15. Ikeya K, Hanai H, Sugimoto K, Osawa S, Kawasaki S, Iida T, et al. The Ulcerative Colitis Endoscopic Index of Severity More Accurately Reflects Clinical Outcomes and Long-term Prognosis than the Mayo Endoscopic Score. J Crohns Colitis. 2016;10(3):286-95.
16. Pabla BS, Schwartz DA. Assessing Severity of Disease in Patients with Ulcerative Colitis. Gastroenterol Clin North Am. 2020;49(4):671-88.
17. Jauregui-Amezaga A, Geerits A, Das Y, Lemmens B, Sagaert X, Bessissow T, et al. A Simplified Geboes Score for Ulcerative Colitis. Journal of Crohn's and Colitis. 2016;11(3):305-13.
18. Jovanovic M, Gajovic N, Jurisevic M, Markovic B, Maric V, Jovanovic M, et al. Fecal sST2 correlates with disease severity of ulcerative colitis. Vojnosanitetski pregled. 2018;76:26-.
19. Scoville EA, Allaman MM, Brown CT, Motley AK, Horst SN, Williams CS, et al. Alterations in Lipid, Amino Acid, and Energy Metabolism Distinguish Crohn's Disease from Ulcerative Colitis and Control Subjects by Serum Metabolomic Profiling. Metabolomics. 2018;14(1):17.
20. Ding W, Zou G-L, Zhang W, Lai X-N, Chen H-W, Xiong L-X. Interleukin-33: Its Emerging Role in Allergic Diseases. Molecules. 2018;23(7):1665.
21. Drake LY, Kita H. IL-33: biological properties, functions, and roles in airway disease. Immunol Rev. 2017;278(1):173-84.
22. Miller AM. Role of IL-33 in inflammation and disease. J Inflamm (Lond). 2011;8(1):22.
23. Guo H, Bossila EA, Ma X, Zhao C, Zhao Y. Dual Immune Regulatory Roles of Interleukin-33 in Pathological Conditions. Cells. 2022;11(20):3237.
24. Griesenauer B, Paczesny S. The ST2/IL-33 Axis in Immune Cells during Inflammatory Diseases. Front Immunol. 2017;8:475.
25. Lang T, Mansell A. The negative regulation of Toll-like receptor and associated pathways. Immunology & Cell Biology. 2007;85(6):425-34.
26. Xiong X, Liao X, Qiu S, Xu H, Zhang S, Wang S, et al. CXCL8 in Tumor Biology and Its Implications for Clinical Translation. Front Mol Biosci. 2022;9:723846.
27. Wera O, Lancellotti P, Oury C. The Dual Role of Neutrophils in Inflammatory Bowel Diseases. Journal of Clinical Medicine. 2016;5(12):118.
28. Meniailo ME, Malashchenko VV, Shmarov VA, Gazatova ND, Melashchenko OB, Goncharov AG, et al. Interleukin-8 favors pro-inflammatory activity of human monocytes/macrophages. Int Immunopharmacol. 2018;56:217-21.
29. Ha H, Debnath B, Neamati N. Role of the CXCL8-CXCR1/2 Axis in Cancer and Inflammatory Diseases. Theranostics. 2017;7(6):1543-88.
30. Monserrat-Mesquida M, Quetglas-Llabres M, Capo X, Bouzas C, Mateos D, Pons A, et al. Metabolic Syndrome is Associated with Oxidative Stress and Proinflammatory State. Antioxidants (Basel). 2020;9(3).

IMPORTANCE OF POPULATION EDUCATION IN IMPLEMENTATION OF COMPULSORY IMMUNIZATION AGAINST POLYOMYELITIS IN CHILDREN

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ABSTRACT

Poliomyelitis is a contagious disease characterized by the appearance of fever, malaise, scratching in the throat, gastrointestinal signs, and after a few days the appearance of muscle paralysis, as well as respiratory and vasomotor disorders. Today, this disease is very rare, due to systemic active immunization. The aim of this study was to assess the level of knowledge and attitudes toward poliomyelitis and importance of immunization against it.

A specially designed survey questionnaire was used for interviewing purposes. Data analysis and processing were performed using a statistical data processing package (SPSS for Windows, version 20). A chi-square test was used from the statistical tests.

The educated profile of the respondents was as follows: medical sciences - 37 (50%), natural sciences and mathematics - 6 (8,1%), social sciences - 5 (6,8%), technical sciences - 19 (25,7%) and arts - 7 (9,5%). The study findings indicate a high level of awareness and knowledge of the population regarding polio, as well as good health awareness of the population about immunization i.e. polio vaccination. 34 respondents from the field of medical education answered the question exactly how the vaccine protects against the disease. In the field of natural mathematical sciences, a total of 3 gave the correct answer, which makes 50% of the total number of persons (6) in the mentioned field. In addition, majority of the study sample (70,3%) is aware that child should be vaccinated. Furthermore, 91,9% of respondents agree that education of parents regarding children vaccination is of great importance for whole community.

Based on findings of present study it can be concluded that efforts still need to be made in education of the wider population toward polio and the importance of vaccination. In addition to healthcare professionals, the entire community should participate in this strategic task.

Keywords: *Polio, immunization, vaccine, subjects, education, vaccination calendar.*

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INTRODUCTION

Poliomyelitis (*Hene-Medin* disease, *acute anterior poliomyelitis*) is an acute infectious and contagious disease caused by Poliovirus. Polioviruses belong to the group of Enteroviruses, of the genus Enteroviruses, a family of Picorn viruses. There are three types of Polioviruses type 1, 2 and 3 (1). Type 1 contributes most to the appearance of paralysis. Man is the main reservoir and the main transmitter of the virus. The disease most commonly affects children between the ages of three and eight, while it is much less common in adults but has more serious consequences. The disease occurs individually and in epidemics. The routes of transmission of the infection are oral-fecal. The incubation time ranges from 3 to 35 days. The virus is excreted in the stool for up to six weeks from the onset of the disease (2).

The infection can flow without any symptoms or as a general infection, such as meningitis or paralysis. Paralytic polio occurs in only 0.1-1% of infected persons (3, 4). The clinical presentaton of the paralytic form shows the appearance of several stages: the preparative stage, the paralysis stage and the recovery stage. In children, the disease posses often biphasic flow. The first stage of the disease or minor illness lasts 1-3 days, followed by a latent period of 2-5 days during which the patients are symptom free. Subsequently, a paralytic phase of the disease develops, preceded by meningitis. In adults, the disease is usually a single-phase course, with slower development of paralysis (5). One in 200 infected persons develops paralysis, and 5-10% of those with paralysis have a fatal outcome due to muscle paralysis (6).

The diagnosis is made on the basis of anamnestic data, a clinical signs (asymmetrical, flaky palsies followed by muscle atrophy), epidemiological data, isolation of the oropharynx and stool virus and serological analyzes. Examination of cerebrospinal fluid may be similar to that of viral meningitis. Confirmation of the diagnosis is most likely to be achieved by demonstrating viral RNA by chain polymerization in stool, blood or cerebrospinal fluid (7). The basic rule in the treatment of this disease is hospitalization and absolute rest. The treatment of polio is symptomatic. The principles of polio treatment are the removal of symptoms (analgesics for muscle and head pain, oral administration of vitamins C and B), the treatment of secondary infections, and rehydration. After the acute phase, active physical therapy and rehabilitation are performed. Extremities affected by paralysis are placed in a physiological position to prevent contractures. In patients with respiratory paralysis, artificial respiration using respirators must be administered. For therapeutic purposes, in addition to appropriate physical and supportive therapy, tests are also being conducted to administer nerve growth factor (IGF-1), in order to stimulate the appearance of new axonal shoots and muscle synapses. Recovery in children is faster and more complete than in adults (7).

Today, this disease is very rare, thanks to systemic active immunization. The multiple-dose vaccine provides lifelong protection (7). The last polio epidemics in our country were from 1959-1962. Since 1962., systemic immunization against polio has been introduced, which has led to the eradication of this disease in our country. Already in 1960., the introduction of the Sabin vaccine in Yugoslavia began. In the following years, the vaccination was mandatory for children in other countries of the world (8). The number of polio cases worldwide has decreased by 99% since 1988., due to administration of the vaccine. The last case in our country was registered in 1996. Five vaccines are available: inactivated (IPV) and four live oral: trivalent (OPV), bivalent (bOPV) and two monovalent (mOPV1 and mOPV3) (9, 10).

Nowdays, these vaccines have been replaced by the Pentaxim vaccine. Pentaxim is a registered divalent vaccine against diphtheria, tetanus, pertussis, polio and invasive infections caused by *Haemophilus Influenzae* type b. It is used for primary vaccination of infants from the third month of age and for revaccination, one year after primary vaccination, i.e. during the second year of the child's life, at 7 and 14 years of age (9, 10). The generally recommended vaccination schedule includes primary vaccination consisting of three injections at intervals of one to two months, starting at the third month of life (at 3, 4, and 6 months). Revaccination is carried out in a single dose at 2, 7 and 14 years of age. Reactions after administration of the vaccine are usually mild and transient. More serious consequences are rare and can occur after administration of any vaccine. Redness, swelling, stabbing pain, mild fever and irritability are common.

Although polio is thought to been eradicated (11), appearance of sporadic cases and possibility of entries from surrounding countries cause strong concerns. Insufficient comprehension and misunderstanding regarding vaccination are still present in many countries, especially of the undeveloped ones (12, 13). Another problem which often occurs is lack of confidence on vaccine safety (11-13). Occasional estimation of people`s understanding towards this disease as well as importance of vaccination may ensure overcoming of these concerns. Therefore, assessment of knowledge and attitudes regarding polio could be of wider social significance.

According to all mentioned above, the aim of present research was to assess the level of knowledge and attitudes toward poliomyelitis and importance of immunization against it.

MATERIAL AND METHODS

This cross-sectional research was conducted in "Pinus" pharmacy, Cuprija, in the period 20.05-30.06.2018.

The investigation was conducted as a cross-sectional study and included 74 subjects, aged >18, both genders. Subjects were classified into three age groups: 1. from 20 to 30 years old; 2. from 30 to 40 years old; and 3. over 50 years old. Gender distribution in whole study group was as follows: 20 male (27%) and 54 female (73%).

A specially designed survey questionnaire was used for interviewing purposes. Participation in the study was voluntary and anonymous. The questionnaire was specifically designed for the purpose of this research and consisted of 15 closed-ended questions: socio-demographic data of respondents, respondents' attitudes about the causes and occurrence of polio, knowledge about the importance of immunization in childhood as the most effective measure of prevention and eradication of poliomyelitis.

Data analysis and processing were performed using a statistical data processing package (SPSS for Windows, version 20). A chi-square test was used from the statistical tests. A $p < 0.05$ value was used as the level of statistical significance of the differences. The results obtained are presented in tables (expressed as absolute numbers and as a percentage) and by means of a graph.

RESULTS

Table 1 shows structure of the study sample by age. The age group from 20 to 30 consists of 32 respondents (43,2%), the age group from 30 to 40 years consists of 15 respondents (20,3%) and the age group over 50 consists of 27 respondents (36,5%)).

Table 2 represents educational structure of the study sample. The educated profile of the respondents was as follows: medical sciences - 37 (50%), natural sciences and mathematics - 6 (8,1%), social sciences - 5 (6,8%), technical sciences - 19 (25,7%) and arts - 7 (9,5%).

According to the type of settlements, 52 respondents (70,3%) live in urban areas while 22 respondents (29,7%) live in rural environment (Table 3).

Graph 1 represents respondent's awareness and knowledge of polio. When asked if they know what polio is, almost all respondents answered affirmatively (71/74 respondents) - all from medical sciences (37 respondents), and majority from other education areas: technical sciences (18), natural sciences (6), social sciences (5) and arts (5). Negative answer were found in 1 respondent from technical sciences, and 2 from arts. Statistical significance is $p = 0.012$ ie. $p < 0.05$

When asked whether respondents know how the vaccine protects against the aforementioned disease, 42 respondents answered correctly (56,8%), 11 respondents answered incorrectly (14,9%) and 21 respondents did not know the answer (28,4%). 34 respondents (out of a total of 37) from the field of medical education answered the question exactly how the vaccine protects against the disease (Graph 2). In the field of natural and mathematical sciences, a total of 3 gave the correct answer, which makes 50% of the total number of persons (6) in the mentioned field. Statistical significance was also observed ($p < 0.05$).

Graph 3 shows awareness and knowledge whether a child should not be vaccinated. The correct answer was given by 52 respondents (70,3%), the incorrect answer was given by 10 respondents (13,5%), while I do not know answer was given by 12 respondents (16,2%). It can be noticed that correct answer was the most present in the subjects from the field of medical sciences, which is partly expected. Statistical significance for this question is $p < 0.05$.

Graph 4 represents awareness and knowledge regarding revaccination. 33 respondents (44,6%) gave an accurate answer to what the revaccination was, 12 respondents (16,2%) gave an incorrect answer, and 29 respondents (39,2%) did not know the answer, compared to the total number of 74 respondents. Graph 4 also shows the individual representation of correct and incorrect answers by different fields of education ($p < 0.05$).

When asked whether you think it is necessary to educate parents about the importance and risk of not vaccinating a child, 68 subjects responded positively (91,9%), while the remaining 6 answered negatively (8,1%). Statistical significance for this question is $p = 0.008$ ie. $p < 0.05$ (Graph 5).

Table 1. Structure of the study sample by age

Age group	Number of respondents	%
from 20 to 30 years old	32	43,2
from 30 to 40 years old	15	20,3
over 50 years old	27	36,5

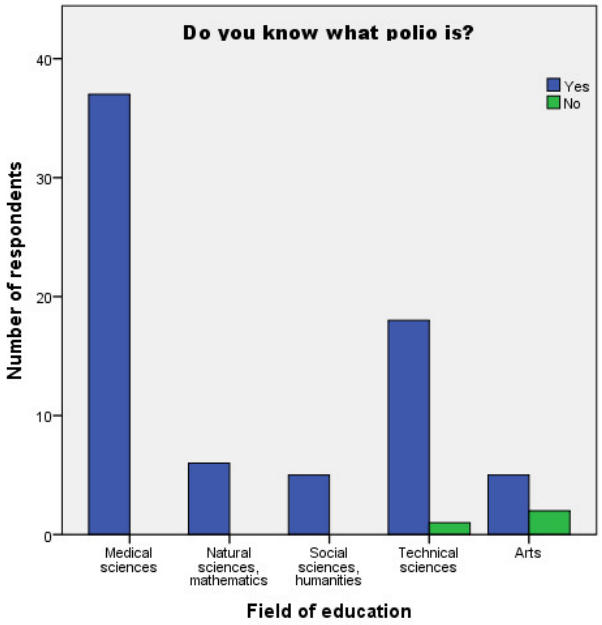
Table 2. Structure of the study sample by field of education

Educational profile of respondents	Medical sciences	Natural sciences and mathematics	Social Sciences	Technical sciences	Arts
Number of respondents	37	6	5	19	7
%	50%	8,1%	6,8%	25,7%	9,5%

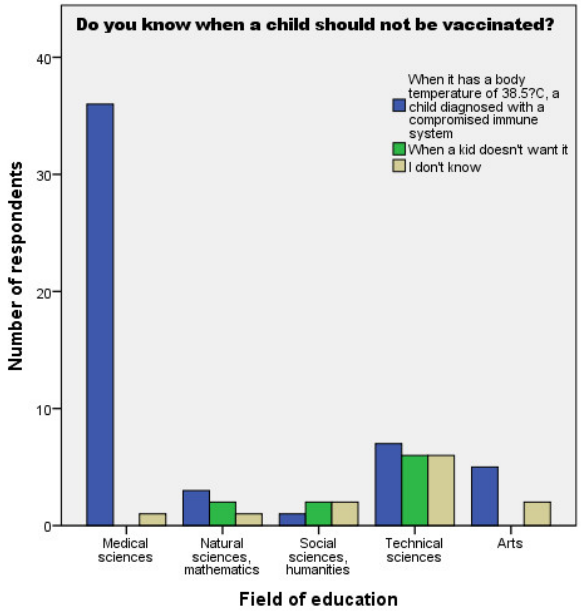
Table 3. Structure of the study sample by type of settlement

Type of settlement	Number of respondents	%
Urban area	52	70,3
Rural area	22	29,7

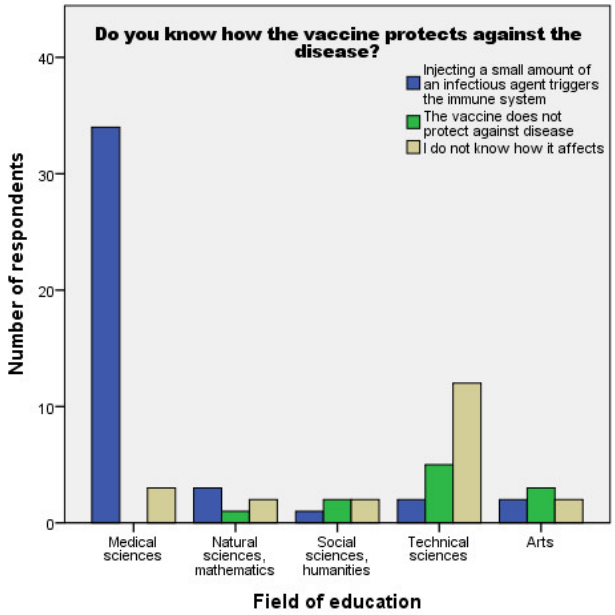
Graph 1. Respondent's awareness and knowledge of polio



Graph 2. Importance of immunization i.e. polio vaccines



Graph 3. Awareness and knowledge when a child should not be vaccinated



DISCUSSION

Despite it was assumed that polio is eradicated in our country, according to a report from the Batut Institute of Public Health in the period from 1996 to 2012, 30 cases of polio were reported (24 were caused by wild polio virus and 6 cases were classified as vaccine associated polio) (14, 15). These data are important in terms of raising the awareness about the necessity of education about polio and its immunization. Exactly, the severity of the clinical feature and the risk of recirculation of this virus was the guiding idea for the creation of this study. For the purpose of this article we have chosen five most important questions which are relevant for present topic.

At the beginning it can be noticed that almost all respondents were aware of main polio characteristics and the majority of them were from medical sciences. This is logical result having in mind that these knowledge are the most accessible to them. Our findings show that all these informations should be more available to a wide population structure and not just medical ones. Today it is well known that polio viruses are transmitted through direct contact with diseased person, by faeces and pharyngeal secretions, or most often via infected hands. The receptors for this virus are expressed exclusively on human cells (16, 17), which means

that eradication is absolutely possible with adequate prevention measures (2). Polio as an infectious disease can manifest itself as an abortive manifestation of fever, sore throat, headache, vomiting and abdominal pain. In such patients, the neurological finding is neat. Another manifestation of the disease is non-paralytic poliomyelitis, which is characterized by signs of leptomeningeal affection and may occur as a stand-alone disease or as the last clinical manifestation of the prepolytic stage of paralytic poliomyelitis. Studies show that only one in 150 infected persons develop paralytic polio. The disease can range from weakness of individual muscles to complete quadriplegia (2, 4).

Beside basic knowledges regarding polio, another important question is significance of immunization and it`s mechanism of protection. This question is more difficult and required deeper knowledges. Results of present study pointed out that more than half of subjects understand basic principles of vaccine action, while other half does not know at all or incorrectly know. Moreover, as expected, only respondents from medical field completely correct answered on this question. Based on our study population it can be suggested that high perecent of people is not or poorly educated referring to vaccination. As mentioned in recent studies of similar design this is mainly typical for underdeveloped or countries in transition (12, 13).

Among all measures for the prevention of infectious diseases, immunization is the most effective and economically justifiable measure that has directly affected the incidence and mortality of infectious diseases worldwide (18). Many diseases which have been the first-rate health problems in the developed world today have been eliminated or reduced to a single occurrence. In many developed countries, thanks to the positive attitudes of society towards immunization and the commitment to achieving safe immunization coverage, conditions have been created for poliomyelitis to be eliminated (19). In addition, educational level of population in these countries regarding polio or other infectous disease is high (18, 19).

The Strategic Plan for the Eradication of Poliomyelitis is an activity aimed at mass immunization of children to maintain high vaccination coverage among children, the use of additional vaccine doses during national immunization days, and the implementation of effective poliomyelitis surveillance systems (20, 21). On this way, universal control of this disease is achieved (21, 22). Controversial opinions, discussions and movements are the cornerstones of our time. In a study similar to present one (23) subjects from both genders were of the opinion that behind the immunization lay a conspiracy theory regarding population or fertilization control. Moreover, some investigations indeed noted that administration of polio vaccine could be associated with infertility in girls (24). Anti-vaccine movements and boycotts of the polio immunization campaign have had a negative impact on the continued education of the population about the importance of mandatory vaccination. Although immunization coverage across Europe is over 90%, about 600,000 children remain unvaccinated a year (25, 26).

On the other hand, our results have shown that two thirds of our subjects possess knowledges about contraindications for child vaccination and again most of them are from medical education. The results of different studies indicate a discrepancy between attitudes and the extent of coverage by immunization of children. In the absence of knowledge about the consequences of non-vaccination, one in five parents surveyed found it less harmful for children to contract an infectious disease than to be vaccinated against it (27).

Increasing public awareness of the negative attitudes of vaccination has raised concerns that the vaccine is considered useless, ineffective, unnecessary and dangerous to health, which would impair the homeostasis of the organism and endanger health. Concerns have also been intensified by fears of side effects that occur after polio vaccination. Lack of faith in the efficacy and safety of the vaccine, distrust related to post-vaccine reactions require that vaccination promotion activities should be undertaken as soon as possible. Insufficient confidence in immunization against polio and other infectious diseases has hampered routine and legally binding immunizations that apply to all WHO member states.

Our results also indicate that almost half of subjects comprehend concept of revaccination, while other half does not know at all or incorrectly know. Like in previous cases, the most number of accurate answers were from medical field, while respondents from social sciences and arts were the least familiar with this issue. As known, revaccination is any subsequent administration or repeated administration of the appropriate vaccine dose after the first immunization, i.e. vaccinations according to the childhood vaccination calendar. The results of various researches show that parents are generally not aware of the complexity of the immunization calendar. In a study by Goldstein et al, an insight into the health records of children of previously surveyed parents, who claimed that their children were regularly vaccinated with all age-prescribed vaccines, revealed the existence of more than 30% of incompletely immunized children (28).

In a another investigation, mothers' attitudes toward mandatory child vaccination were examined. It was found that there was no significant difference in the immunization status of children of mothers who thought that vaccine-preventable diseases were dangerous and mothers who considered the opposite (29). The results of other studies indicate a marked discrepancy between parental beliefs about the importance of immunization and the immunization status of children. It has been shown that the children of parents who think that disease prevention is very important are less fully immunized (23%) than the children of parents who do not percieve the benefit of immunization as a measure of protection against vaccine preventable diseases (24, 25). In some U.S. states, vaccination providers have achieved significant success in increasing their immunization response

by introducing a system of texting to parents reminding them of the day, time and type of immunization (30).

On the basis of all mentioned above it is necessary to educate parents about the importance and risk of not vaccinating a child. Although more than 90% of our respondents realize the importance of this issue, there were few of them with the opposite attitude. The other survey results (not presented) show that most parents often use different media when it comes to polio vaccination topics. It was also noted that the most reliable way of informing parents was equally through presentation and broadcasting, though statistics show us that informing via books, scientific journals, etc. should not be neglected. In France and the United Kingdom, the decision to immunize children is made by parents. The decision made by parents is the result of prior knowledge or experience of vaccine prophylaxis. Very often, due to the impact of negative attitudes about immunization (often from different sources), parents often delay or fail to immunize their children (30).

Parents' negative attitudes toward vaccination that precede their child's immunization rejection contribute to creating conditions for the outbreak of infectious diseases that have been eliminated in the population. The results of studies conducted in the U.S. indicate that if vaccination is discontinued under the conditions of vaccination spread, the epidemiological situation would be unfavorable (30). The role of parents, parenting attitudes and their beliefs and knowledge about vaccination of children in early childhood and adolescence are very important in making the right decisions (31). The follow-up of novelties in the field of immunization practice through the media, various seminars and educations by health care professionals will prevent many contagious diseases that have had a high rate of morbidity, comorbidity and mortality in the past (32, 33). The support and effort that healthcare professionals, volunteers, and institutions promoting public health play in raising awareness of vaccine administration, surveillance, and eradication of disease are very important.

CONCLUSION

Based on findings of present study it can be concluded that efforts still need to be made in education of the wider population toward polio and the importance of vaccination. In addition to healthcare professionals, the entire community should participate in this strategic task.

It is also valuable to highlight the significance of polio vaccination and to provide insight into the novelty of preventing this disease. This is considered to be a secure key to success in complete eradicating of polio and other infectious diseases. Through conversation, reflection and educational lectures on vaccination, parents and the entire population gain positive views on the vaccination of children.

CONFLICT OF INTEREST

None of the authors of the present study has any actual or potential conflicts of interest to disclose, including financial, personal, or other relationships with specific persons or organizations.

REFERENCES

1. Mehndiratta MM, Mehndiratta P, Pande R. Poliomyelitis: historical facts, epidemiology, and current challenges in eradication. Neurohospitalist. 2014;4(4):223-229.
2. Wolbert JG, Higginbotham K. Poliomyelitis. In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2020 Jan.
3. Mueller S, Wimmer E, Cello J. Poliovirus and poliomyelitis: a tale of guts, brains, and an accidental event. Virus Res 2005; (2); 175–93.
4. Bao J, Thorley B, Isaacs D, Dinsmore N, Elliott EJ, McIntyre P, Britton PN. Polio - The old foe and new challenges: An update for clinicians. J Paediatr Child Health. 2020 Oct;56(10):1527-1532..
5. Greene SA, Ahmed J, Datta SD, Burns CC, Quddus A, Vertefeuille JF, Wassilak SGF. Progress Toward Polio Eradication – Worldwide. Morb Mortal Wkly Rep. 2019 May 24;68(20):458-462.
6. Arita M. Poliovirus Studies during the Endgame of the Polio Eradication Program. Jpn J Infect Dis. 2017 Jan 24;70(1):1-6.
7. Zoll GJ, Melchers WJ, Kopecka H, Jambroes G, van der Poel HJ, Galama JM. General primer-mediated polymerase chain reaction for detection of enteroviruses: application for diagnostic routine and persistent infections. J Clin Microbiol. 1992 Jan;30(1):160-5.
8. Borjanovic S, Ristic S. Immunization of the population against infectious diseases. Belgrade. 1981
9. Hampton LM, Farrell M, Ramirez-Gonzalez A, Menning L, Shendale S, Lewis I, Rubin J, Garon J, Harris J, Hyde T, Wassilak S, Patel M, Nandy R, Chang-Blanc D. Cessation of use of trivalent oral polio vaccine and introduction of inactivated poliovirus vaccine worldwide, 2016. Wkly. Epidemiol. Rec. 2016; 91: 421-32.
10. Jorba J, Diop OM, Iber J, Henderson E, Zhao K, Sutter RW, Wassilak SGF, Burns CC. Update on vaccine-derived polioviruses - Worldwide, January 2017-June 2018. Morb. Mortal. Wkly. Rep. 2018; 67: 1189-94.
11. Khan MU, Ahmad A, Aqeel T, Salman S, Ibrahim Q, Idrees J, Khan MU. Knowledge, attitudes and perceptions towards polio immunization among residents of two highly affected regions of Pakistan. BMC Public Health. 2015;15:1100.
12. Mehmood K, Hashmi Inam Qadir J, Rashid AZ, Bashir RA, Khosa ZA, Khalid S, Hashmi KTK. Polio vaccination; knowledge, attitude, and practice in district Abottabad, Khyber Pakhtunkhwa, Pakistan. Professional Med J. 2014;21:1026–32.
13. Naeem M, Adil M, Abbas SH, Khan A, Khan MU, Naz SM. Coverage and causes of non-immunization in

national immunization days for polio; a consumer and provider perspective study in Peshawar. J Postgrad Med Inst. 2012;26:48–54.
14. Radovanovic Z. Field epidemiology - epidemic research. Belgrade: Faculty of Medicine; 2000.
15. Vukovic Z, Loncarevic G, Mugosa B. Epidemiological surveillance of diseases prevented by immunization. Manual 1. Podgorica: UNICEF - Institute for Health Care. 2001
16. Boot HJ, Kasteel DT, Buisman AM, Kimman TG. Excretion of wild-type and vaccine-derived poliovirus in the feces of poliovirus receptor-transgenic mice. J Virol 2003; 77; 6541–6545
17. Freistadt MS, Fleit HB, Wimmer E. Poliovirus receptor on human blood cells: a possible extraneural site of poliovirus replication. Virology 1993; 195; 798–803
18. Al Awaidy ST, Khamis F. Wild Poliovirus Type 1 in Oman: A re-emerging threat that requires urgent, targeted and strategic preparedness. Sultan Qaboos Univ Med J. 2020 Feb;20(1):e1-e4.
19. Von Mutius E, Weiland SK, Fritzsch C,Duhme H, Keil U. Increasing prevalence of hay fever and atopy among children in Leipzig, East Germany. Lancet 1998; 351; 862-866.
20. Report on the conducted immunization on the territory of the Republic of Serbia in 2015. Institute of Public Health of Serbia "Dr Milan Jovanović Batut". Belgrade 2016; p. 428.
21. Kisanga A, Abiuda B, Walyaula P, Losey L, Samson O. Evaluation of the Functionality and Effectiveness of the CORE Group Polio Project's Community-Based Acute Flaccid Paralysis Surveillance System in South Sudan. Am J Trop Med Hyg. 2019 Oct;101(4_Suppl):91-99.
22. Kimman TG, Boot H. The polio eradication effort has been a great success— Let's finish it and replace it with something even better. Lancet Infect Dis 2006; 6; 675–678.
23. Yahya M. (2006) Polio vaccines: difficult to swallow: the story of a controversy in northern Nigeria. Working paper series, 261. Brighton: IDS.
24. Houtrouw SM, Carlson KL. The relationship between maternal characteristics, maternal vulnerability beliefs, and immunization compliance. Issues in Comprehensive Pediatric Nursing 1993; 16; 41-50.
25. Aylward RB, Hull HF, Cochi SL, Sutter RW, Olive JM, Melgaard B. Disease eradication as a public healthstrategy: a case study of poliomyelitis eradication. Bull WHO. 2000; 78 (3); 285−97.
26. Muscat M, Bang H, Wohlfahrt J, Glismann S, Mølbak K. EUVAC.NET Group. Measles in Europe: an epidemiological assessment. Lancet 2009; 373 (9661); 383-389.
27. Taylor JA, Cufley D. The association between parental health beliefs and immunization status among children followed by private pediatricans. Clinical Pediatrics 1996; 35; 18-22.
28. Goldstein ST, Zhou F, Hadler SC, Bell BP, Mast EE, Margolis HS. A mathematical model to estimate global hepatitis B disease burden and vaccination impact. International Journal of Epidemiology 2005; 34: 1329– 1339
29. Kharbanda OE, Stockwell MS, Fox HW, Rickert IV. A qualitative evaluation of parental readiness for text message immunization reminders. American Journal ofPublic Health 2009; 99; 2176–2178.
30. Abbotts B, Osborne LM. Immunization status and reasons for immunization delay among children using public health immunization clinics. American Journal of Diseases of Children 1993; 147; 965-968.
31. Atkinson W, Wolfe S, Hamborsky J, eds. Centers for Disease Control and Prevention. Poliomyelitis, in: Epidemiology and Prevention of Vaccine-Preventable Diseases. 12th ed., 2nd printing. Washington DC: Public Health Foundation 2012; 249–262.
32. Koopman JS, Henry CJ, Park JH, Eisenberg MC, Ionides EL, Eisenberg JN. Dynamics affecting the risk of silent circulation when oral polio vaccination is stopped. Epidemics. 2017;20:21-36.
33. Orr D, Baram-Tsabari A, Landsman K. Social media as a platform for health-related public debates and discussions: the Polio vaccine on Facebook. Isr J Health Policy Res. 2016;5:34.

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OVEREXPRESSION OF SENESCENCE-ASSOCIATED BETA-GALACTOSIDASE (*SA-B-GAL*) AS A PROGNOSTIC MARKER OF INVASIVE BREAST CARCINOMA

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ABSTRACT

Malignant transformation in invasive breast cancer (IBC) is the result of the accumulation of successive mutations in critical regions of the genome. Another important mechanism for controlling malignant progression is cellular senescence. Although some research suggests its protective role, recent studies have shown that senescence has a significant impact on the development and progression of cancer. The aim of our work is to examine the potential prognostic value of a specific senescence marker, β galactosidase (GLB1). The investigation encompassed a cohort of 147 individuals diagnosed with IBC. In each case of IBC, the occurrence of Non-Invasive Lesions (NIL) was recorded. The assessment of GLB1 expression was conducted by quantifying the percentage of nuclear expression in epithelial cells of both IBC and NIL. Upon examination of the data, it was ascertained that the expression of GLB1 was markedly elevated in IBC in comparison to NIL, with statistical significance ($p<0.001$). Furthermore, disparities in GLB1 expression across various molecular subtypes of breast cancer were observed ($p<0.001$), presenting the most pronounced expression in the HER2-positive (HER2+) subtype of IBC, whereas the Triple-Negative Breast Cancer (TNBC) subtype exhibited the minimal GLB1 expression. Additionally, a substantial variance in GLB1 expression was noted contingent on the presence of HER2 expression ($p<0.001$). We can conclude that examining the presence of cellular senescence using GLB1 can facilitate the differentiation of NIL from IBC, as well as indicate the prognosis of the disease itself.

Keywords: *Breast Neoplasms, Cellular Senescence, beta-Galactosidase, Molecular Subtype, Immunohistochemistry.*

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INTRODUCTION

According to the data reported by GLOBOCAN in the year 2020, IBC constitutes the predominant malignancy diagnosed globally, with an incidence rate of approximately 48 cases per 100,000 individuals. Additionally, it represents the foremost cause of mortality attributed to cancer, with a death rate of approximately 14 per 100,000 population (1). Cancer itself represents an etiologically, histopathologically and genetically heterogeneous disease with a hereditary predisposition (2). The malignant transformation observed in IBC is the result of an accumulation of successive mutations within critical genomic regions. These regions are typically integral to the regulation of cellular processes such as growth and division, DNA repair mechanisms, and apoptosis. This accumulation disrupts the normal cellular regulatory mechanisms, leading to the uncontrolled proliferation characteristic of malignant cells (3). Despite advances in the discovery of molecular diversity and efforts in the treatment of IBC, the incidence and mortality rates are still very high. Emerging biomarkers are currently under investigation for their potential application in diagnostic evaluations, as well as in the prognostication and monitoring of disease recurrence (4, 5).

Cellular senescence is characterized by an irreversible cessation of cell division, which is accompanied by the copious secretion of cytokines and various bioactive substances, a phenomenon commonly known as the senescence-associated secretory phenotype (SASP) (6). Senescent cells can have, depending on the context, beneficial or detrimental roles in various physiological and pathological processes. Although numerous studies suggest a protective role of senescence in terms of the onset and progression of cancer, recent studies have established that senescence, especially SASP, has a significant impact on the onset and progression of cancer (7). Research has elucidated that senescent tumor stroma fibroblasts can facilitate the proliferation of pre-neoplastic or neoplastic cells, as evidenced in studies (8, 9). The conditioned medium from senescent fibroblasts exhibits a similar capacity to promote tumor cell growth, akin to that observed in co-culture with senescent fibroblasts (10, 11) indicating that SASP may promote tumor cell proliferation, at least *in vitro*, via paracrine factors. A fundamental method for detecting cellular senescence is the application of histochemical techniques to identify senescence-associated β-galactosidase (SA-β-GAL) (12), a technique that highlights this enzyme's activity stemming from acid lysosomal β-galactosidase, which, in senescent cells, is detected at nearly neutral pH due to its overexpression. The study of β-galactosidase activity predominantly resides within the field of oncopathology (13), where this enzyme is observable in both benign and precancerous conditions, as well as malignant tumors (14). As literature data accumulated, it became apparent that the role of SA-β-GAL in oncogenesis is multifaceted and complex. Numerous studies have shown active SA-β-GAL in primary tumor cells, with its activity recorded in 100% of primary ovarian cancer cases prior to chemotherapy (15). In malignant colon tumors, the level of SA-β-GAL was twice as high as in normal tissue (16). SA-β-GAL is also active in precancerous colon adenomas (17). Given its presence in both tumor cells and precancerous lesions, SA-β-GAL emerges as a potentially valuable prognostic marker. Therefore, the aim of our work is to investigate the potential prognostic value of SA-β-GAL in NIL and IBC.

Therefore, the aim of our study is to investigate the potential prognostic value of SA-β-GAL in NIL and IBC. In this paper, we investigated the expression of lysosomal beta-galactosidase using the GLB1 antibody, which shows the activity of SA-β-GAL. Namely, research by Lee et al. and Kurz et al., published in prestigious journals, demonstrates that lysosomal β-galactosidase is the source of SA-β-GAL activity, indicating that an increased protein level of lysosomal β-galactosidase accompanies the appearance of SA-β-GAL activity in senescent cells (18, 19). Kurz et al. have shown that the SA-β-GAL activity detected in senescent cells can be attributed to an increase in the level of a classical lysosomal enzyme (19). The *GLB1* gene was found to be the source of senescence-associated β-galactosidase activity (18), and expression correlated with SA-β-GAL activity both in vitro and in vivo (20).

MATERIALS AND METHODS

Study Design

This investigation adhered to the ethical standards delineated in the Declaration of Helsinki, receiving clearance from the Ethics Committee under the reference number 01/17/2290. The research cohort comprised 147 individuals diagnosed with breast cancer, all of whom received their diagnosis and underwent treatment at the University Clinical Center in Kragujevac, Serbia.

For the purpose of conducting a comprehensive pathological examination, specimens collected through surgical procedures such as tumorectomy, quadrantectomy, and mastectomy, which included the dissection of regional lymph nodes, were stained using Hematoxylin and Eosin (H&E). To preclude the potential induction of senescence in tumor cells by prior therapeutic interventions, specimens from patients who received neoadjuvant treatments, such as chemotherapy or radiotherapy, were systematically excluded from the analysis. The examination included a detailed observation for the presence of non-invasive lesions, encompassing in situ lobular and ductal carcinoma, lobular and ductal atypical hyperplasia, alongside normal ductal and acinar epithelium within the IBC samples and adjacent tissues. The IBC cases were systematically categorized into three primary histological classifications: ductal, lobular, and a collective category for other histological variants. Concurrently, a comprehensive evaluation of critical macroscopic, pathological, and prognostic indicators was undertaken. This evaluation encompassed tumor dimensions, lymph node involvement, histological classification and grade, as well as the examination of intra and peritumoral mononuclear infiltration, necrosis, and indicators of vascular, lymphatic, and perineural invasion (21).

Furthermore, the classification of IBC into four molecular categories—Luminal A, Luminal B, HER2 positive, and Triple-Negative Breast Cancer (TNBC)—was conducted, alongside the determination of the disease stage (22).

Immunohistochemical (IHC) procedure

The immunohistochemical analysis was meticulously executed following a standardized protocol. Initially, tissue specimens were preserved in a 10% solution of Neutral Buffered Formalin, stabilized at a pH of 7.0, and subsequently embedded in paraffin. For each participant, a representative tissue section from the paraffin block was selected for IHC processing. Sections, with a thickness of 4 μm, were affixed to adhesive slides (SuperFrost® Plus, VWR, Leuven, Belgium), followed by deparaffinization in xylene and gradual rehydration through a series of alcohol solutions of diminishing concentrations. Prior to antibody incubation, epitope retrieval was conducted, succeeded by the inhibition of endogenous peroxidase activity using a 3% hydrogen peroxide solution for a duration of 5 minutes. The tissue specimens were then exposed to a mixture of primary monoclonal and polyclonal antibodies, maintained at room temperature for the suggested timeframes. The antibodies employed included, but were not limited to, mAb GLB1 (OTI1C9, 1:150, MA5-26152, INVITROGEN, USA), mAb ER (1D5, RTU, IR657, DAKO, Denmark), mAb PR (PgR636, RTU, IR068, DAKO, Denmark), pAb HER2 (1:1200, AO485, DAKO, Denmark), and Ki67 (1:200, MIB-1, IR626, DAKO, Denmark). Following the primary antibody application, sections underwent incubation with a biotinylated secondary antibody, available commercially, at room temperature for the specified period (En Vision FLEX HRP, RTU, K8000). The IHC reactions were developed using 3,3'-diaminobenzidine tetrahydrochloride (DAB), with final contrasting performed using Mayer's hematoxylin (Hematoxylin M, HEMM-OT-1L, Biognost, Croatia), and the slides were sealed with Canada balsam. Negative controls excluded the primary antibody incubation, whereas known IBC expressions served as positive benchmarks. The slide examination was carried out under 100x, 200x, and 400x magnifications using a light microscope (AxioScop 40, Carl Zeiss, Germany), with key sections captured via a digital camera (AxioCam ICc1, Carl Zeiss, Germany).

Evaluation of IHC Staining

The evaluation of IHC staining was meticulously performed by two independent pathologists, who were not privy to the clinical follow-up data during their analysis..

The analysis of estrogen receptor (ER) and progesterone receptor (PR) expression utilized the Allred scoring system, which aggregates the proportion of tumor cells exhibiting positive nuclear staining and the intensity of the IHC staining (23). This scoring system yields a range from 0 to 8 for each sample evaluated.

For the assessment of the human epidermal growth factor receptor 2 (HER2) expression, the process adhered to established guidelines (24). The classification of all Inflammatory Breast Cancer (IBC) samples into HER2 negative (0 and 1+) and HER2 positive (3+) categories was based on the continuity and intensity of the membrane staining. Samples with equivocal HER2 expression (2+) underwent further testing using the silver in situ hybridization (SISH) technique, following which, they were definitively categorized as either HER2 positive or negative IBCs..

The expression of Ki67, a marker of cellular proliferation, was quantified as the percentage of positively staining tumor cells out of a total of 100 cells counted in the area of highest tumor proliferation. Based on a predetermined threshold value for Ki67 expression established by our laboratory, IBC samples were stratified into three categories reflecting proliferative activity: low (Ki67 <15%), medium (Ki67: 15-30%), and high (Ki67 >30%) (25, 26).

Expression of GLB1 was quantified by assessing the percentage of cytoplasmic expression in epithelial cells of invasive breast carcinoma and normal-adjacent tissue. By analyzing the expression, we defined the cut-off for GLB1. Based on the obtained results, we divided all invasive tumors into GLB1 positive (>27.5%) and GLB1 negative (≤27.5%). At the same time, GLB1 expression was read in stromal fibroblasts and classified into positive (≥1%) and negative (no staining).

Statistical data processing

For the statistical analysis of the collected data, the SPSS software (version 22.0, SRSS Inc., Chicago, IL) was employed. The methodology encompassed a variety of statistical techniques, including descriptive statistics for summarizing the data, the Mann-Whitney test and Kruskal-Wallis test for non-parametric comparisons between groups, and the $\chi 2$ test for categorical data analysis. Additionally, the Pearson or Spearman correlation coefficient was utilized to measure the strength and direction of associations between variables. The Receiver Operating Characteristic (ROC) curve analysis was conducted to evaluate the diagnostic performance of the tests, which involved determining the cut-off value, sensitivity, and specificity of the tests under consideration. The determination of these parameters enabled the assessment of the test's practical reliability in statistical analysis. The significance level was set with all reported p-values being two-sided, and a p-value of less than 0.05 was considered indicative of statistical significance.

RESULTS

General Characteristics

The study's experimental cohort comprised 147 females diagnosed with IBC, with an average age of 58 years. The age range within this group varied from a minimum of 29 years to a maximum of 84 years. There was no statistically significant correlation between age and expression of GLB1 in the examined groups, except in the in situ group (ρ=0.227, p=0.047) where a positive statistically significant correlation

was shown, indicating that the expression of GLB1 increases with age. A notable observation within this cohort was the concurrent presence of in situ carcinoma (ISC) alongside IBC in 79 of the patients. Furthermore, atypical hyperplasia (AH) was identified in 82 patients, while 109 cases displayed areas of glandular parenchyma devoid of any signs of epithelial proliferation or atypia, referred to as normal epithelium (NE) of ducts and acini. The mean size of the cancerous tumors within this group was recorded at 22.5 mm. Detailed clinico-pathological characteristics pertaining to the IBC cases are systematically presented in Table 1 for further examination and analysis.

Table 1. Clinicopathological characteristics of breast cancer

Variables		**N**	**%**
Side	left	66	44.9
	right	81	55.1
Histological type	lobular	18	12.4
	ductal	123	84.8
	other	4	2.8
Histological grade	HG1	17	11.9
	HG2	73	51
	HG3	53	37.1
Nuclear grade	NG1	17	15.2
	NG2	64	57.1
	NG3	31	27.7
Mitotic index	grade 1	19	42.2
	grade 2	20	44.4
	grade 3	6	13.3
Tumor necrosis	absent	26	21.7
	present	94	78.3
Desmoplasia	low	17	16.3
	medium	55	52.9
	high	32	30.8
Periductal elastosis	low	19	20.0
	medium	20	44.4
	high	16	35.6
Perineural invasion	absent	101	68.7
	present	46	31.3
Lymphatic invasion	absent	72	48.9
	present	75	51.1
Vascular invasion	absent	113	76.9
	present	34	23.1
HER2	negative	115	79.3
	positive	30	20.7
Ki67	low	30	20..9
	medium	42	29.4
	high	71	49.7
Molecular subtypes	Lum A	30	20.4
	Lum B	76	51.7
	HER2 +	19	12.9
	TNBC	22	15
T status	T1	48	35.8
	T2	64	47.8
	T3	9	6.7
	T4	13	9.7
N status	N0	50	37.3
	N1	48	35.8
	N2	19	14.2
	N3	17	12.7

Expression of *GLB1* in relation to cytological changes in epithelium

The expression of GLB1 exhibits a significant incremental pattern, starting from NE, progressing through AH and ISC, and peaking in IBC. Quantitatively, the average *GLB1* expression levels were noted to be 15% in NE, 24% in AH, 29% in ISC, and 39% in IBC, indicating a significant upward trend (Kruskal-Wallis test, p<0.001). Notably, the comparison between the ISC and AH groups did not reveal a statistically significant difference in GLB1 expression levels (Mann-Whitney U test, p=0.211). However, statistically significant differences were observed between all other groups, underscoring distinct expression profiles across different stages and forms of breast tissue alterations (as illustrated in Figure 1A). The immunohistochemical expression of *GLB1* across various histo- and cytomorphological changes is detailed in Figures 1B through 1E, providing a visual representation of these findings.

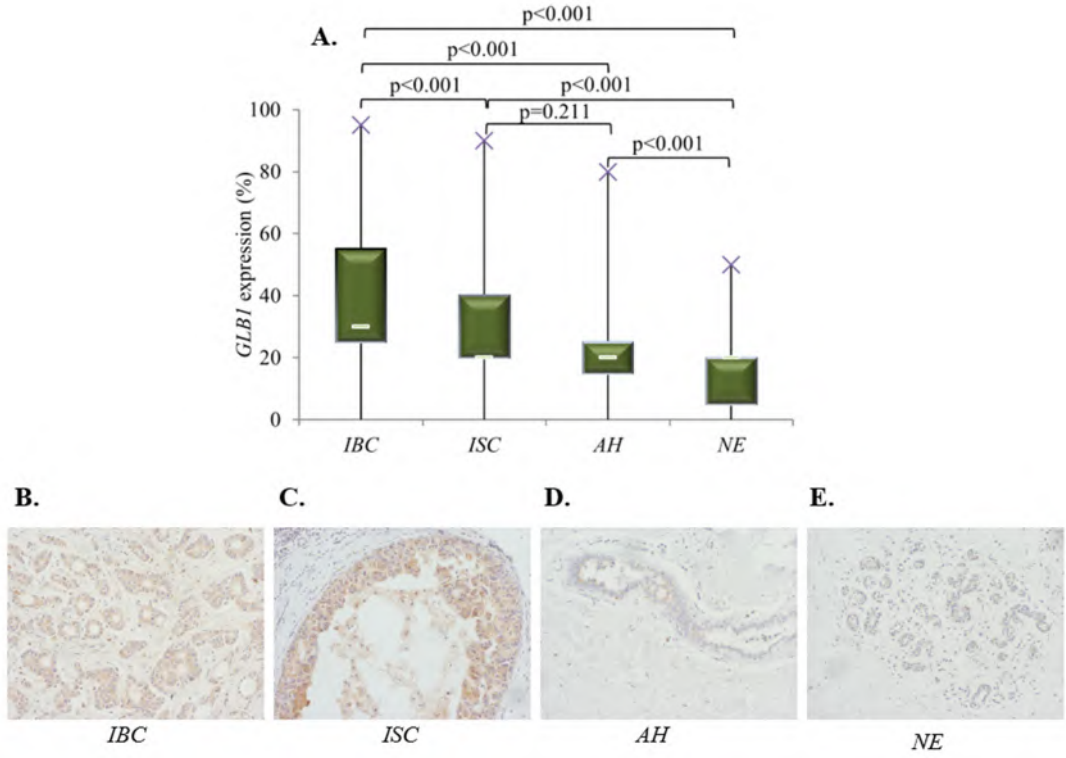

Figure 1.Expression of GLB1 in association with cytological alterations within the epithelium.

Panel A highlights a statistically significant disparity in GLB1 expression among all evaluated groups, with the exception of the comparison between in situ carcinoma (ISC) and atypical hyperplasia (AH), where no significant difference was detected (Mann-Whitney U, p=0.211). The data is presented in terms of the median expression levels. The subsequent panels provide microscopic visualizations of GLB1 expression across different histological and cytological variations, captured through immunohistochemical analysis at an original magnification of 200x: Panel B showcases GLB1 expression in IBC; Panel C illustrates its expression in ISC; Panel D in AH; and Panel E in normal epithelium (NE), thereby offering a comprehensive visual assessment of GLB1 expression relative to the progression of epithelial cytological changes.

In exploring the relationship between GLB1 expression among the four groups specified, in connection with cytological changes in the epithelium, a pattern was discerned where an elevation in GLB1 expression within any given group was paralleled by an increase in expression levels across the remaining groups. Specifically, a rise in GLB1 expression within IBC cells was correlated with heightened expression in NIL cells. Additionally, an escalation in GLB1 expression in ISCs was observed alongside increases in AH and NE, suggesting a cascading effect of GLB1 expression enhancement across the spectrum of epithelial cell changes, as determined by Spearman's rank correlation coefficient (ρ). This trend underscores a broader systemic response or a potential interconnected regulatory mechanism influencing GLB1 expression across varying stages of epithelial transformation (as depicted in Figure 2).

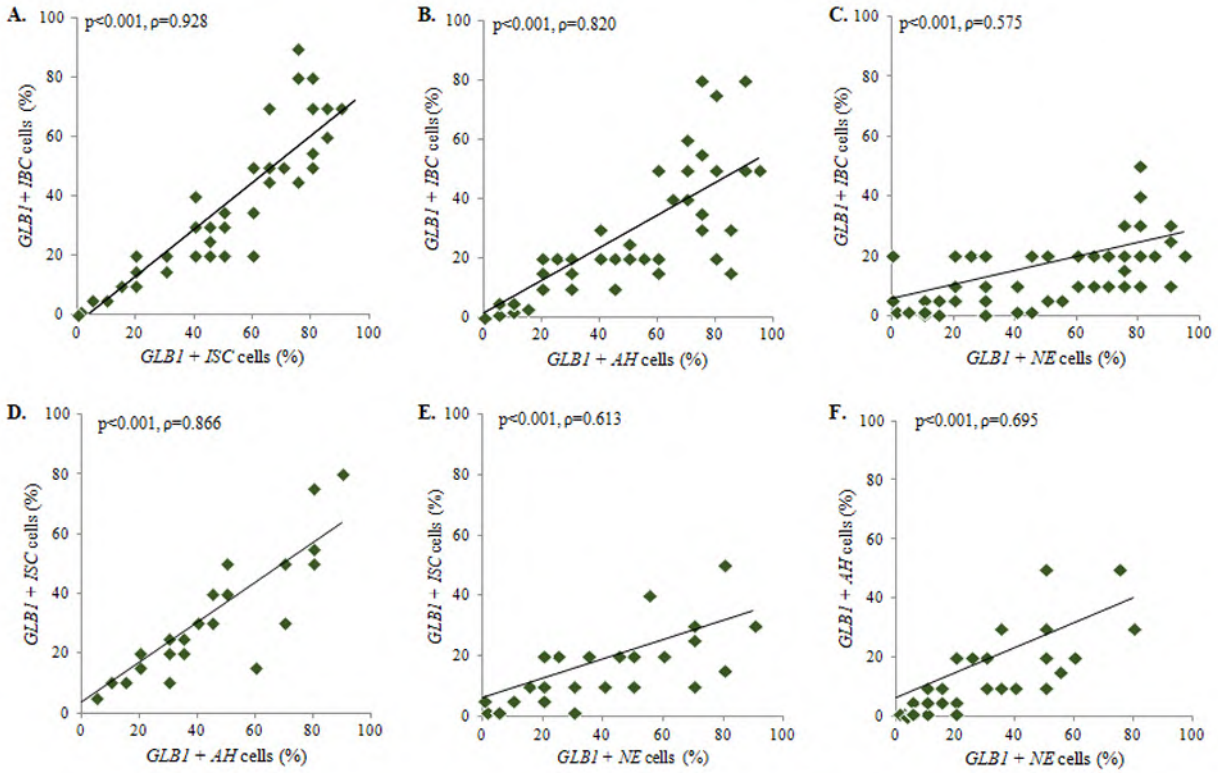

Figure 2. Correlation of GLB1 expression between groups in relation to cytological changes in epithelium.

A robust positive correlation was consistently observed across all groups, as evidenced by the statistical outcomes: Panel A illustrates the correlation between Invasive Breast Cancer (IBC) and in situ carcinoma (ISC) with a Spearman's rho (ρ) of 0.928 ($p<0.001$); Panel B depicts the correlation between IBC and atypical hyperplasia (AH) with a ρ of 0.820 ($p<0.001$); Panel C showcases the correlation between IBC and normal epithelium (NE) with a ρ of 0.575 ($p<0.001$); Panel D represents the correlation between ISC and AH with a ρ of 0.866 ($p<0.001$); Panel E between ISC and NE with a ρ of 0.613 ($p<0.001$); and Panel F highlights the correlation between AH and NE with a ρ of 0.695 ($p<0.001$).

GLB1 expression in tumor cells in relation to the molecular subtype of IBC

The analysis revealed a statistically significant variance in GLB1 expression across the different molecular subtypes of IBC, confirmed by the Kruskal-Wallis test ($p<0.001$). The HER2+ subtype of IBC exhibited the most elevated GLB1 expression levels, in contrast, the TNBC subtype demonstrated the lowest expression levels of GLB1. Comparatively, the Luminal A (Lum A) and Luminal B (Lum B) subtypes of IBC did not show a statistically significant difference in GLB1 expression (Mann-Whitney U, $p=0.128$). However, a discernible and significant disparity in GLB1 expression was noted among all other subgroup comparisons, as detailed in Figure 3.

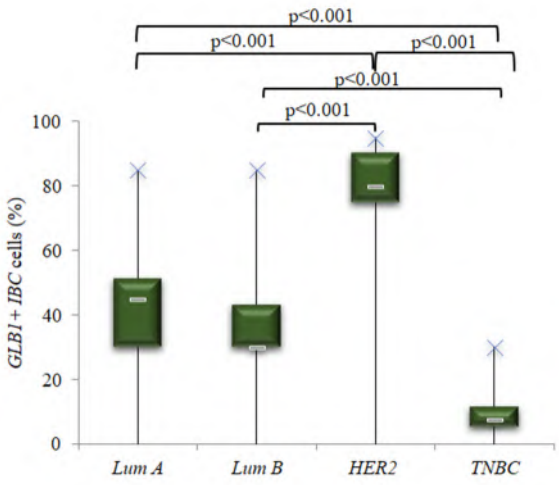

Figure 3. GLB1 expression in different molecular subtypes of *IBC*.

A statistically significant difference was shown between all investigated groups except between Lum A and Lum B subtypes of IBC (*Mann-Whitney U, p=0.128*). The result is presented as the median.

HER2-overexpressing IBCs had significantly higher GLB1 expression values compared to GLB1-negative IBCs. In terms of expression relative to Ki67 expression, there was no significant difference between those with low, moderate, or high expression, a notable increase in the proportion of Ki67-positive cells was observed in samples exhibiting

GLB1 expression (Figure 4). Other clinicopathological features of IBC were not associated with GLB1 expression.

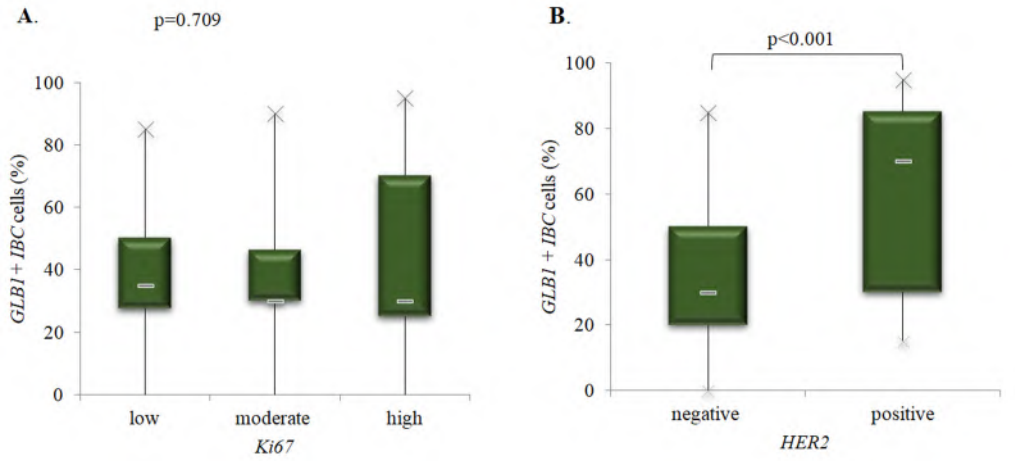

Figure 4. *Impact of Ki67 and HER2 Expression on GLB1 Levels in Tumor Cells*

Panel A demonstrates that GLB1 expression in tumor cells is not influenced by the level of Ki67 expression, with the findings conveyed through median values (Kruskal-Wallis, p=0.709), indicating no statistically significant association between GLB1 expression and the proliferation marker Ki67. Conversely, Panel B illustrates a substantial difference in GLB1 expression that is contingent upon HER2 expression. This notable variance is also represented through median values, showcasing a statistically significant distinction in GLB1 expression levels in relation to HER2 status (Mann Whitney U, p<0.001).

It was found that there is no significant correlation between estrogen and progesterone receptor expression and *GLB1* expression in *IBC* (*Spearman ρ*) (Figure 5).

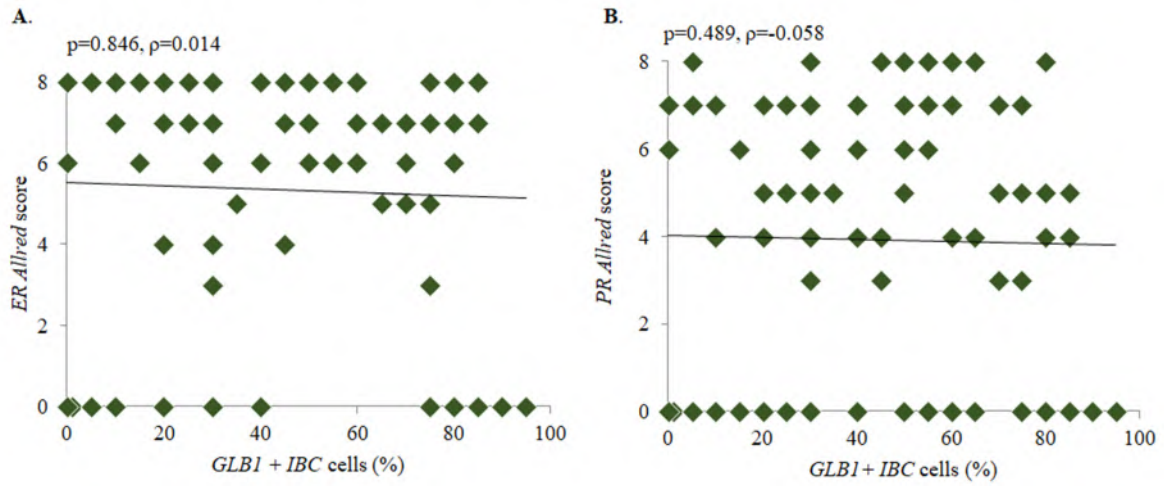

Figure 5. **GLB1 expression dependent on ER and PR expression**.

The expression of ER and PR was analyzed through the Allred score. There is no statistically significant expression of GLB1 relative to the expression of **A**. ER (p=0.846, ρ=0.014) and **B**. PR (p=0.489, ρ=-0.058).

GLB1 expression as a marker of breast cancer progression

The obtained ROC curve shown in Figure 6 indicates that increased expression of GLB1 can be a reliable marker of IBC progression (AUC =0.740; p<0.001). The results of the analysis indicate that the threshold value of 27.5% of GLB1 positive tumor cells enables the separation of patients with

IBC from patients with NIL (sensitivity 74.1%, specificity 80.0%).

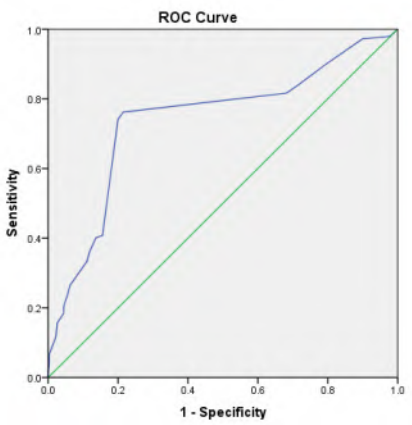

Figure 6. Receiver Operating Characteristic (ROC) curve analysis of GLB1 expression levels in Non-Invasive Lesions and Inflammatory Breast Cancer.

The Area Under the Curve (AUC) was calculated to be 0.740, indicating a good level of diagnostic accuracy. The sensitivity of the test—its ability to correctly identify those with IBC—was 74.1%, while the specificity—its ability to correctly identify those without IBC (or with NIL)—stood at 80.0%. The threshold value for GLB1 was determined to be 27.5%.

After the analysis and the threshold value obtained, all IBCs were classified into a group with negative (≤27.5%) and positive (>27.5%) GLB1 expression (Figure 7A). Figure 7B-C shows the immunohistochemical expression of GLB1 in relation to the threshold value.

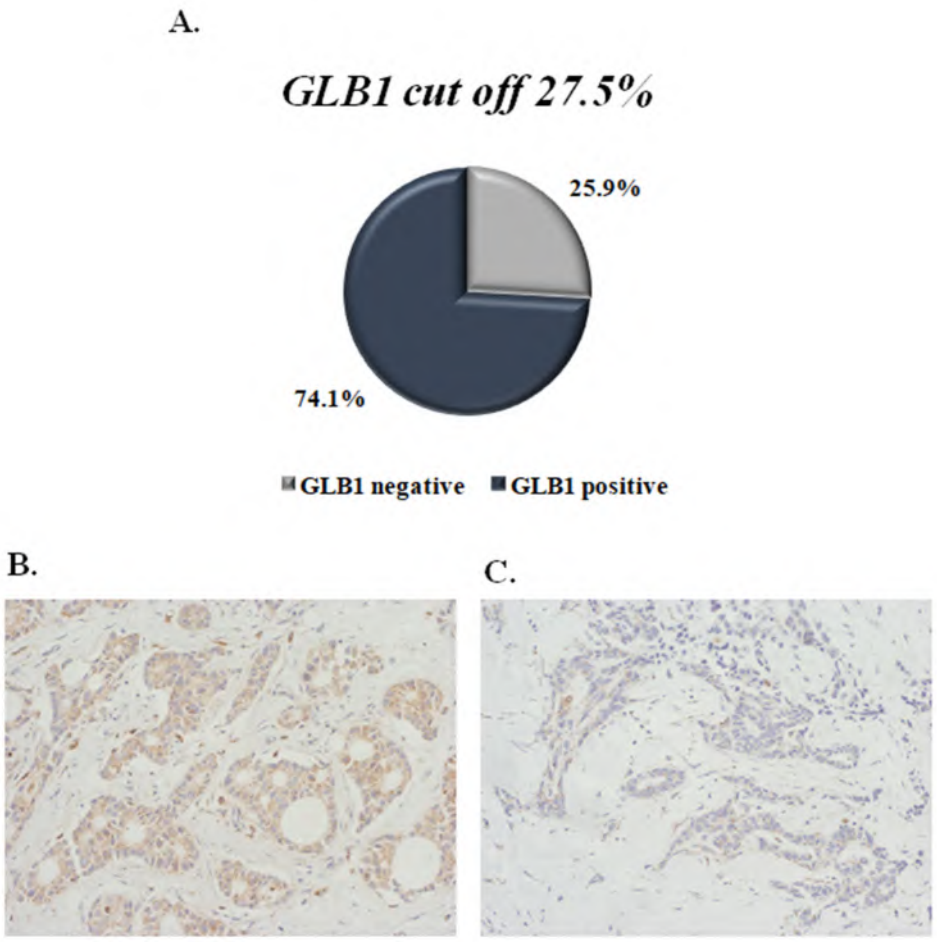

Figure 7. A. Frequency of GLB1+ and GLB1- IBC in relation to GLB1 expression threshold. Microscopic image of GLB1 expression relative to threshold: B. GLB1+ and C. GLB1- (immunohistochemical analysis, original magnification 200x).

The association of GLB1 expression with the following clinical-pathological characteristics was shown: histological grade, nuclear grade, molecular subtype of IBC, HER2 status (Table 2). A total of 94.7% of IBCs that were negative for GLB1 expression were also HER2 negative, while 26.2% of GLB1 positives were HER2 positive. The association of HER2 expression was also shown when molecular subtypes of IBC were observed, i.e. in GLB1 negative there was no HER2+ subtype of IBC, while in GLB1 positive the percentage was 17.4%. On the contrary, the highest percentage, i.e. 55.1% of GLB1 negative belongs to the TNBC molecular subtype, while only one IBC, i.e. 0.9%, belongs to this molecular subtype of GLB1 positive.

Table 2. Association between GLB1 expression in IBC and examined clinicopathological characteristics

Variables		**GLB1 cut off 27,5%**		**Chi-Square**	**p**
Mononuclear infiltrate	absent	2 (6,5%)	2 (3,2%)	3,892	0,273
	low	10 (32,3%)	33 (53,2%)		
	medium	14 (45,2%)	21 (33,9%)		
	high	5 (16,1%)	6 (9,7%)		
Stromal fibroblasts	negative	4 (22,2%)	13 (17,3%)	0,020	0,887
	positive	14 (77,8%)	62 (82,7%)		
Histological type	lobular	4 (10,8%)	14 (13,0%)	0,120	0,942
	ductal	32 (86,5%)	91 (84,3%)		
	other	1 (2,7%)	3 (2,8%)		
Histological grade	HG1	2 (5,3%)	15 (14,3%)	10,016	0,007
	HG2	14 (36,8%)	59 (56,2%)		
	HG3	22 (57,9%)	31 (29,5%)		
Nuclear grade	NG1	2 (6,7%)	15 (18,3%)	8,080	0,018
	NG2	14 (46,7%)	50 (61,0%)		
	NG3	14 (46,7%)	17 (20,7)		
Tumor necrosis	absent	5 (16,7%)	21 (23,3%)	0,262	0,609
	present	25 (83,3%)	69 (76,7)		
Perineural invasion	absent	27 (71,1%)	74 (67,9%)	0,025	0,874
	present	11 (28,9%)	35 (32,1%)		
Lymphatic invasion	absent	14 (36,8%)	58 (53,2%)	2,402	0,121
	present	24 (63,2%)	51 (46,8%)		
Vascular invasion	absent	31 (81,6%)	82 (75,2%)	0,639	0,424
	present	7 (18,4%)	27 (24,8%)		
Molecular subtypes	Lum A	5 (13,2%)	25 (22,9%)	67,563	0,000
	Lum B	12 (31,6%)	64 (58,7%)		
	HER2 +	0 (0,0%)	19 (17,4%)		
	TNBC	21 (55,3%)	1 (0,9%)		
HER2	negative	36 (94,7%)	79 (73,%)	6,249	0,012
	positive	2 (5,3%)	28 (26,2%)		
Ki67	low	7 (20,0%)	23 (21,3%)	0,433	0,805
	medium	9 (25,7%)	33 (30,6%)		
	high	19 (54,3%)	52 (48,1%)		
T status	T1	10 (28,6%)	38 (38,4%)	2,457	0,507
	T2	18 (51,4%)	46 (46,5%)		
	T3	4 (11,4%)	5 (5,1%)		
	T4	3 (8,6%)	10 (10,1%)		
N status	N0	9 (25,0%)	41 (41,8%)	4,862	0,182
	N1	13 (36,1%)	35 (35,7%)		
	N2	8 (22,2%)	11 (11,2%)		
	N3	6 (16,7%)	11 (11,2%)		

In relation to the histological grade, IBCs that are GLB1 negative are mostly of histological grade 3 57.9% (22), while the highest percentage of GLB1 positive belongs to grade 2, i.e. 56.2% (59). Also, in relation to the nuclear grade, in GLB1-negative cases, the percentage of IBCs that are grade 2 and grade 3 is equal, i.e. 46.7% (14), while in GLB1-positive cases, 61.0% (50) belong to grade 2.

DISCUSSION

To evaluate the presence of senescent cells across different breast tissue conditions, β-galactosidase expression was quantified utilizing the GLB1 antibody. Confirmation of senescence in the same tissue samples had been previously established in our earlier studies (27, 28). These investigations revealed elevated expression levels of two critical markers of senescence, specifically p16 and p21 - molecules integral to cell cycle regulation (29). Consequently, these findings substantiate the reliability of β-galactosidase, detected via GLB1, as an effective marker for identifying senescent cells. The analysis indicated that β galactosidase levels were highest in IBC, decreasing progressively through ISC, AH, to the lowest levels observed in NE. This gradient of β galactosidase expression highlights the variable distribution of senescent cells, reflecting the cellular aging process across a spectrum of breast tissue changes, from benign to malignant pathologies. The investigation of β galactosidase activity extended to conditions such as fibroadenoma, proliferative fibrocystic mastopathy, and infiltrative breast cancer, emphasizing its relevance in the context of breast tissue alterations and its potential as a biomarker for cellular senescence (16). Research findings indicate that β galactosidase activity varies significantly across different tissue types and pathological states. In fibroadenoma cells, β galactosidase activity remains within normal ranges, reflecting the benign nature of these tumors. In contrast, there is an increase in β galactosidase activity in proliferating duct cells within fibrocystic mastopathy, indicating elevated cellular senescence in association with proliferative alterations. The highest level of β galactosidase activity is observed in cells of infiltrative breast carcinoma, emphasizing the pronounced senescence in malignant tissues (16). Moreover, β galactosidase is found in 60% of hepatocellular carcinoma tissues, compared to its detection in only 20% of normal liver cases. Additionally, in individuals with fibrosis due to chronic viral hepatitis C, β galactosidase activity is observed in 50% of cases. These findings highlight the differential expression of β galactosidase across various liver conditions, emphasizing its significance as a potential marker for cellular senescence in both oncogenic and non-oncogenic contexts (30). *Alexandraki* et al examined β galactosidase in normal tissue, adenomas and pituitary carcinomas and came to the conclusion that the high expression of this marker was in adenomas and carcinomas, compared to normal pituitary tissue (31). The findings reveal a pronounced correlation between β galactosidase activity and the malignant potential of cells, indicating that higher levels of this marker are closely associated with increased malignancy.

Interestingly, an examination of GLB1 expression across different molecular subtypes of IBC, we observed the highest percentage of GLB1 positive tumor cells in HER2 positive IBC, then in Luminal A, then in Luminal B, while the lowest expression was in TNBC. These observations align with findings reported by Cotarello et al. (32), who also identified a differential expression pattern of β-galactosidase among these subtypes. In a subsequent study by these authors, a significantly elevated expression of β-galactosidase was observed in Luminal A IBC samples when compared to Luminal B. However, no expression of this marker was detected in the primary tumors of the HER2-positive and TNBC subtypes, though it was present in metastases corresponding to these subtypes (33). The detection of tumor cells expressing GLB1 suggests that their presence is attributable either to mechanisms intrinsic to the tumor cells themselves or to the tumor microenvironment. It is recognized thatdifferent subtypes of IBC are determined by different subsets of epigenetic and genetic abnormalities (34, 35). Considering the role of senescence in inhibiting oncogene-induced transformation, it is feasible to hypothesize that variances in the senescence capability among different IBC subtypes may be attributed to inherent differences in driver mutations and other genetic defects.

Furthermore, it's possible that these IBC subtypes differ in their capacity to recruit immune cells responsible for eliminating senescent cells. The induction of senescence in response to oncogenic stress largely depends on the integrity of the p53-p21 and p16-pRb tumor suppressor pathways. Any defects in these pathways could undermine the cell's ability to undergo senescence, potentially facilitating cancer progression (36, 37).

Mutations in the p53 gene, prevalent in approximately 37% of all IBC cases, exhibit distinct patterns across IBC subtypes. Notably, TNBCs demonstrate mutations in the p53 gene in approximately 80% of cases (38). In Triple-Negative Breast Cancers, the predominance of p53 gene mutations includes nonsense and frameshift mutations that disrupt the genetic sequence. These mutations lead to the complete loss of tumor suppressor function of p53 or the emergence of oncogenic characteristics. Thus, the loss of tumor suppressor function due to mutations in the p53 gene in TNBC could explain the limited presence of GLB1-expressing senescent cancer cells observed in our study. Within luminal A and B breast carcinomas, mutations in the p53 gene are chiefly characterized by missense mutations, which do not invariably result in the total loss of p53 tumor suppressor functionality. Moreover, these mutations are significantly less frequent compared to those observed in TNBC, with around 12% of luminal A and 29% of luminal B tumors exhibiting p53 gene mutations. (35). Consequently, it is noteworthy that, within our research, luminal A tumors exhibited higher GLB1 expression compared to luminal B tumors. This observation suggests that the lesser frequency and nature of p53 gene mutations in luminal A breast tumors might be contributing factors to the increased prevalence of senescent cells within this IBC subtype. Additionally, a significant portion of HER2-

positive breast tumor samples in our study demonstrated GLB1 expression. It has been observed that senescent cells, through their SASP and influenced by continuous HER2 signaling, may impede the elimination of senescent cells and foster prometastatic conditions (39). Therefore, the high expression of GLB1 in cancer cells in our HER2-positive IBC cells may suggest that oncogenic HER2-induced senescence results in a secretory phenotype that reduces the elimination of senescent cells by inhibiting immune cell activation. This leads to their accumulation within the tumor. It is also possible that this secretory phenotype could increase the ability of non-senescent cells to proliferate and metastasize.

Senescent cells are characterized by a permanent cessation of the cell cycle, implying that cells expressing GLB1, indicative of senescence, should not proliferate. To investigate whether GLB1-expressing cells exhibit growth arrest, we assessed their expression of Ki67, a marker of cell proliferation. Although the analysis did not yield statistically significant differences between the parameters, a higher percentage of Ki67-positive cells was noted in those exhibiting GLB1 expression. This observation is consistent with findings reported by others (32), suggesting an indirect correlation where high GLB1 expression in IBC cells may be associated with a poorer prognosis of the disease.

Our findings reveal that the application of specific antibodies targeting β-galactosidase (GLB1) indicated its expression in tumor stromal fibroblasts. This suggests that β-galactosidase expression in vivo could represent an indicator of senescence initiation within the tumor stroma. Evidence from multiple studies supports the notion that senescent fibroblasts can facilitate tumorigenesis, highlighting the complex role of cellular senescence in the tumor microenvironment (40, 41). It is the tumorigenic activity of senescent stromal fibroblasts that is attributed to SASP (42). While the specific impact of the SASP varies depending on the context, its paracrine influence in advanced cancer generally serves to either directly augment the proliferative and metastatic capacity of neoplastic cells or indirectly facilitate their dissemination by fostering a conducive environment. This leads to local tissue remodeling, highlighting the dual role of SASP in modifying the tumor microenvironment and influencing cancer progression (43). SASP is believed to constitute approximately 50% of the tumor-promoting actions of senescent fibroblasts, leaving the origins of the remaining 50% of this activity as yet unidentified (44, 45). Notably, various stimuli traditionally employed to induce fibroblast senescence, including oxidative stress and brief exposure to hydrogen peroxide, have been recently recognized to also trigger autophagy and the differentiation into myofibroblasts. (46-48).

The remaining 50% of the tumor-promoting effects attributed to senescent fibroblasts could potentially be linked to their autophagic or catabolic states, compromised mitochondrial function, and a transition towards aerobic glycolysis. This metabolic shift leads to the generation of mitochondrial by-products including L-lactate, ketones, glutamine, and free fatty acids. Such by-products might serve as metabolic substrates for adjacent tumor cells, supporting their anabolic growth and contributing to the complex interplay within the tumor microenvironment (49). It can be deduced that specific subtypes of senescent fibroblasts possess the capacity to foster tumor proliferation without necessarily exhibiting a SASP (50).

CONCLUSIONS

Our observations indicate that the accumulation of GLB1 in tumors is nonlinear, which can be used to assess the progression of precancerous and cancerous lesions. We have also shown that senescent tumor cells exist in advanced IBC and that the proportion of these cells varies significantly depending on the IBC subtype. High percentages of GLB1 positive tumor cells exist in HER2+ breast cancer samples, while in TNBC there are no or very few GLB1 positive tumor cells, which primarily depends on the additional genetic and epigenetic characteristics of each subtype of IBC. We further demonstrated high expression of this marker in fibroblasts of the tumor stroma, which may indicate that the microenvironment itself affects the progression of IBC. Therefore, further evaluation of GLB1 expression is justified, which will give a significant encouragement to the development of IBC diagnostics and understanding of its morphogenesis, as well as help in determining the individual approach to a patient treatment.

AUTHOR CONTRIBUTIONS

Conceptualization, M.I.; methodology D.J. and S.M; validation, M.V and D.A.; formal analysis, J.S and M.S..; investigation, D.J.; data curation, M.I.; writing-original draft preparation, D.J.; writing-review and editing, M.I and D.J.; visualization D.J., S.M.; supervision, D.K., M.D.S. and M.I. All authors have read and agreed to the published version of the manuscript.

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INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Clinical Center Kragujevac, Serbia, number: 01/17/2290, date: June 14 2017.

INFORMED CONSENT STATEMENT

Informed consent was obtained from all subjects involved in the study.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Sung, H.; Ferlay, J.; Siegel, R. L.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F., Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* 2021, *71* (3), 209-249.
2. Beňačka, R.; Szabóová, D.; Guľašová, Z.; Hertelyová, Z.; Radoňák, J., Classic and New Markers in Diagnostics and Classification of Breast Cancer. *Cancers (Basel)* 2022, *14* (21).
3. Alhmoud, J. F.; Woolley, J. F.; Al Moustafa, A. E.; Malki, M. I., DNA Damage/Repair Management in Cancers. *Cancers (Basel)* 2020, *12* (4).
4. Litton, J. K.; Burstein, H. J.; Turner, N. C., Molecular Testing in Breast Cancer. *Am Soc Clin Oncol Educ Book* 2019, *39*, e1-e7.
5. Sauter, E. R., Reliable Biomarkers to Identify New and Recurrent Cancer. *Eur J Breast Health* 2017, *13* (4), 162-167.
6. Schmitt, C. A.; Wang, B.; Demaria, M., Senescence and cancer - role and therapeutic opportunities. *Nat Rev Clin Oncol* 2022, *19* (10), 619-636.
7. Morales-Valencia, J.; David, G., The Contribution of Physiological and Accelerated Aging to Cancer Progression Through Senescence-Induced Inflammation. *Front Oncol* 2021, *11*, 747822.
8. Lau, L.; David, G., Pro- and anti-tumorigenic functions of the senescence-associated secretory phenotype. *Expert Opin Ther Targets* 2019, *23* (12), 1041-1051.
9. Guo, Y.; Ayers, J. L.; Carter, K. T.; Wang, T.; Maden, S. K.; Edmond, D.; Newcomb, P. P.; Li, C.; Ulrich, C.; Yu, M.; Grady, W. M., Senescence-associated tissue microenvironment promotes colon cancer formation through the secretory factor GDF15. *Aging Cell* 2019, *18* (6), e13013.
10. Bavik, C.; Coleman, I.; Dean, J. P.; Knudsen, B.; Plymate, S.; Nelson, P. S., The gene expression program of prostate fibroblast senescence modulates neoplastic epithelial cell proliferation through paracrine mechanisms. *Cancer Res* 2006, *66* (2), 794-802.
11. Coppe, J. P.; Boysen, M.; Sun, C. H.; Wong, B. J.; Kang, M. K.; Park, N. H.; Desprez, P. Y.; Campisi, J.; Krtolica, A., A role for fibroblasts in mediating the effects of tobacco-induced epithelial cell growth and invasion. *Mol Cancer Res* 2008, *6* (7), 1085-98.
12. Huang, W.; Hickson, L. J.; Eirin, A.; Kirkland, J. L.; Lerman, L. O., Cellular senescence: the good, the bad and the unknown. *Nat Rev Nephrol* 2022, *18* (10), 611-627.
13. Chen, S. W., L.; Ma, X.; Wu, Y.; Hou, S. Kill two birds with one stone: A near-infrared ratiometric fluorescent probe for simultaneous detection of _-galactosidase in senescent and cancer cells. Sens. Actuators B Chem. 2022, 367, 132061
14. Zieba, J.; Ksiazkiewcz, M.; Janik, K.; Banaszczyk, M.; Peciak, J.; Piaskowski, S.; Lipinski, M.; Olczak, M.; Stoczynska-Fidelus, E.; Rieske, P., Sensitivity of neoplastic cells to senescence unveiled under standard cell culture conditions. *Anticancer Res* 2015, *35* (5), 2759-68.
15. Pakuła, M.; Mały, E.; Uruski, P.; Witucka, A.; Bogucka, M.; Jaroszewska, N.; Makowska, N.; Niklas, A.; Moszyński, R.; Sajdak, S.; Tykarski, A.; Mikuła-Pietrasik, J.; Książek, K., Correction: Pakuła et al. Deciphering the Molecular Mechanism of Spontaneous Senescence in Primary Epithelial Ovarian Cancer Cells. Cancers 2020, 12, 296. *Cancers (Basel)* 2023, *15* (3).
16. Bosmann, H. B.; Hall, T. C., Enzyme activity in invasive tumors of human breast and colon. *Proc Natl Acad Sci U S A* 1974, *71* (5), 1833-7.
17. Bartkova, J.; Rezaei, N.; Liontos, M.; Karakaidos, P.; Kletsas, D.; Issaeva, N.; Vassiliou, L. V.; Kolettas, E.; Niforou, K.; Zoumpourlis, V. C.; Takaoka, M.; Nakagawa, H.; Tort, F.; Fugger, K.; Johansson, F.; Sehested, M.; Andersen, C. L.; Dyrskjot, L.; Ørntoft, T.; Lukas, J.; Kittas, C.; Helleday, T.; Halazonetis, T. D.; Bartek, J.; Gorgoulis, V. G., Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature* 2006, *444* (7119), 633-7.
18. Lee, B. Y.; Han, J. A.; Im, J. S.; Morrone, A.; Johung, K.; Goodwin, E. C.; Kleijer, W. J.; DiMaio, D.; Hwang, E. S., Senescence-associated beta-galactosidase is lysosomal beta-galactosidase. *Aging Cell* 2006, *5* (2), 187-95.
19. Kurz, D. J.; Decary, S.; Hong, Y.; Erusalimsky, J. D., Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *J Cell Sci* 2000, *113 (Pt 20)*, 3613-22.
20. Ewald, J. A.; Desotelle, J. A.; Church, D. R.; Yang, B.; Huang, W.; Laurila, T. A.; Jarrard, D. F., Androgen deprivation induces senescence characteristics in prostate cancer cells in vitro and in vivo. *Prostate* 2013, *73* (4), 337-45.
21. Tan, P. H.; Ellis, I.; Allison, K.; Brogi, E.; Fox, S. B.; Lakhani, S.; Lazar, A. J.; Morris, E. A.; Sahin, A.; Salgado, R.; Sapino, A.; Sasano, H.; Schnitt, S.; Sotiriou, C.; van Diest, P.; White, V. A.; Lokuhetty, D.; Cree, I. A., The 2019 World Health Organization classification of tumours of the breast. *Histopathology* 2020, *77* (2), 181-185.
22. Goldhirsch, A.; Wood, W. C.; Coates, A. S.; Gelber, R. D.; Thürlimann, B.; Senn, H. J., Strategies for subtypes--dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. *Ann Oncol* 2011, *22* (8), 1736-47.
23. Allred, D. C.; Harvey, J. M.; Berardo, M.; Clark, G. M., Prognostic and predictive factors in breast cancer by

immunohistochemical analysis. *Mod Pathol* 1998, *11* (2), 155-68.
24. Wolff, A. C.; Hammond, M. E.; Hicks, D. G.; Dowsett, M.; McShane, L. M.; Allison, K. H.; Allred, D. C.; Bartlett, J. M.; Bilous, M.; Fitzgibbons, P.; Hanna, W.; Jenkins, R. B.; Mangu, P. B.; Paik, S.; Perez, E. A.; Press, M. F.; Spears, P. A.; Vance, G. H.; Viale, G.; Hayes, D. F., Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. *Arch Pathol Lab Med* 2014, *138* (2), 241-56.
25. Focke, C. M.; Bürger, H.; van Diest, P. J.; Finsterbusch, K.; Gläser, D.; Korsching, E.; Decker, T., Interlaboratory variability of Ki67 staining in breast cancer. *Eur J Cancer* 2017, *84*, 219-227.
26. Gnant, M.; Thomssen, C.; Harbeck, N., St. Gallen/Vienna 2015: A Brief Summary of the Consensus Discussion. *Breast Care (Basel)* 2015, *10* (2), 124-30.
27. Jovanovic, D. V.; Mitrovic, S. L.; Milosavljevic, M. Z.; Ilic, M. B.; Stankovic, V. D.; Vuletic, M. S.; Dimitrijevic Stojanovic, M. N.; Milosev, D. B.; Azanjac, G. L.; Nedeljkovic, V. M.; Radovanovic, D., Breast Cancer and p16: Role in Proliferation, Malignant Transformation and Progression. *Healthcare (Basel)* 2021, *9* (9).
28. Jovanovic, D.; Mitrovic, S.; Alic, D.; Besic, D.; Knezevic, D.; Dimitrijevic, J.; Ilic, M., Prognostic Significance of p21 Protein in Breast Cancer. *Experimental and Applied Biomedical Research (EABR)* 2023, *0* (0).
29. Domen, A.; Deben, C.; Verswyvel, J.; Flieswasser, T.; Prenen, H.; Peeters, M.; Lardon, F.; Wouters, A., Cellular senescence in cancer: clinical detection and prognostic implications. *J Exp Clin Cancer Res* 2022, *41* (1), 360.
30. Paradis, V.; Youssef, N.; Dargère, D.; Bâ, N.; Bonvoust, F.; Deschatrette, J.; Bedossa, P., Replicative senescence in normal liver, chronic hepatitis C, and hepatocellular carcinomas. *Hum Pathol* 2001, *32* (3), 327-32.
31. Alexandraki, K. I.; Munayem Khan, M.; Chahal, H. S.; Dalantaeva, N. S.; Trivellin, G.; Berney, D. M.; Caron, P.; Popovic, V.; Pfeifer, M.; Jordan, S.; Korbonits, M.; Grossman, A. B., Oncogene-induced senescence in pituitary adenomas and carcinomas. *Hormones (Athens)* 2012, *11* (3), 297-307.
32. Cotarelo, C. L.; Schad, A.; Kirkpatrick, C. J.; Sleeman, J. P.; Springer, E.; Schmidt, M.; Thaler, S., Detection of cellular senescence within human invasive breast carcinomas distinguishes different breast tumor subtypes. *Oncotarget* 2016, *7* (46), 74846-74859.
33. Cotarelo, C. L.; Schad, A.; Schmidt, M.; Hönig, A.; Sleeman, J. P.; Thaler, S., Detection of Cellular Senescence Reveals the Existence of Senescent Tumor Cells within Invasive Breast Carcinomas and Related Metastases. *Cancers (Basel)* 2023, *15* (6).
34. Testa, U.; Castelli, G.; Pelosi, E., Breast Cancer: A Molecularly Heterogenous Disease Needing Subtype-Specific Treatments. *Med Sci (Basel)* 2020, *8* (1).
35. Comprehensive molecular portraits of human breast tumours. *Nature* 2012, *490* (7418), 61-70.
36. Mijit, M.; Caracciolo, V.; Melillo, A.; Amicarelli, F.; Giordano, A., Role of p53 in the Regulation of Cellular Senescence. *Biomolecules* 2020, *10* (3).
37. Kudlova, N.; De Sanctis, J. B.; Hajduch, M., Cellular Senescence: Molecular Targets, Biomarkers, and Senolytic Drugs. *Int J Mol Sci* 2022, *23* (8).
38. Li, J. P.; Zhang, X. M.; Zhang, Z.; Zheng, L. H.; Jindal, S.; Liu, Y. J., Association of p53 expression with poor prognosis in patients with triple-negative breast invasive ductal carcinoma. *Medicine (Baltimore)* 2019, *98* (18), e15449.
39. Angelini, P. D.; Zacarias Fluck, M. F.; Pedersen, K.; Parra-Palau, J. L.; Guiu, M.; Bernadó Morales, C.; Vicario, R.; Luque-García, A.; Navalpotro, N. P.; Giralt, J.; Canals, F.; Gomis, R. R.; Tabernero, J.; Baselga, J.; Villanueva, J.; Arribas, J., Constitutive HER2 signaling promotes breast cancer metastasis through cellular senescence. *Cancer Res* 2013, *73* (1), 450-8.
40. Yang, M.; Jiang, Z.; Yao, G.; Wang, Z.; Sun, J.; Qin, H.; Zhao, H., GALC Triggers Tumorigenicity of Colorectal Cancer via Senescent Fibroblasts. *Front Oncol* 2020, *10*, 380.
41. Al-Khalaf, H. H.; Ghebeh, H.; Inass, R.; Aboussekhra, A., Senescent Breast Luminal Cells Promote Carcinogenesis through Interleukin-8-Dependent Activation of Stromal Fibroblasts. *Mol Cell Biol* 2019, *39* (2).
42. Mavrogonatou, E.; Pratsinis, H.; Kletsas, D., The role of senescence in cancer development. *Semin Cancer Biol* 2020, *62*, 182-191.
43. Mavrogonatou, E.; Pratsinis, H.; Papadopoulou, A.; Karamanos, N. K.; Kletsas, D., Extracellular matrix alterations in senescent cells and their significance in tissue homeostasis. *Matrix Biol* 2019, *75-76*, 27-42.
44. Krtolica, A.; Parrinello, S.; Lockett, S.; Desprez, P. Y.; Campisi, J., Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc Natl Acad Sci U S A* 2001, *98* (21), 12072-7.
45. Krtolica, A.; Campisi, J., Cancer and aging: a model for the cancer promoting effects of the aging stroma. *Int J Biochem Cell Biol* 2002, *34* (11), 1401-14.
46. Yun, H. R.; Jo, Y. H.; Kim, J.; Shin, Y.; Kim, S. S.; Choi, T. G., Roles of Autophagy in Oxidative Stress. *Int J Mol Sci* 2020, *21* (9).
47. Wu, Z.; Wang, H.; Fang, S.; Xu, C., Roles of endoplasmic reticulum stress and autophagy on H2O2-induced oxidative stress injury in HepG2 cells. *Mol Med Rep* 2018, *18* (5), 4163-4174.
48. Huang, X.; He, C.; Hua, X.; Kan, A.; Mao, Y.; Sun, S.; Duan, F.; Wang, J.; Huang, P.; Li, S., Oxidative stress induces monocyte-to-myofibroblast transdifferentiation through p38 in pancreatic ductal adenocarcinoma. *Clin Transl Med* 2020, *10* (2), e41.
49. Capparelli, C.; Guido, C.; Whitaker-Menezes, D.; Bonuccelli, G.; Balliet, R.; Pestell, T. G.; Goldberg, A. F.; Pestell, R. G.; Howell, A.; Sneddon, S.; Birbe, R.; Tsirigos, A.; Martinez-Outschoorn, U.; Sotgia, F.; Lisanti, M. P., Autophagy and senescence in cancer-

associated fibroblasts metabolically supports tumor growth and metastasis via glycolysis and ketone production. *Cell Cycle* 2012, *11* (12), 2285-302.

50. Coppé, J. P.; Rodier, F.; Patil, C. K.; Freund, A.; Desprez, P. Y.; Campisi, J., Tumor suppressor and aging biomarker p16(INK4a) induces cellular senescence without the associated inflammatory secretory phenotype. *J Biol Chem* 2011, *286* (42), 36396-403.

PROFESSIONAL DRIVERS' KNOWLEDGE ABOUT THE INFLUENCE OF MEDICINES THAT MAY IMPAIR DRIVING

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ABSTRACT

More knowledge about the impact of medication on driving are indicative of a lower likelihood of having a motor vehicle crash. The aim of this study was to investigate knowledge of professional drivers about the influence of driving impairing medicines in Serbia and Bosnia and Herzegovina.

This multicenter cross-sectional study was conducted in 6 cities in Serbia and Bosnia and Herzegovina, during first trimester of 2017, with 221 professional drivers, using questionnaire with 35 statements, where participants expressed their agreement according to Likert scale, from completely disagree to completely agree.

The average score related to the drivers' knowledge was 131,58 (range from 49 to 175), 22,6% were unaware that some medicines may influence psychophysical abilities and ability to drive. A high percentage of participants in the study don't know that a negative impact on the driving ability can be the result of the use of medicines from groups for which it is unexpected to have such effects, medicines that are dispensed without a medical prescription, herbal remedies, dietary supplements and medicines that affects eyesight or hearing. More than half didn't know that medicines labeled with warning symbols Δ, ▲ and § are not allowed to be used immediately before or during driving.

Professional drivers' knowledge about driving impairing medicines is not satisfactory. Labeling system of these medicines is inadequate. These findings could help to identify drivers, who are at increased risk for using potentially impairing medicines, to inform and educate them, and to prevent driving under the influence of medicines.

Keywords: *Professional drivers, knowledge, driving impairing medicines, warning symbols.*

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INTRODUCTION

Driving a motor vehicle continues to be the most popular means of transportation (1), although it is a multifaceted task (2), and complex and risky activity that requires good physical and mental health state and the ability (3) to interact simultaneously with both the vehicle and the external environment (4). Vehicles, which are characteristics of civilization, have turned into a big problem in various social and public health respects due to increasing the number of the road and city accidents with high mortality rate (5). Motor vehicle accidents represent a major health problem worldwide (6), and especially those due to prescription drug impairment have increased in the past decade (7). Drugged driving is a safety issue of increasing public concern (8), as well as driving under the influence of prescription and over-the-counter medication (9). Especially, drugs used in combination with alcohol are likely to produce a significant threat to traffic safety (10). Most often it is because medicines can cause drowsiness, make drivers less attentive or slow reflexes that some drugs increase the risk of accidents, but they can also affect the ability of judgment, impair the view or cause dizziness making the road dangerous (11). One recent study demonstrated that patients are not able to predict accurately their level of driving impairment (12). The impact of a drug on driving is an important consideration when designing a medication regimen (13), because drugs with potential to impair driving were consumed by a third of the general population (2). One in five drivers reported recent use of medication that may impair driving (14). Both illicit and licit drugs that affect the central nervous system (CNS) have the potential to impair driving performance (15). Knowing how medications affect the ability to drive is clearly a safety issue that is relevant to patients, physicians, drug manufacturers, drug legislators, and the general public (16). The DRUID project has as its aim to make a wide-reaching attempt, by using a uniform study design, to close the gap that still exists in knowledge about traffic safety in relation to the use of psychoactive drugs (17) and was an integrative effort to reduce the danger of alcohol, illicit drugs, and medicines in traffic (18).

According to national Act about road traffic safety professional driver is a person who is employed to drive a vehicle which transports people (within the public transport or else) or goods (19). Commercial drivers may also exhibit particular forms of unsafe driving behavior to cope with workload and time pressure (20). These drivers are at a greater risk of accident involvement (21), as commercial vehicles involved in approximately quarter of all road traffic fatalities (22). Impaired driving among commercial drivers is of particular public health importance (23). They are subjected to countless factors that influence their professional practice, among which the intake of psychoactive substances stands out (24). Truck drivers are especially vulnerable to psychoactive substance abuse (25). Higher utilization of sedating agents was positively associated with being a commercial driver (26). A significant proportion of commercial drivers reported use of stimulant as the most helpful fatigue management strategy (27), and was also involved in drugs and alcohol abuse while driving (28). In spite of comprehensive drug testing in the trucking industry, some drivers are continuing to take illicit and other drugs with the potential of having a negative effect on their driving ability (29). Knowledge of professional drivers about driving impairing medicines is not satisfactory (30), as well as in general population of drivers (26; 31) and old drivers (32). Fifty-one percent (51.5%) of professional drivers were unaware of the existence of medicines that impair driving (30). More careful attitudes or more knowledge about the impact of medication on driving are indicative of a lower likelihood of having a motor vehicle crash (26). Evidence suggests that patients ignore or do not read prescription labels that warn of driving impairment (33). Regardless of the excellent results obtained by DRUID project and other studies, some data about this important issue are missing. There is a lack of data about drivers' knowledge and attitudes about driving impairing medicines, especially in a very significant and vulnerable population such as professional drivers, as well as data about factors that influence their knowledge and attitudes.

THE AIM OF THE PAPER

The aim of this study was to investigate knowledge of professional drivers about the influence of driving impairing medicines in Serbia and Bosnia and Herzegovina.

PATIENTS AND METHODS

Study settings

This multicenter cross-sectional study was conducted in 6 cities in Serbia and Bosnia and Herzegovina, during the first trimester of 2017. Approval for conducting the study was obtained by the Ethics Committee of the Faculty of Medical Sciences, University of Kragujevac. All participants gave their written consent after they have been thoroughly acquainted with the research protocol, respecting all ethical norms and principles according to Helsinki declaration and standards of Good Clinical Practice.

Study population

The study population was made of professional drivers, both genders, age from 20 to 65, who were employed in taxi associations, transport organizations, delivery services, auto-traffic companies where they drive car/truck/van/bus, or they spend most of their working time to operate the machinery, such as industrial workers, workers who handle cranes, forklifts and agricultural machinery. Participants, who expressed unwillingness to comply with the study protocol and who didn't currently work as professional drivers, because their driver's license were subtracted due to a traffic violation, health issues or for some other reasons, were excluded from the study.

Development of the questionnaire

The knowledge of professional drivers was examined using the questionnaire which consists of 35 items. The questionnaire for assessing professional drivers' knowledge about influence of medicines on driving abilities (QPDK-IMDA) was developed through several steps, according to the guidelines (34), by members of the research team. After the professional drivers were determined as an object of measurement, an item pool was generated through literature review about drug use among professional drivers and their awareness of adverse effects on driving ability. Next step was revision and correction of the initial pool of items, made by the three-member expert committee. Then, the initial pool of items was tested on 4 professional drivers (in Šabac, Serbia) for clarity and comprehension. Few minor changes were made after pilot, and then final Serbian version of the questionnaire was prepared for use. The questionnaire was filled by the researchers who interviewed the participants. Respondents expressed their agreement with the offered assertions according to the Likert scale: "completely disagree" (1), "partially disagree" (2), "neither agree nor disagree" (3), "partially agree" (4), and "completely agree" (5) (the questionnaire is provided as an Additional file 1).

Collected data

During the study data about the following factors that could affect the knowledge and attitudes of professional drivers about influence of medicines on driving ability were collected: socio-demographic characteristic (gender, age, educational and marital status and living area), length of driving experience, inclination to traffic violations and accidents, the presence of chronic diseases, the use of driving impairing medicines, alcohol and narcotic drugs and understanding the warning symbol on the outer package of medicines were collected as independent variables.

Dependent variables explored in the study were professional drivers' knowledge about influence of alcohol and narcotics, medicines that may impair driving, specific groups of medicines (medicines used in treatment of psychiatric, neurological, cardiovascular, gastrointestinal and infectious diseases, medicines used in treatment of severe pain, allergies, flu and colds and with an effect the vision or hearing), including non-proscription medicines, herbal remedies and dietary supplements, as well as knowledge about adverse drug reactions that affect the ability to drive, the system of labeling outer and inner medicines package as well as patients informational leaflet and information that should be given to drivers when issuing driving impairing medicines.

Statistical analysis

The predicted power of the study (1-ß) was 95%, error type 1 (α) was 5%. The minimum number of participants, calculated using Gpower 3.1 (effect size f2 = 0.15, number of predictors 16) was 204. All calculations in this study were performed by SPSS software, version 20. Before performing statistical analysis, the normality of the data distribution was examined by Kolmogorov-Smirnoff test. Data about the basic characteristics of the respondents were processed using descriptive statistics. For each item in the questionnaire, mean score, standard deviation and variance were calculated. Reliability and internal consistency of the questionnaire were established by calculating Cronbach's alpha coefficient and split half Cronbach's alpha. Kruskal-Wallis test was performed in order to compare respondents' knowledge scores in different groups formed on the basis of their demographic characteristics, driving behavior, health status, using of medicines on daily basis, potentially factors that may influence knowledge about driving impairing medicines. With this test, we obtained an indication whether the knowledge of the professional drivers statistically significantly differed in certain groups. Statistically significant results were those in which the probability of null hypothesis was less than 5% ($p<0.05$).

RESULTS

In the study, 168 (76,02%) professional drivers participated from Serbia and 53 (23,98%) from Bosnia and Herzegovina, based on availability of participants for the study. The average age of the participants was 42,82 years (ranging from 21 to 65, standard deviation 11,26). Male sex was 216 (97,74%) of respondents, and only 5 (2,26%) were females. Response rate in the study was 94,01%. From 235 respondents at the beginning, 221 completed the study.

The knowledge of participants about driving impairing medicines, specific groups of medicines, including non-proscription medicines, herbal remedies and dietary supplements, as well as knowledge about labeling system of this medicines and adverse drug reactions that affect driving ability, was estimated by QPDK-IMDA. The average score related to the drivers' knowledge was 131,58 (range from 49 to 175; standard deviation 32,12; median 141,00). Cronbach's Alpha for the QPDK-IMDA was 0,984 (split half Cronbach's Alpha was 0,972 and 0,973). The main characteristics of the respondents and the results of comparing their knowledge scores in different groups formed on the basis of demographic characteristics are given in the Table 1.

Table 1. The main characteristics of the respondents

		FREQUENCY	PERCENTAGE	KRUSKAL-WALLIS test p	MEDIAN
GENDER	Male	216	97,74	0,648	141,00
	Female	5	2,26		133,00
EDUCATION LEVEL	Unfinished primary school	5	2,26	0,000	73,00
	Primary school	41	18,55		77,00
	High school	122	55,20		143,00
	Higher school	38	17,19		150,00
	Faculty	15	6,79		146,00
AGE	From 20 to 29	29	13,12	0,000	144,00
	From 30 to 39	65	29,41		144,00
	From 40 to 49	61	27,60		145,00
	From 50 to 59	45	20,36		86,00
	From 60 to 65	21	9,50		94,00
MARITAL STATUS	Not married (without children)	50	22,62	0,003	144,50
	Not married (with children)	19	8,60		131,00
	Married (without children)	16	7,24		147,00
	Married (with children)	134	60,63		139,50
	Other	2	0,90		147,00
LIVING AREA	Urban	97	43,89	0,000	147,00
	Suburban	48	21,72		141,50
	Rural	76	34,39		117,50
COUNTRY	Serbia	168	76,02	0,085	140,50
	Bosnia and Herzegovina	53	23,98		145,00
CITY	Šabac	120	54,30	0,000	140,00
	Belgrade	19	8,60		146,00
	Vranje	29	13,12		137,00
	Brod	16	7,24		124,00
	Derventa	15	6,79		145,00
	Brcko	22	9,95		163,50

The results of comparing the scores of professional drivers' knowledge in different groups formed on the basis of their driving behavior, health status, using of medicines on daily basis, potentially factors that may influence professional drivers' knowledge, are given in the Table 2.

Table 2. Potentially factors that may influence professional drivers' knowledge

		FREQUENCY	PERCENTAGE	KRUSKAL-WALLIS test p	MEDIAN
How long have you got a driving license?	Less than 1 year	0	0,00	0,000	
	1 to 5 years	9	4,07		141,00
	5 to 10 years	20	9,05		145,50
	10 to 20 years	76	34,39		145,50
	More than 20 years	116	52,49		133,50
Do you drink alcohol?	Yes	106	47,96	0,000	146,00
	No	115	52,04		127,50
How often do you drink alcohol?	Frequently (once per week or more)	34	32,08	0,000	71,50
	Occasionally (once per 2 months)	64	60,38		136,50
	Rarely (less than once per 2 months)	8	7,55		147,00

		FREQUENCY	PERCENTAGE	KRUSKAL-WALLIS test p	MEDIAN
Have you ever been driving under the influence of alcohol?	Yes, only once	24	10,86	0,000	148,00
	Yes, 2 to 5 times	30	13,57		137,50
	Yes, 5 to 10 times	8	3,62		141,00
	Yes, more than 10 times	56	25,34		121,00
	No	103	46,61		80,50
Have you ever been driving under the influence of a psychoactive substance?	Yes, only once	3	1,36	0,000	142,00
	Yes, 2 to 5 times	6	2,71		125,00
	Yes, 5 to 10 times	2	0,90		114,50
	Yes, more than 10 times	10	4,52		126,50
	No	200	90,50		66,50
Which psychoactive substances you used?	Medicines	12	85,72	0,264	70,00
	Narcotic drugs	1	7,14		136,00
	Other	1	7,14		66,00
Have you ever been treated for alcohol and / or psychoactive substance addiction?	Yes	8	3,62	0,000	141,00
	No	213	96,38		69,00
Have you ever had a traffic accident?	Yes, only once	67	30,32	0,000	145,00
	Yes, 2 to 5 times	35	15,84		139,00
	Yes, 5 to 10 times	10	4,52		131,00
	Yes, more than 10 times	1	0,45		79,50
	No	108	48,87		68,00
Have you made traffic violations so far and have you been punished by the competent state authorities?	Yes, only once	37	16,74	0,000	142,00
	Yes, 2 to 5 times	58	26,24		148,00
	Yes, 5 to 10 times	23	10,41		144,50
	Yes, more than 10 times	46	20,81		138,00
	No	57	25,79		75,00
Do you suffer from a chronic illness?	Yes	45	20,36	0,000	142,00
	No	176	79,64		85,00
Which chronic illnesses have respondents?	Neurological and psychiatric diseases	9	20,00	0,097	77,00
	Cardiovascular diseases	30	66,67		82,00
	Other diseases	6	13,33		142,50
Do you use prescribed therapy daily?	Yes	40	18,10	0,000	142,00
	No	181	81,90		82,00
Does the respondent in therapy have a driving impairing medicine?	Yes, only one	15	37,50	0,006	73,00
	Yes, several	7	17,50		68,00
	No	18	45,00		140,50
Do you recognize the warning symbol on the outer package of medicines?	Yes	72	32,58	0,000	133,00
	No	149	67,42		151,00

Driving impairing medicines were used by 9,95% of all participants. Among 18,10% of participants who were used prescribed therapy on daily basis, 37,50 have one, 17,50% have several, while 45,00% didn't have any driving impairing medicines. The most common was the use of anxiolytics (18 drivers) and antidepressants (7 drivers). Anxiolytic benzodiazepines were represented in 45,00% of the respondents who use the therapy every day. Detailed results of driving impairing medicines in daily therapy of study participants are given in the Table 3.

Table 3. Drugs that may impair driving in daily therapy of study participants

GROUP	INN	FREQUENCY	PERCENTAGE (all participants)	PERCENTAGE (participants who use daily therapy)
ANXIOLYTICS	alprazolam	8	3,62	20,00
	bromazepam	5	2,26	12,50
	diazepam	4	1,81	10,00
	prazepam	1	0,45	2,50
ANTIDEPRESSANTS	sertraline	2	0,90	5,00
	venlafaxine	2	0,90	5,00
	maprotiline	1	0,45	2,50
	paroxetine	1	0,45	2,50
	amitriptyline	1	0,45	2,50
ANTIEPILEPTICS	clonazepam	2	0,90	5,00
HYPNOTICS	midazolam	1	0,45	2,50
	zolpidem	1	0,45	2,50
ANTIMIGRENICS	sumatriptan	2	0,90	5,00
PROPULSIVES	metoclopramide	1	0,45	2,50
CENTRAL ANTIHYPERTENSIVE	methyldopa	1	0,45	2,50

The vast majority of respondents were aware that the driving under the influence of alcohol and psychoactive substances is considered as violation according to the Act about road traffic safety. The fact that alcohol influence psychophysical abilities and the ability to drive motor vehicle or machines knew 84,1% of respondents, while 86,4 of them knew for such effects of narcotic drugs. About quarter of professional drivers wasn't familiar with fact that some medicines may influence driving ability (22,6%) and that some medicines have strongly influence on psychophysical ability and the ability to drive motor vehicle or machines (24,9%). Similar results were for medicines used in the treatment of psychiatric (25,3%), neurological diseases (32,1%) and severe pain (34,4%). The influence of medicines from other groups on driving ability was not so familiar: medicines used in the treatment of allergies, flu and cold (52,0%), infectious (66,5%), cardiovascular (59,7%) and gastrointestinal diseases (74,7%).

A high percentage of respondents didn't know that medicines that are dispensed without a medical prescription (64,7%), herbal remedies (73,3%) and dietary supplements (76,9%) can also influence psychophysical abilities and the ability to drive motor vehicle or machines. The influence of medicines that affects eyesight or hearing was known for 65,2% and 55,2% of respondents.

Majority of study participants knew that medications that have adverse drug reactions such as drowsiness, dizziness and mood swings (82,8%) and medications that reduce the power of observation, the power of reasoning and the rate (76,5%) of reaction can influence psychophysical abilities and the ability to drive motor vehicle or machines.

Only 32,6% of professional drivers recognized warning symbols on the outer package of medicines (Δ, ▲, §), while more than half didn't know that medicines labeled with warning symbols Δ (50,7%), ▲ (52,5%), § (66,1%) are not allowed to be used immediately before or during driving. Majority of them (81,0%) agreed that medicines that may adversely influence the psychophysical abilities and the ability to drive motor vehicles and machines should be marked with clearer and more understandable symbols on the outer package, 83,8% agreed that in the patient's informational leaflet must be such information, while 72,0% agreed that medicines that are dispensed without a medical prescription, herbal remedies and dietary supplements, should be adequately labeled, by warning symbols, as medicines with a prescription regimen.

The most of respondents thought that it was necessary to contact a health professional for further information about driving impairing medicines (80,1%), must read patients' informational leaflet in the drugs box (76,9%) and that health professional should give the additional information and educational leaflet (72,9%) during the process of dispensing or selling those medicines. Also, three quarters of them (75,1%) considered that it is necessary to periodically conduct educational campaigns in the written and electronic media about the negative impact of medicines on driving ability.

Over 70% of surveyed professional drivers agreed that they should never drive motor vehicles or machines under the influence of alcohol, driving impairing medicines and narcotic drugs (78,7%), if they feel tired, stressed, have fever or severe pain (74,2%). Also, they agreed that if drowsiness, dizziness, vision or hearing disturbances are felt during driving, it is necessary to immediately discontinue driv-

ing (79,2%) and not try to remove those symptoms by using energy and /or alcoholic beverages (74,7%).

DISCUSION

Our study has shown that professional drivers' knowledge about driving impairing medicines, specific groups of medicines, including non-proscription medicines, herbal remedies and dietary supplements, as well as knowledge about labeling system of this medicines and adverse drug reactions that affect driving ability is insufficient. Also, we found that there is a statistically significant difference between professional drivers' knowledge scores in different groups formed on the basis of their demographic characteristics, driving behavior, using of medicines on daily basis and recognition of warning symbols. This study also demonstrated that usage of driving impairing medicines in the examined population of drivers is high.

About quarter of participators in this study were unaware that some medicines may influence or strongly affect psychophysical abilities and ability to drive. This is much better than in the study of Kagashe i Saleman (30), where more over the half is not familiar with those adverse effects of medicines. The problem may be, that a high percentage of participants in the study don't know that a negative impact on the driving ability can be the result of the use of medicines from groups for which it is unexpected to have such effects, like medicines used in the treatment of allergies, flu and cold, infectious, cardiovascular and gastrointestinal diseases, medicines that are dispensed without a medical prescription, herbal remedies, dietary supplements and medicines that affects eyesight or hearing. Studies conducted by Del Rio and Alvarez (6) and Girotto et al. (25) have confirmed that drivers often use medicines from these groups for acute problems or chronic illness. The full extent of impaired driving due to prescription drug use has yet to be elucidated, especially with prescription agents that are not traditionally thought of as impairing (7). The number of professional drivers who knew that medicines labeled by warning symbols (Δ,▲,§) have driving impairment as adverse effects was not satisfactory. Respondents who were recognized warning symbols have high score of knowledge than those who don't. This indicates the necessity of changing the labeling system of driving impairing medicines and implements more understandable symbols that clearly indicate that the drugs have a negative impact on driving (35-37), with which most respondents agreed. Proper warning labels can be useful as a support tool to provide tailor-made information to patients consuming DIMs (38). They also think that information about driving impairment must be indicated in the patients' information leaflet and that all driving impairing medicines must be labeled in the same way. More than 70% of professional drivers in our study agreed that health professionals are relevant source of information about driving impairing medicines, which is slightly better than in the study of Smyth et al. (39). In the study of Kagashe and Saleman (30), about half of participants said that they had never been warned on the effects of medicines on driving. Similar results were shown in a study done by Brooke et al. (40) where patients were not informed about their fitness to drive. Study by Laaksonen et al. (41) shown that patients want to be informed about their medications, the risks of their administration and adverse effects. So it is the responsibility of the pharmacist to attract the attention of the patient during the provision these medicines, to inform and educate them. (11). Dispensing support tools with information on the potential impairing effect of a medicine on the fitness to drive increases awareness, reported risk communication behavior as well as knowledge of pharmacists on this topic (42). Respondents mostly support the launch of educational campaigns in the written and electronic media about the negative impact of medicines on driving ability, with the aim of raising awareness of all categories of drivers on this topic. A systematic review done by Elder et al. (43) confirmed that mass media campaigns that are carefully planned, well executed, attain adequate audience exposure, and are implemented in conjunction with other ongoing prevention activities, are effective in reducing alcohol-impaired driving and alcohol-related crashes. Over one in five respondents didn't agree with fact that they shouldn't operate motor vehicles and machines if they feel physically or emotionally unable to drive. This is slightly better than in the study of Alonso et al. (3), where 37.5% decided to use the vehicle although they were not in perfect conditions to drive.

A statistically significant difference between professional drivers' knowledge scores were found in groups based on participants age, level of education, marital status, living area and city of living. Also, results shown difference in groups formed by length of driving experience, drivers' behavior (use of alcohol, narcotic drugs, inclination to traffic violations and accidents), the presence of chronic diseases, the use of driving impairing medicines and understanding the warning symbol on the outer package of medicines. Only country, sex, type of used narcotic drugs and types of chronic diseases as grouping factor did not show any difference in drivers' knowledge. This is similar with results of studies of Monteiro et al. (31) and MacLennan et al. (32), who also showed that drivers' knowledge depends on age and educational level. Studies of Okamura et al. (26) and Elayeh et al. (44) demonstrated that less knowledge about medication and road safety, were suggestive of higher likelihood of at-fault crash involvement.

About 10% of all participants use driving impairing medicines. More over the half (55%) among drivers who used prescribed therapy on daily basis have at least one, while 17,5% used two or more driving impairing medicines. These data support the fact that drivers are not sufficiently informed about the effects of medication on driving, which can negatively contribute to traffic safety. In the study Girotto et al. (25), with professional drivers, 72.9% and 18.6% used one and two drugs, respectively. In the study Smith et al. (39), 73.2% of respondents used some of the driving impairing medicines, and 56.1% used them in the previous year, while in the study Kelley-Baker et al. (14),

almost 20% of respondents used some of the driving impairing medicines in the previous two days, all of this was found in the general population of drivers. About 8% of all respondents and almost the half of respondents who used therapy every day has anxiolytic benzodiazepines, which is significantly more than in study by conducted Labat et al. in France (45), where only 0,4% used these medications and in study of Herrera-Gómez at al. (46), where 1,13% of drivers used these medicines every day. This is probably due to the excessive presence of benzodiazepines in therapy. Study done by Drummer and Yap showed a tendency for benzodiazepine-positive drivers to have an increased crash risk (47).

The limitation of the study

This study has the characteristic constraints that have cross-sectional studies. The questionnaire used in the study was not previously validated (validation of the questionnaires was performed during this study). Limitations of this study could be in the domain of interpreting the results. According to our knowledge, very few studies have attempted to address the drivers' knowledge and attitudes about the effects of medicines on the ability to drive and use machines, as well as the analysis of factors that influence the knowledge and the attitude of professional drivers, so adequate comparisons with other research is limited. One of the limitations of the study could be the sincerity of the participants during research and their random sampling for the research.

CONCLUSION

Driving under the influence of alcohol, narcotics and driving impairing medicines is an important risk factor for the safety of traffic, especially for professional drivers; however, their knowledge about this important issue is not satisfactory. The questionnaire developed in this study could help to identify drivers who are at increased risk for using potentially impairing medicines. Health professionals are in a position to ensure that patients fully understand the risk of impaired driving, to increase driver awareness of the negative effects of medicines on driving ability and by providing additional information to medicinal drug users may prevent driving under the influence of medicines.

CONFLICT OF INTEREST STATEMENT

We have no conflicts of interest to disclose.

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REFERENCES

1. Kowalski K, Jeznach A, Tuokko HA. Stages of driving behavior change within the Transtheoretical Model (TM). J Safety Res 2014; 50: 17-25.
2. Gutierrez-Abejón E, Herrera-Gómez F, Criado-Espegel P, Alvarez FJ. Use of driving-impairing medicines by a Spanish population: a population-based registry study. BMJ Open 2017; 7(11): e017618.
3. Alonso F, Esteban C, Sanmartín J, Useche SA. Consistency between the subjective perception of feeling indisposed, the decision to drive and driving performance. Sci J Public Health 2016; 4(6): 482-8.
4. Driver and Vehicle Licensing Agency. Assessing Fitness to Drive–a Guide for Medical Professionals. London: Department for transport; 2016.
5. Haghi A, Ketabi D, Ghanbari M, Rajabi, H. Assessment of human errors in driving accidents; analysis of the causes based on aberrant behaviors. Life Sci J 2014; 11(9): 414-20.
6. Del Río MC, Alvarez FJ. Medication and fitness to drive. Pharmacoepidemiol Drug Saf. 2003; 12(5): 389-94.
7. Sigona N, Williams KG. Driving under the influence, public policy, and pharmacy practice. J Pharm Pract 2015; 28(1): 119-23.
8. Brady JE, Li G. Trends in alcohol and other drugs detected in fatally injured drivers in the United States, 1999–2010. Am J Epidemiol 2014; 179(6): 692-9.
9. Rudisill TM, Zhu M, Kelley GA, Pilkerton C, Rudisill BR. Medication use and the risk of motor vehicle collisions among licensed drivers: A systematic review. Accid Anal Prev 2016; 96: 255-70.
10. Kelly E, Darke S, Ross J. A review of drug use and driving: epidemiology, impairment, risk factors and risk perceptions. Drug Alcohol Rev 2004; 23: 319-44.
11. Duquet N. Drugs and driving [Conduite et médicaments]. J Pharm Belg 2013; (2): 4-11.
12. Verster JC, Roth T. Drivers can poorly predict their own driving impairment: a comparison between measurements of subjective and objective driving quality. Psychopharmacology 2012; 219(3): 775-81.
13. Hetland A, Carr DB. Medications and impaired driving. Ann Pharmacother 2014; 48(4): 494-506.
14. Kelley-Baker T, Waehrer G, Pollini RA. Prevalence of Self-Reported Prescription Drug Use in a National Sample of U.S. Drivers. J Stud Alcohol Drugs 2017; 78(1): 30-8.
15. Walsh JM, Verstraete AG, Huestis MA, Mørland J. Guidelines for research on drugged driving. Addiction 2008; 103(8): 1258-68.
16. Ramaekers JG. Drugs and Driving Research in Medicinal Drug Development. Trends Pharmacol Sci 2017; 38(4): 319-21.
17. Albrecht M. The "Driving under the Influence of Drugs, Alcohol and Medicines"(DRUID) project of the European Commission. Dtsch Med Wochenschr 2008; 133 Suppl 2: S45-6.
18. European Monitoring Centre for Drugs and Drug Addiction. Driving Under the Influence of Drugs, Alcohol and Medicines in Europe — findings from the DRUID project. Luxembourg: Publications Office of the European Union; 2012.

19. Act about road traffic safety. Official Gazette of Republic of Serbia. 2018; 24.
20. Newnam S, Greenslade J, Newton C, Watson B. Safety in occupational driving: Development of a driver behaviour scale for the workplace context. Appl Psychol-Int Rev 2011; 60: 576–99.
21. Davey J, Wishart D, Freeman J, Watson B. An application of the driver behaviour questionnaire in an Australian organisational fleet setting. Transport Res Part F Traffic Psychol Behav 2007; 10(1): 11-21.
22. Poulter DR, Chapman P, Bibby PA, Clarke DD, Crundall D. An application of the theory of planned behaviour to truck driving behaviour and compliance with regulations. Accid Anal Prev 2008; 40(6): 2058-64.
23. Vearrier D, Vearrier L, McKeever R, Okaneku J, LaSala G, Goldberger D, McCloskey K. Issues in driving impairment. Dis Mon 2016; 62(4): 72-116.
24. Girotto E, Mesas AE, de Andrade SM, Birolim MM. Psychoactive substance use by truck drivers: a systematic review. Occup Environ Med 2014; 71(1): 71-6.
25. Girotto E, Guidoni CM, González AD, Mesas AE, Andrade SM. Continued use of drugs and working conditions among truck drivers. Cien Saude Colet 2016; 21(12): 3769-76.
26. Okamura K, Fujita G, Kihira M, Kosuge R. Patterns of use, knowledge, and perceived effects of sedating medication on driving: a questionnaire survey of Japanese drivers who use sedating medication. Transport Res Part F Traffic Psychol Behav 2018; 54: 276-89.
27. Williamson A. Predictors of psychostimulant use by long-distance truck drivers. Am J Epidemiol 2007; 166(11): 1320-6.
28. Mir MU, Razzak JA, Ahmad K. Commercial vehicles and road safety in Pakistan: exploring high-risk attributes among drivers and vehicles. Int J Inj Contr Saf Promot 2013; 20(4): 331-8.
29. Couper FJ, Pemberton M, Jarvis A, Hughes M, Logan BK. Prevalence of drug use in commercial tractor-trailer drivers. J Forensic Sci 2002; 47(3): 562-7.
30. Kagashe G, Seleman K. Knowledge, attitude and practice of commercial drivers in Dar es Salaam with regard to medicines that impair driving. Trop J Pharm Res 2009; 8(4): 297-302.
31. Monteiro SP, van Dijk L, Verstraete AG, Alvarez FJ, Heissing M, de Gier JJ. Predictor for patient knowledge and reported behaviors regarding driving under the influence of medicines: a multi-country survey. BMC Public Health 2012; 12: 59.
32. MacLennan PA, Owsley C, Rue LW, McGwin G. Older adults' knowledge about medications that can impact driving Washington DC, United States of America: AAA foundation for traffic safety; 2009.
33. Veldhuijzen DS, Van Wijck AJM, Verster JC, et al. The impact of patients' psychotropic drug knowledge and warning labels on the decision whether to drive a car or not. Traffic Inj Prev 2006; 7(4): 360-4.
34. DeVellis RF. Scale Development: Theory and Applications (Applied Social Research Methods), 3rd edition. London: SAGE publications; 2011.
35. Ravera S, Monteiro SP, de Gier JJ, van der Linden T, Gómez-Talegón T, Alvarez FJ; DRUID Project WP4 Partners. A European approach to categorizing medicines for fitness to drive: outcomes of the DRUID project. Br J Clin Pharmacol 2012; 74(6): 920-31.
36. Fierro I, Gómez-Talegón T, Alvarez FJ. The Spanish pictogram on medicines and driving: The population's comprehension of and attitudes towards its use on medication packaging. Accid Anal Prev 2013; 50: 1056-61.
37. Monteiro S, Huiskes R, van Dijk L, van Weert J, de Gier J. How effective are pictograms in communicating risk about driving-impairing medicines? Traffic Inj Prev 2013; 14(3): 299-308.
38. Emich B, van Dijk L, Monteiro SP, de Gier JJ. A study comparing the effectiveness of three warning labels on the package of driving-impairing medicines. Int J Clin Pharm 2014; 36(6): 1152-9.
39. Smyth T, Sheehan M, Siskind V. Hospital outpatients' responses to taking medications with driving warnings. Traff Inj Prev 2013; 14(1): 18-25.
40. Brooke BT, Southward RD. An audit on advice on fitness to drive during accident and Emergency Department attendance. Emerg Med J 2006; 23(2): 103-4.
41. Laaksonen R, Duggan C, Bates I. Desire for information about drugs: relationships with patients' characteristics and adverse effects. Pharm World Sci 2002; 24(5): 205-10.
42. Legrand SA, Boets S, Meesmann U, Verstraete AG. Medicines and driving: evaluation of training and software support for patient counselling by pharmacists. Int J Clin Pharm 2012; 34(4): 633-43.
43. Elder RW, Shults RA, Sleet DA, Nichols JL, Thompson RS, Rajab W; Task Force on Community Preventive Services. Effectiveness of mass media campaigns for reducing drinking and driving and alcohol-involved crashes: a systematic review. Am J Prev Med 2004; 27(1): 57-65.
44. Elayeh E, Bulatova N, Basheti I, Abu Farha R, Al-Rawi N, Abu Snaineh A,Alahwal I. The use and safety of medications known to affect driving in Jordan: A cross-sectional study. Traffic Inj Prev 2016; 17(3): 238-44.
45. Labat L, Fontaine B, Delzenne C, Doublet A, Marek MC, Tellier D, Tonneau M,Lhermitte M, Frimat P. Prevalence of psychoactive substances in truck drivers in the Nord-Pas-de-Calais region (France). Forensic Sci Int 2008; 174(2-3): 90-4.
46. Herrera-Gómez F, Gutierrez-Abejón E, Criado-Espegel P, Álvarez FJ. The Problem of Benzodiazepine Use and Its Extent in the Driver Population: A Population-Based Registry Study. Front Pharmacol 2018; 9: 408.
47. Drummer OH, Yap S. The involvement of prescribed drugs in road trauma. Forensic Sci Int 2016; 265: 17-21.

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SOCIOECONOMIC DISPARITIES IN THE SELF-PERCEIVED ORAL HEALTH, MISSING TEETH AND DENTURES IN THE ADULT POPULATION OF SERBIA

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ABSTRACT

Oral health is integral part of general health. The aim of this study was to assess the self-perceived oral health, presence of dentures and missing teeth in the adult population of Serbia and to determinate the socio-economic inequalities. The study represents a secondary analysis of data obtained from the fourth National Health Survey of the Republic of Serbia. For the purpose of this study, data of 12.439 adult populations aged 20 years and older was used. Descriptive methods were used to display the data and chi-square test was used to compare differences between different groups. All results where the probability is less than 5% were considered statistically significant. Self-perceived oral health of the highest percentage of respondents was rated as good, almost every third respondent lacks 1 to 5 teeth and most common dental restoration was total dentures. Condition of teeth and gums was assessed as very bad, more often by male respondents, aged 65 and over, married, lowest education and poorest. Regarding the lack of teeth females reported the lack of all teeth, in age group 65 and over, who were married. All types of dentures were more often used by women, married or cohabiting, while implants were the most common in respondents aged 35 to 64. Implementation of educational programs and preventive measures would contribute in raising awareness of importance of oral health in older age groups. The findings of our study suggest that actions should address socioeconomic factors in order to reduce health inequalities.

Keywords: *Self-perceived oral health, socioeconomic characteristics, National Health Survey of Serbia.*

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INTRODUCTION

Oral health refers to health of the teeth, gums, and entire oral-facial system. Good oral health is in direct relation with eating, speaking, communication, and also influence self-confidence. It is under the influence of many internal and external factors, such as diseases, type of diet, bad habits, drugs and medication and many more, so the proper prevention is necessary to be involved in each age group, focusing on keeping the oral health on the highest level (1).

Oral diseases are a major public health problem for countries and populations worldwide, although they are often not publicly recognized as such. Oral diseases are a group of different disease entities with their own etiology and burden, and different possibilities for their prevention, care and rehabilitation (2).

Those diseases are not discriminating towards the gender, income or country development, but they do tend to be more prominently common in the developing countries because the public health system often does not include the dental health insurance (3,4). There are broad diapason of these diseases that could lead to loss of one or more teeth, like dental caries (in any type of dentition), paradontopathy, gum diseases, but also the injures in the sport and other accidents (5). Lost teeth, anterior or the posterior brings along problems with self-esteem, mastication, and esthetic is endangered due to the visibility of lost anterior teeth. So there are many components that must be observed. It is showed that missing teeth can impact the older and younger people in different ways where older group associate losing teeth with aging and younger group decrease social interaction due to poorer estetics (6).

Untreated caries of permanent teeth is the most widespread dental disease, followed by paradontopathy and those are the main reasons of why people have poor self-perception about their dental health (7). Dental caries is the chronic disease of had tissue of the teeth that with different symptomatology. It is not uncommon to be combined with the soft tissue problems, like gingivitis (gum disease) and combination of those two deepened the symptoms that lead to gum bleeding, bad smell, and overall increase the poor oral hygiene. People of any age with these problems will have issues in interaction with other people, at their job, self-esteem and eventually that can, in the long term, influence their mental health, and lot of studies links it with depression, anxiety and lost teeth with lover cognition levels (8).

For example, the parodontopathy is the disease that usually follows the older age, especially generalised parodontopathy. This leaves elderly people missing the group of teeth or all of them. The car accidents are statistically more common in the younger and middle age people and sport accidents and dental caries are more common in the adolescents and high school children. Loosing teeth can happen anytime, and treatments options may differ from age groups, but the common fact is that the missing teeth must be replaced due to short-terms and long-term complications (9). There are different therapies for replacing missing teeth, and they can vary from dental bridge and dentures to the implant therapy. Depending of the personality type, those therapy plans can be differently accepted and many people, because of the way of how they perceived replacement of lost teeth can be very problematic and not realistic in their demands (10). That's why many older people choose not to wear the dentures, decline the therapy options or demand the implant therapy, although usually there are not indicated for demanded therapy.

Most oral diseases are preventable through self-care or simple, evidence-based and cost-effective population-wide measures, including action on the broader social, economic and political determinants of health, enabling significant reductions in disease burden and limiting negative impacts (11).

The dramatic increase in the global number of oral disease cases represents a huge burden on health systems. The main causes of oral health inequalities are often complex and linked to historical, country-specific, economic, cultural, social or political factors. The conditions in which people are born, grow, live, work and age and the structural drivers of those conditions - the unfair distribution of power, money and resources in society - are the basic social determinants of inequality in oral health (12).

The aim of this study was to assess the self-perceived oral health, the presence of dental prosthetics and missing teeth number in the adult population of Serbia and determinate the socio-economic inequalities within.

MATERALS AND METHODS

The study represents a secondary analysis of data obtained from the fourth National Health Survey of the Republic of Serbia, which was conducted by the Ministry of Health of the Republic of Serbia in accordance with the recommendations for the implementation of the European Health Survey in 2019. (13).

The research was conducted as a descriptive, cross-sectional analytical study on a representative sample of the population of Serbia. The primary target population consisted of all persons aged 15 and over living in private (non-institutional) households in the Republic of Serbia. The excluded persons were the ones living in collective households (student dormitories, dormitories for children and youth with disabilities, homes for socially endangered children, retirement homes for seniors, care homes for the elderly and infirm, adult disability homes, monasteries, convents, etc.). Stratification was performed according to the type of area (urban and other) and the four regions: the Belgrade region, the Vojvodina region, the Sumadija and Western Serbia region, and the Southern and Eastern Serbia region. For the purpose of this study, data on the adult population aged 20 years and older were used and total of 12.439 participants were included.

.Ethical standards in the Health Research of the Serbian population are in accordance with the international Declaration of Helsinki, adopted at the General Assembly of the World Medical Association in 1964, and improved by amendments as of 2013, as well as the legislation of the Republic of Serbia. In order to respect the privacy of the research subjects and the confidentiality of the information collected about them, all necessary steps were taken in accordance with the General Data Protection Regulation (GDPR), a new European legal framework that prescribes the way to use citizens' personal data, as well as with the national Law on the Protection of Personal Data, the Protection Strategy personal data and the Law on Official Statistics with the application of the principle of statistical confidentiality.

The research instruments were: a household info panel, which was used to collect information about all household members, i.e. about the socio-economic characteristics of the household itself, and a questionnaire for self-completion.

For the purposes of this research, the following socioeconomic variables were used: gender, age, marital status and region, education, employment status, and well-being index, while the variables related to oral health were self-rated oral health, missing teeth, and the presence of dentures.

All data of interest are presented and analyzed by adequate mathematical-statistical methods appropriate for the data type. Chi-square test ($\chi 2$) test was applied to test the difference in the frequency of categorical variables while the descriptive statistics of numerical variable was used for presenting the mean and standard deviation and categorical variables were presented as percentage. Multinomial logistic regression was also performed. All results with the probability that is equal to, or less than 5% ($p \leq 0.05$) were considered statistically significant. Statistical analysis was performed using a commercial, standard IBM SPSS software package Version 19.0. (The Statistical Package for the Social Sciences software) (Version 19.0., SPSS Inc., Chicago, IL).

RESULTS

The research was conducted in 2019, during which a total of 12.439 people aged 20 and over were surveyed. The average age of all subjects was 52.8±17.7 years, with women being significantly older than men. The largest number of respondents were married or cohabiting (63.2%) and from the region of Šumadija and Western Serbia (32.0%). The largest number of respondents completed secondary school (56.4%), whereby women significantly more often had primary and lower education than men ($p<0.001$). The material condition of the respondents, assessed on the basis of the welfare index, shows that the largest percentage belongs to the poor category (40.4%). When it comes to the work status of the respondents, it turns out that almost two thirds of the respondents (61.4%) were unemployed or inactive. Men were employed significantly more often (42.9%) than women (32.2%) ($p<0.001$). The sociodemographic characteristics of the respondents by gender and overall are shown in Table 1.

Table 1. Socidemographic characteristics by gender and total

Variables	Gender				Total		p
	Male		Female				
	n	%	n	%	n	%	
Mean age ±Standard deviation	51.7±17.5		53.8±17.8		52.8±17.7		<0.001
Age groups							
20-34	1260	20.9	1149	17.9	2409	19.4	<0.001
35-44	991	16.4	958	15.0	1949	15.7	
45-54	991	16.4	998	15.6	1989	16.0	
55-64	116	18.7	1261	19.7	2387	19.2	
65-74	1072	17.8	1225	19.1	2297	18.5	
75+	592	9.8	816	12.7	1408	11.3	
Marital status							
Marriage/cohabiting	3941	65.3	3903	61.1	7844	63.2	<0.001
Never married or cohabiting	1409	23.4	830	13.0	2239	18.0	
Divorce. separation or death of partner	671	11.1	1659	26.0	2330	18.8	

Variables	Gender				Total		p
	Male		Female				
	n	%	n	%	n	%	
Region							
Belgrade region	1363	22.6	1549	24.2	2912	23.4	0.108
Region of Vojvodina	1343	22.3	1450	22.6	2793	22.5	
Sumadia and West Serbia region	1977	32.8	2000	31.2	3977	32.0	
South and East Serbia region	1349	22.4	1408	22.0	2757	22.2	
Education level							
Elementary school or less	1174	19.5	1896	29.6	3070	24.7	<0.001
High school	3739	62.1	3270	51.0	7009	56.4	
Higher school or Faculty	1112	18.5	1240	19.4	2352	18.9	
Index of wellbeing							
Poor and poorest layer	2414	40.0	2608	40.7	5022	40.4	0.334
Middle layer	1206	20.0	1319	20.6	2525	20.3	
Rich and wealthy layer	2412	40.0	2480	38.7	4892	39.3	
Employment status							
employed	2586	42.9	2062	32.2	4648	37.4	<0.001
unemployed	1168	19.4	1103	17.2	2271	18.3	
Inactive/retired	2171	36.0	3192	49.8	5636	43.1	
Total	6032	48.5	6407	51.5	12439	100	

In the self-assessment of oral health (condition of the mouth and teeth), the largest number of respondents rated it as good (32.7%), followed by average (22.9%), bad (18.9%), and very good (14.5%).
Regarding the lack of teeth, almost every third respondent lacks 1 to 5 teeth (31.7%), while 17.6% of respondents have all teeth. Every fourth respondent (23.0%) stated that they did not have more than 10 teeth, while every ninth respondent did not have any teeth (11.2%).
Total dentures are the most common dental restoration that the largest number of our respondents have (14.6%), followed by partial dentures that 11.9% of respondents have, 9.4% of them have fixed dental restorations, and only 3.4% have implants.
If we look at the self-assessment of the condition of the teeth and gums in relation to the demographic characteristics of the respondents in Table 2, it is noted that the condition of the teeth and gums is assessed as very bad to a slightly higher degree by male respondents (6.1%), ($p<0.001$), aged 65 and over (64.0%), ($p<0.001$), who are in a married/cohabiting (58.9%), ($p<0.001$), and who were from the Šumadija region and Western Serbia (31.5%), ($p<0.001$). Also, it is noted that the condition of the teeth and gums is assessed as very bad to the greatest degree by respondents with the lowest education (54.5%), ($p<0.001$), the poorest (55.9%) ($p<0.001$), and inactive in terms of work status (77.5%), ($p<0.001$).

Table 2. Self-perceived oral health in adult population according to the socio-demographic characteristics

Variables	Self-perceived health of teeth and gums												p
	No answer		Very good		Good		Average		Bad		Very bad		
	n	%	n	%	n	%	n	%	n	%	n	%	
Gender													
Male	309	4.8	904	14.1	2081	32.5	1463	22.8	1262	19.7	388	6.1	<0.001
Female	363	6.0	897	14.9	1990	33.0	1385	23.0	1094	18.1	303	5.0	

Variables	Self-perceived health of teeth and gums												p
	No answer		Very good		Good		Average		Bad		Very bad		
	n	%	n	%	n	%	n	%	n	%	n	%	
Age groups													
20-34	209	31.7	990	55.0	962	23.6	183	6.4	59	2.5	6	0.9	<0.001
35-44	97	14.7	404	22.4	950	23.3	335	11.8	144	6.1	19	2.7	
45-54	94	14.3	194	10.8	798	19.6	545	19.1	305	12.9	51	7.4	
55-64	81	12.3	127	7.1	673	16.5	732	25.7	598	25.4	173	25.0	
65-74	56	8.5	62	3.4	502	12.3	719	25.2	733	31.1	221	32.0	
75+	122	18.5	24	1.3	186	4.6	334	11.7	517	21.9	221	32.0	
Marital status													
Marriage/ cohabiting	307	46.9	897	50.0	2736	67.3	1981	69.7	1506	63.9	411	58.9	<0.001
Never married or cohabiting	220	33.6	810	45.1	814	20.0	241	8.5	129	5.5	24	3.5	
Divorce. separation or death of partner	128	19.5	88	4.9	513	12.6	620	21.8	720	30.6	255	37.0	
Region													
Belgrade region	124	18.8	619	34.4	973	23.9	628	22.1	435	18.5	129	18.7	<0.001
Region of Vojvodina	193	29.3	389	21.6	879	21.6	598	21.0	549	23.3	181	26.2	
Sumadia and West Serbia region	144	21.9	506	28.1	1456	35.8	947	33.3	701	29.8	218	31.5	
South and East Serbia region	198	30.0	287	15.9	763	18.7	675	23.7	671	28.5	163	23.6	
Education level													
Elementary school or less	207	31.6	91	5.1	566	13.9	766	26.9	1060	45.0	376	54.5	<0.001
High school	339	51.8	1076	59.8	2535	62.3	1684	59.1	1093	46.4	276	40.0	
Higher school or Faculty	109	16.6	633	35.2	970	23.8	397	13.9	203	8.6	38	5.5	
Index of wellbeing													
Poor and poorest layer	297	45.1	534	29.7	1403	34.5	1188	41.7	1211	51.4	386	55.9	<0.001
Middle layer	111	16.8	338	18.8	869	21.3	585	20.5	484	20.5	133	19.2	
Rich and wealthy layer	251	38.1	929	51.6	1799	44.2	1075	37.7	661	28.1	172	24.9	
Employment status													
employed	229	35.3	1023	57.6	2046	50.8	836	29.8	433	18.6	78	11.4	<0.001
unemployed	116	17.9	330	18.6	836	20.8	522	18.6	391	16.8	76	11.1	
Inactive/retired	303	46.8	422	23.8	1142	28.4	1146	51.6	1509	64.7	531	77.5	

Regarding the lack of teeth (including implants), it is noted that 62.9% of female persons reported the lack of all teeth, significantly more often than men who reported the lack of teeth in 37.3%, followed by respondents of the oldest age group 65 and over (74.5%), who were married/cohabiting (54.5%) and who were from Vojvodina (30.7%). Looking at the lack of all teeth (including implants) according to the socioeconomic characteristics of the respondents, we see that they are most often respondents with the lowest education (50.5%), from the poorest (46.4%) and inactive in terms of work status (83.5%) (Table 3).

Table 3. Missing teeth (including implants) in adult population according to socio-demographic characteristics

Variables	Missing teeth (including dental implants)												p
	No answers		I have all natural teeth		I'm missing 1-5 teeth		I'm missing 6-10 teeth		I'm missing more than 10 teeth but still have some of mine		I don't have any natural teeth		
	n	%	n	%	n	%	n	%	n	%	n	%	
Gender													
Male	62	53.0	1124	51.3	2054	52.1	959	49.7	1315	45.9	518	37.1	<0.001
Female	55	47.0	1065	48.7	1888	47.9	970	50.3	1551	54.1	878	62.9	
Age groups													
20-34	6	5.1	1288	58.8	1003	25.4	71	3.7	35	1.2	6	0.4	<0.001
35-44	11	9.4	479	21.9	109	27.7	241	12.5	109	3.8	19	1.4	
45-54	17	14.5	233	10.6	852	21.6	439	22.8	378	13.2	70	5.0	
55-64	32	27.4	118	5.4	605	15.3	573	29.7	790	27.6	269	19.3	
65-74	34	29.1	49	2.2	314	8.0	441	22.5	952	33.2	507	36.9	
75+	17	14.5	22	1.0	78	2.0	164	8.5	602	21.0	525	37.6	
Marital status													
Marriage/ cohabiting	77	67.5	1029	47.2	2659	67.6	1404	72.9	1916	66.9	759	54.5	<0.001
Never married or cohabiting	11	9.6	1056	48.5	860	21.9	141	7.3	127	4.4	44	3.2	
Divorce, separation. death of partner	26	22.8	94	4.3	416	10.6	381	19.8	823	28.7	590	42.4	
Region													
Belgrade region	75	64.1	712	32.5	871	22.1	452	23.4	508	17.1	294	21.1	<0.001
Region of Vojvodina	20	17.1	458	20.9	843	21.4	358	18.6	686	23.9	428	30.7	
Sumadia and West Serbia region	10	8.5	689	31.5	1314	33.3	698	36.2	916	32.0	350	25.1	
South and East Serbia region	12	10.3	330	15.1	914	23.2	421	21.8	756	26.4	324	23.2	
Education level													
Elementary school or less	20	17.9	135	6.2	518	13.1	487	25.2	1206	42.1	704	50.5	<0.001
High school	55	49.1	1305	59.6	2515	63.8	1190	61.7	1374	48.0	570	40.9	
Higher school or Faculty	37	33.0	748	34.2	909	23.1	252	13.1	285	9.9	121	8.7	
Index of wellbeing													
Poor and poorest layer	31	26.5	685	31.3	1509	38.3	758	39.3	1391	48.5	648	46.4	<0.001
Middle layer	27	23.1	442	20.2	759	19.3	417	21.6	582	20.3	298	21.3	
Rich and wealthy layer	59	50.4	1062	48.5	1674	42.5	754	39.1	893	31.2	450	32.2	
Employment status													
employed	37	31.9	1230	57.1	2164	55.7	629	33.2	469	16.5	119	8.6	<0.001
unemployed	18	15.5	427	19.8	917	23.6	373	19.7	427	15.0	109	7.9	
Inactive/retired	61	52.6	498	23.1	806	20.7	895	47.2	1949	68.5	1154	83.5	

The presence of dental restorations in the adult population, according to demographic characteristics, is such that all types of prostheses are more often worn by women, married or cohabiting, from the region of Šumadija and western Serbia, while in terms of age, the oldest age group of 65 and over most often had total or partial dentures and fixed prosthesis, while implants were more often done in respondents aged 35 to 64 (69.2%). According to socioeconomic characteristics, the presence of

dental restorations in the adult population was most often in respondents with secondary education. Total dentures were most often worn by the poorest (41.5%), while partial, fixed and implants were most often worn by wealthiest respondents (40.8%, i.e. 51.9% and 54.6%), while when it comes to work status, inactive ones mostly have total dentures (81.0%) had partial dentures (66.1%), while respondents who were employed mostly had implants (Table 4).

Table 4. Occurrence of teeth recoupments in the adult population according to the socio-demographic characteristics

Variables	Occurrence of teeth recoupments											
	Total removable dentures		p	Partial removable dentures		p	Fixed dentures - bridge		p	Implants		p
	Yes	No		Yes	No		Yes	No		Yes	No	
Gender												
Male	35.0	50.6	<0.001	37.4	49.6	<0.001	41.4	48.7	<0.001	44.9	48.0	<0.001
Female	65.0	49.4		62.6	50.4		58.6	51.3		55.1	52.0	
Age groups												
20-34	0.2	13.3	<0.001	1.4	12.6	<0.001	0.3	14.2	<0.001	9.2	11.0	<0.001
35-44	1.9	17.1		5.4	15.9		1.8	16.2		21.2	14.1	
45-54	6.8	19.4		14.2	17.7		5.8	18.5		22.4	16.9	
55-64	22.2	22.0		29.7	20.8		23.2	23.1		25.6	21.9	
65-74	36.3	18.8		33.1	20.1		33.6	19.0		15.8	22.2	
75+	32.6	9.4		16.3	13.0		35.3	9.2		5.9	13.8	
Marital status												
Marriage/cohabiting	59.1	68.2	<0.001	71.1	65.9	<0.001	72.3	65.9	<0.001	71.5	66.4	<0.001
Never married or cohabiting	2.9	13.5		3.1	13.0		5.9	12.3		13.2	11.5	
Divorce, separation. death of partner	38.8	18.3		25.7	21.2		21.8	21.8		15.3	22.1	
Region												
Belgrade region	21.2	21.4	<0.001	25.3	20.7	<0.001	34.3	19.7	<0.001	20.2	21.4	<0.001
Region of Vojvodina	27.0	21.9		26.2	22.2		17.3	23.5		25.2	22.7	
Sumadia and West Serbia region	33.6	31.8		28.2	32.8		31.1	32.3		36.0	32.0	
South and East Serbia region	18.3	24.9		20.3	24.3		17.4	24.5		18.6	23.9	
Education level												
Elementary school or less	43.9	25.4	<0.001	28.6	28.7	<0.001	15.7	30.3	<0.001	15.3	29.2	<0.001
High school	44.9	58.0		55.8	55.7		58.9	55.3		56.7	55.7	
Higher school or Faculty	11.2	16.6		15.6	15.6		25.4	14.4		28.0	15.1	
Index of wellbeing												
Poor and poorest layer	41.5	42.5	<0.001	36.8	43.3	<0.001	27.5	44.2	<0.001	30.6	42.8	<0.001
Middle layer	22.2	19.9		22.4	20.0		20.6	20.3		14.8	20.6	
Rich and wealthy layer	36.4	37.6		40.8	36.8		51.9	35.5		54.6	36.6	

Variables	Occurrence of teeth recoupments											
	Total removable dentures		p	Partial removable dentures		p	Fixed dentures - bridge		p	Implants		p
	Yes	No		Yes	No		Yes	No		Yes	No	
Employment status												
employed	10.9	38.7	<0.001	22.3	35.7	<0.001	37.3	33.3	<0.001	51.7	33.0	<0.001
unemployed	8.1	20.4		11.6	19.3		13.0	18.9		16.7	18.3	
Inactive/retired	81.0	40.9		66.1	45.0		49.7	47.8		31.7	48.8	

Multinomial logistic regression analysis was performed to see the predictors of poor oral health with variables of dental status - different types of dentures and number of missing teeth. We concluded that statistically significant predictors of self-perceived poor oral health were people wearing total dentures, partial dentures, fixed prosthetics, implants and people missing 1-5, 6-10 and more than 10 teeth (p = 0.00). People wearing total and partial dentures were 2.2 (OR = 2.2) and 1.7 (OR = 1.7) times more likely to self-perceived health as bad. Also, people wearing fixed prosthetics and implants also were more likely to perceived oral health as bad (OR = 1.3 and OR = 4.0). Missing teeth are also contributing in a large amount in how people self-perceive oral health, mainly people missing 1 to 5, 6 to 10 and more than 10 teeth with odds ratio 25.3, 13.8 and 4.5 more likely (Table 5).

Table 5. Predictors of poor self-perceived oral health using multinomial logistic regression

Self-perceived poor oral health	B	Std. Error	Wald	df	Sig.	OR	95% CI for OR	
							Lower Bound	Upper Bound
Total denture	0.8	0.1	43.9	1.0	0.00	2.2	1.8	2.8
Partial denture	0.6	0.1	13.7	1.0	0.00	1.7	1.3	2.3
Fixed prostatic	0.2	0.2	1.1	1.0	0.30	1.3	0.8	1.9
Implant	1.4	0.6	5.2	1.0	0.00	4.0	1.2	13.0
Missing 1-5 teeth	3.2	0.4	64.5	1.0	0.00	25.3	11.5	55.6
Missing 6-10 teeth	2.6	0.2	135.6	1.0	0.00	13.8	8.8	21.4
Missing more than 10 teeth	1.5	0.1	155.3	1.0	0.00	4.5	3.5	5.7

*reference category is very poor

DISCUSSION

As the starting point of digestion and place where the most “estetic” organs are placed - teeth, oral cavity is of a great importance in various aspects of people’s everyday life and overall health. Socioeconomic factors are the ones that are mostly investigated regarding the influence on the good oral health, and poorer people with lower education, and also the elderly people are more endangered of have oral health issues and are prone to losing their teeth due to multiple comorbidities that are following the older age (14, 15). In Daviovic results, female, of the age between 35 and 65+ were more from urban living place had more like hood of having dentures (16). This findings are similar to ours where we also determinate female gender, in slighter older age groups 55-75, mostly married with high school level of education as the ones more commonly wearing the dentures - either partial or total.

Good health system definitely increases the better oral health if it’s included in the healthcare system. But this is something that is problem even in the more developed countries, and the countries that are developing are the ones mostly in the problem due to combination of all named factors (17). That’s why the global situation of the dental status is not so bright in developing countries. They tend to have higher incidence and prevalence of gum and hard tissue diseases and this is consequentially followed with the teeth loss (18). Losing only one tooth can be problematic because it may cause the movement of other teeth and can influence the therapy procedures of patients. Lesser number of lost teeth is something more common among younger population, where the leading cause of teeth loss are traumas due to accidents, fights and hard teeth tissue disease, like dental caries (19). One in five adults aged 20-64 years had one or more permanent teeth with untreated decay (20). The mean number of missing teeth increases with age, from 0.7 teeth at 20-34 years to 7.4 at 75 years and older. More than 1 in 10 adults aged 65-74 years had lost all their teeth and prevalence of edentulous among adults increased from 1.2% at 35-49 years to 19.7% at 75 years or older (21). In the study of Milosavljevic et al, the highest average number of missing teeth

among the military insured's was in the group of 35-44 years and it was roughly 6.5 while the most missing teeth in that age group was one to five (22). In our adult population, the highest percentage of people missed 1 to 5 teeth (31.2%) and number of teeth did decrease with age of participants and 37.5% of people older than 75 were edentulous. The soft tissue disease, paradontopathy, of oral cavity are more common in older and elderly people, and in combination with their current health status where certain medicaments and oral health conditions like xerostomia can increase degradation of oral health (23).

Study of Davidovic et al showed more than one third of their respondents describe their oral health as poor and very poor and over the half of them reported missing from one to ten teeth. Especially the younger adults, without partner, economically more stable and with a higher level of education perceived their oral health as good and they also had more natural teeth present (16). This is in line with our results, nearly one third or respondents described their oral health as poor or very poor (24.5%) and nearly half (47.2%) reported missing one to ten teeth. Lost teeth lead to problems with mastication and digestion, appearance, self-esteem, problems with temporomandibular joint, in case they are not treated properly and on time (24). There are also studies that investigated the impact of tooth loss to the people's cognitive degradations (25). Nowadays there are many different options to compensate teeth loss, form dental bridges over the partial and total dentures to the many different options for dental implants, but still there are many disparities in the choice therapies in adults. Dental implants are becoming more utilized in the older adults even they are costly, because of their long-term durability (26). Half of the older adults, over the age of 65 had nearly 25% of dentures as their rehabilitation therapy for their lost teeth by Daviovic results (16). The most common therapy in rehabilitant lost teeth in our study was partial and total prosthetics - 26.5%. In German seniors aged 65-74 years was noted declining number of missing teeth from late 90s up to early 2000 and more than 35% had removable dentures and had on average 10 remaining tooth and higher incidence of periodontal diseases in comparison to the younger people (27, 28).

Although there is much on-going improvements in oral health, many people still suffer from chronic oral conditions and lack of access to the dental care they need which is also the situation in Serbia. Poor dental care affects the health care system and influence and dental health needs of people in the lower-income countries tend to overpower the number of physicians and increase the out of pocket spending (29).

Health of oral mucosa and hard tissues of teeth are crucial in keeping the ideal balance between function and aesthetics, and that is something that often may be underrated. Disease of teeth and oral mucosa are also connected to many different diseases and can influence the psychological aspects and quality of life (30, 31). That's why prevention programs an education about their health must be implemented from early age, due to the different surrounding factors that also influence the opinions of individuals.

Socio-demographic inequalities in self-perceived oral health and dental status are present in the older adults, especially ones age 65 and more in Serbia. Reducing the inequalities among older people, economically less stable and educated can impact their oral health status and dental status which tend to be poorer. This will have impact to the national and individual economic burdens (32, 33).

Limitations of this study are that the factors of losing teeth are not included. It would be helpful to determinate the relationships between dentures, implants and missing teeth with most common reasons for their utilization/losing. Conducting additional research on this topic would be useful in future. Also, the data is limited only to people older than 20 years, where all named socioeconomic factors can influence the younger population self-perceived oral health too, as well as the missing teeth.

CONCLUSION

Self-perceived oral health of the highest percentage of respondents was rated as good, almost every third respondent lacks 1 to 5 teeth and most common dental restoration was total dentures. Condition of teeth and gums was assessed as very bad, more often by male respondents, aged 65 and over, married, lowest education and poorest. Regarding the lack of teeth females reported the lack of all teeth, in age group 65 and over, who were married. All types of dentures were more often used by women, married or cohabiting, while implants were the most common in respondents aged 35 to 64. Statistically significant predictors of self-perceived poor oral health were people wearing total dentures, partial dentures, fixed prosthetics, implants and people missing 1-5, 6-10 and more than 10 teeth. Given that numerous factors from the social and physical environment determine the choices of individuals, the prevention of oral diseases requires a multisectoral and multidisciplinary approach that combines the promotion of healthy lifestyles with activities that affect social and economic determinants, as well as the physical environment. The best results would be achieved by combining population strategies and strategies aimed at the most vulnerable categories of the population. Implementation of educational programs and preventive measures would contribute to raising awareness of the importance of oral health in older age groups. The findings of our study suggest that actions should address socioeconomic factors in order to reduce health inequalities.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

REFERENCES

1. Štefanová E, Baška T, Holubčíková J, Timková S, Tatarková M, Sovičová M, et al. Selected Behavioural Factors Affecting Oral Health in Schoolchildren: Results from the Health Behaviour in School-Aged Children (HBSC) Slovak Study. Int J Environ Res Public Health. 2020;17(20):7516. doi: 10.3390/ijerph17207516.
2. Peres MA, Macpherson LMD, Weyant RJ, Daly B, Venturelli R, Mathur MR, et al. Oral diseases: a global public health challenge. Lancet. 2019;394(10194):249-260. doi: 10.1016/S0140-6736(19)31146-8.
3. Bastani P, Mohammadpour M, Mehraliain G, Delavari S, Edirippulige S. What makes inequality in the area of dental and oral health in developing countries? A scoping review. Cost Eff Resour Alloc. 2021;19(1):54. doi: 10.1186/s12962-021-00309-0.
4. Singh A, Peres MA, Watt RG. The Relationship between Income and Oral Health: A Critical Review. J Dent Res. 2019;98(8):853-860. doi: 10.1177/0022034519849557
5. Rösing CK, Randall C, Giacaman RA. Editorial: Dental caries and periodontal diseases as non-communicable chronic diseases. Front Oral Health. 2023;3:1113029. doi: 10.3389/froh.2022.1113029.
6. Sabharwal A, Stellrecht E, Scannapieco FA. Associations between dental caries and systemic diseases: a scoping review. BMC Oral Health. 2021;21(1):472. doi: 10.1186/s12903-021-01803-w.
7. Wen PYF, Chen MX, Zhong YJ, Dong QQ, Wong HM. Global Burden and Inequality of Dental Caries, 1990 to 2019. J Dent Res. 2022;101(4):392-399. doi: 10.1177/00220345211056247.
8. Tiwari T, Kelly A, Randall CL, Tranby E, Franstve-Hawley J. Association Between Mental Health and Oral Health Status and Care Utilization. Front Oral Health. 2022;2:732882. doi: 10.3389/froh.2021.732882.
9. Grusovin MG, Coulthard P, Worthington HV, George P, Esposito M. Interventions for replacing missing teeth: maintaining and recovering soft tissue health around dental implants. Cochrane Database Syst Rev. 2010;2010(8):CD003069. doi: 10.1002/14651858.CD003069.pub4.
10. Hiltunen K, Vehkalahti MM, Mäntylä P. Is prosthodontic treatment age-dependent in patients 60 years and older in Public Dental Services? J Oral Rehabil. 2015;42(6):454-9. doi: 10.1111/joor.12267.
11. Barranca-Enríquez A, Romo-González T. Your health is in your mouth: A comprehensive view to promote general wellness. Front Oral Health. 2022;3:971223. doi: 10.3389/froh.2022.971223.
12. Duangthip D, Chu CH. Challenges in Oral Hygiene and Oral Health Policy. Front Oral Health. 2020;1:575428. doi: 10.3389/froh.2020.575428.
13. European Health Interview Survey (EHIS wave 3), Methodological Manual, Eurostat, (2018). Available at: https://ec.europa.eu/eurostat/web/products-manuals-and-guidelines/-/KS-02-18-240. (Accessed 15 December, 2024).
14. de Abreu MHNG, Cruz AJS, Borges-Oliveira AC, Martins RdC, Mattos FdF. Perspectives on Social and Environmental Determinants of Oral Health. International Journal of Environmental Research and Public Health. 2021;18(24):13429. https://doi.org/10.3390/ijerph182413429
15. Watt RG, Venturelli R, Daly B. Understanding and tackling oral health inequalities in vulnerable adult populations: from the margins to the mainstream. Br Dent J. 2019;227(1):49-54. doi: 10.1038/s41415-019-0472-7.
16. Davidovic M, Jankovic J, Todorovic J, Bjegovic-Mikanovic V. Socioeconomic inequalities in oral health in Serbia: evidence from a cross-sectional study, European Journal of Public Health. 2022;28(4). https://doi.org/10.1093/ eurpub/cky214.064
17. Stepović M, Vekić B, Dragojević Simić V, Vekić S, Rančić N. Forecasting analysis of selected health and economy related indicators in south eastern European and Balkan countries. Vojnosanitetski Pregled, 2022. DOI: https://doi.org/10.2298/VSP210414089S
18. Petersen PE, Bourgeois D, Ogawa H, Estupinan-Day S, Ndiaye C. The global burden of oral diseases and risks to oral health. Bull World Health Organ. 2005;83(9):661-9.
19. Haworth S, Shungin D, Kwak SY, Kim HY, West NX, Thomas SJ, et al. Tooth loss is a complex measure of oral disease: Determinants and methodological considerations. Community Dent Oral Epidemiol. 2018;46(6):555-562. doi: 10.1111/cdoe.12391.
20. National institute of dental and craniofacial research. Dental Caries (Tooth Decay) in Adults (Ages 20 to 64 Years). Available at: https://www.nidcr.nih.gov/research/data-statistics/dental-caries/adults
21. CDC. 2024 Oral Health Surveillance Report: Selected Findings. Available at: https://www.cdc.gov/oralhealth/php/2024-oral-health-surveillance-report/selected-findings.html
22. Milosavljević M, Daković D, Milosavljević A, Jovanović J, Rančić N. Oral health of 20 to 64 years old military insured's measured by DMFT index: A pilot study. Racionalna terapija. 2013;5(1):9-16. doi: 10.5937/racter5-2659.
23. Capodiferro S, Limongelli L, Favia G. Oral and Maxillo-Facial Manifestations of Systemic Diseases: An Overview. Medicina (Kaunas). 2021;57(3):271. doi: 10.3390/medicina57030271.
24. Leck R, Paul N, Rolland S, Birnie D. The consequences of living with a severe malocclusion: A review of the literature. J Orthod. 2022;49(2):228-239. doi: 10.1177/14653125211042891.
25. Galindo-Moreno P, Lopez-Chaichio L, Padial-Molina M, Avila-Ortiz G, O'Valle F, Ravida A, et al. The impact of tooth loss on cognitive function. Clin Oral Investig.

2022;26(4):3493-3500. doi: 10.1007/s00784-021-04318-4.
26. Corovic S, Janicijevic K, Radovanovic S, Vukomanovic IS, Mihaljevic O, Djordjevic J, et al. Socioeconomic inequalities in the use of dental health care among the adult population in Serbia. Front. Public Health. 2023;11:1244663. doi: 10.3389/fpubh.2023.1244663
27. Srinivasan M, Meyer S, Mombelli A, Müller F. Dental implants in the elderly population: a systematic review and meta-analysis. Clin Oral Implants Res. 2017;28(8):920-930. doi: 10.1111/clr.12898.
28. Nitschke I, Wendland A, Weber S, Jockusch J, Lethaus B, Hahnel S. Considerations for the Prosthetic Dental Treatment of Geriatric Patients in Germany. J Clin Med. 2021;10(2):304. doi: 10.3390/jcm10020304.
29. Stepovic M, Vekic S, Vojinovic R, Jovanovic K, Radovanovic S, Radevic S, et al. Analysis and Forecast of Indicators Related to Medical Workers and Medical Technology in Selected Countries of Eastern Europe and Balkan. Healthcare (Basel). 2023;11(5):655. doi: 10.3390/healthcare11050655.
30. Şenel S. An Overview of Physical, Microbiological and Immune Barriers of Oral Mucosa. Int J Mol Sci. 2021;22(15):7821. doi: 10.3390/ijms22157821
31. Stepovic MM, Rajkovic Z, Stajic D, Sekulic M, Maričić M, Djonovic N. Association between depression levels and self-perceived oral health among medical students. Acta Facultatis Medicae Naissensis 2020; 2(37) doi: 10.5937/afmnai37-25122.
32. Dye BA. The Global Burden of Oral Disease: Research and Public Health Significance. J Dent Res. 2017;96(4):361-363. doi: 10.1177/0022034517693567.
33. Shoaee S, Ghasemi E, Sofi-Mahmudi A, Shamsoddin E, Tovani-Palone MR, Roshani S, et al. Global, regional, and national burden and quality of care index (QCI) of oral disorders: a systematic analysis of the global burden of disease study 1990-2017. BMC Oral Health. 2024;24(1):116. doi: 10.1186/s12903-023-03808-z.

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THE IMPACT OF CROSS-CLAMPING OF THE PORTAL TRIAD ON THE MORPHOLOGY OF THE SMALL INTESTINE

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ABSTRACT

At present, clamping of the portal triad is a widespread surgical procedure in hospitals. Such an operation can prevent pathological changes in the organs. However, the optimal time for clamping remains unclear. To determine the starting time of irreversible morphological changes in the small intestine due to the clamping of the portal triad. The study was carried out on rats (n=94). Animals were randomly subdivided into 4 groups based on the duration of clamping of the portal triad (PT): I control group (CG; without clamping the PT; n=10); II intervention group (6-IG; clamping PT for 6 min; n=28); III intervention group (12-IG; clamping time of the PT for 12 min; n=28); IV intervention group (24-IG; clamping time of the PT for 24 min; n=28). In groups 6-IG, 12-IG, 24-IG, after clamping the portal triad, animals were withdrawn from the experiment after 3 hours, 6 hours, 12 hours, 1 day, 3 days and 7 days. Morphological changes in the small intestine were assessed by measuring the diameter of the lumen of micro-vessels. In addition, the mortality in the groups was analysed as well. In the CG group, the diameter of the arterioles of the small intestine was 34±4 µm, the diameters of pre-capillaries were 15±2µm, the capillaries were 5.4±1 µm, the post-capillaries were 18±2 µm, and the diameter of the lumen of the venues was 40±3 µm. In the 6-IG group (on the 3rd day), the structure of the small intestine showed the recovery signs. By the 7th day, the indicators returned to their original values. In the 12-IG group, the parameters of the small intestine were restored on the seventh day that corresponds to the usual course of the disease. However, in the 24-IG group, changes in these organs persisted until the end of the study. No deaths were reported in the CG and 6-IG animal groups. Mortality among rats of the 12-IG group was 14.3%, while in the 24-IG group with PT clamping for 24 minutes it was 42.8%, respectively. The morphological changes in the microvasculature of the small intestine after 6-minute PT clamping showed a tendency to recover (back to the control parameters). Nevertheless, after 24 minutes of clamping, the changes in the intestinal tissue were irreversible.

Keywords: *Clamping of the portal triad; morphological changes; small intestine; ischemic-reperfusion injury.*

INTRODUCTION

Compression of the portal triad (PT) is the classic method of liver blood flow control (1). Clamping of the AT structures is a frequently performed procedure in liver surgery (2). This method has been mainly associated with the surgical treatment of tumours, trauma, or liver transplantation. However, it can lead to the ischemia-reperfusion injury of inner organs (3). Moreover, it can result in changes in liver blood flow and subsequent tissue damage associated with reperfusion. In fact, tissue damage in local and distant regions affects the outcome of liver surgery. It has been shown that it is related to the duration of hypoxia during the intervention (2).

Apart from that, even a short clamping of the PT can cause stagnation in the portal vein, particularly in the superior and inferior mesenteric veins, thereby reducing visceral outflow (4). In turn, a decrease in blood flow in the intestine can lead to damage to enterocytes and loss of the intestinal barrier because of increased pressure in the microvascular network of intestinal structures (5). Moreover, it can trigger apoptosis of hepatocytes and intestinal cells (6). Previous studies have shown the negative effect of portal vein occlusion on mesenteric blood flow. In addition, it was revealed that such an intervention induces pathological changes in the mesenteric microcirculation and intestinal mucosa at an early stage (7).

The morphological structure of the small intestine is well studied and presented in the literature (8). However, there are insufficient data on changes in the intestine during the period of ligation of the liver PT (9). In fact, the elements of the microvascular system are the most important structures, the location of which can be observed in the submucosa of the small intestine (10). It is well known that 70 % of mesenteric blood flow plays a crucial role in the supply of blood to the mucous and submucosal layers of the small intestine. Moreover, only the remaining portion of the blood flow is dedicated to the muscular and serous layers (11). Therefore, in our work, we examined the morphological changes in the submucosal layer.

The optimal time tolerated (hypoxemia) by the liver during surgery is still controversial. Some reports indicate that the duration of intermittent clamping of the PT (that does not cause clinical complications) is 15 minutes (12). However, there are studies indicating the possibility of continuous clamping of the PT without increasing the risk of complications and risk. The duration of continuous clamping can be up to 90 minutes in the absence of liver pathology or up to 50 minutes in the presence of liver disease (13). Kolahdoozan et al. demonstrated in experiments on animals that the safe time interval for clamping the PT was 30–45 minutes (average) (14).

Up to date, the range of studies reported the detailed effect of PT clamping on liver damage, while pathological changes in the small intestine have not been widely covered yet (4). Nevertheless, it was proved, in the experiments on animal model (in rats), that prolonged clamping of the PT (more than 90 min) increases the risk of death due to the development of multisystem organ damage. Particularly, the lungs, heart and intestines are being damaged too (in addition to the liver) (15). In another study, Sheen-Chen et al. demonstrated significant increase of apoptosis in the small intestine in comparison of 15 minutes of PT clamping and 30 minutes of clamping (6). It has been thought that the development of multi-organ damage is associated with the loss of the intestinal barrier function (16).

Preclinical studies indicated a high risk of damage to the small intestine during clamping of the PT. It has been associated with hypo-perfusion of extra-hepatic structures that lead to the manifestation of bacterial translocation and endo-toxinemia (17-19). However, the different modes of PT clamping cannot provide a clear picture for establishing the optimal time interval to prevent irreversible changes in the structure of the small intestine.

Sebe et al. conducted the study on assessing the histological changes of the small intestine during clamping of the PT in rats (10, 20 and 30 minutes). It was determined that significant morphological changes in the structure of the small intestine (especially in the mucous membrane, in addition to the submucosa, muscle and serous layers) appear only after 30 minutes after clamping (20). However, it should be noted that histological materials were taken immediately after performing PT clamping. This circumstance can indicate only about the initial changes after ischemia, so that the main structural tissue damage has not been properly evaluated (after the subsequent period of reperfusion).

In this regard, there is a need for a thorough examination of histological changes in the small intestine in the early and late periods of reperfusion. It can help to investigate the full depth of tissue damage. This study aimed at the determination of the time of occurrence of morphological changes in the small intestine followed by the clamping of the portal triad.

MATERIALS AND METHODS

Ethical issue

The study was carried out in the Laboratory of Experimental Medicine of the NJSC S.D. Asfendiyarov Kazakh National Medical University (KazNMU), Almaty city, the Republic of Kazakhstan. The study was approved by the ethics committee of KazNMU named after S. D. Asfendiyarov (Minutes No. 7 (84) dated 06/10/2019).

Design of the study

The animals were kept in accordance with the international rules "Guide for the Care and Use of Laboratory Animals" (National Research Council, 2011), as well as with the ethical principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 2006)

The experimental study was conducted on 94 outbred 12-week-old male Wistar Albino rats weighing 190-220 g. Animals before and after the operation were kept in the Vivarium of the B. Atchabarov Research Institute of Fundamental and Applied Medicine (Kazakhstan, Almaty) with a standard diet and care. Laboratory animals were randomly assigned to the following 4 groups:

Group I - Control group (CG): intestinal tissue was resected for use as a control without any manipulation of the PT (n = 10);

Group II - Interventional group (6-IG): clamping of the PT for 6 minutes (n = 28);

Group III - Interventional group (12-IG): clamping of the PT for 12 minutes (n = 28);

Group IV - Interventional group (24-IG): PT clamping for 24 minutes (n = 28)

Surgical procedures

The intervention on laboratory animals was conducted in the operating unit of the Laboratory of the Atchabarov Research Institute of Fundamental and Applied Medicine (Kazakhstan, Almaty).

Surgical intervention was performed with a pre-shaved surgical field, under general anaesthesia (ketamine 80 mg / kg + xylazine 10 mg / kg) (6). The dose and time of administration of the drugs were recorded in the experiment log. The rats were fixed on the operating table in the supine position on the back.

A midline laparotomy was performed in 6-IG, 12-IG, 24-IG groups. The PT was ligated according to the standard technique (19) for 6, 12 and 24 minutes, depending on the group of laboratory animals. Successfully performed PT clamping was determined and assessed by the detection of congestion in the intra-abdominal organs. Further, the operating wound was sutured in layers. After this, the animals were returned to their cages, with no restrictions in activity. After surgical intervention, as a postsurgical analgesic the animals were given an intramuscular injection of diclofenac sodium (5 mg/kg).

Laboratory animals of the 6-IG, 12-IG, 24-IG groups were withdrawn from the experiment after 3 hours, 6 hours for 4 animals, after 12 hours, 1 day, 3 days and 7 days for 5 animals using cervical dislocation (21).

Histology

For histological examination, 2 cm pieces of the small intestine were taken (22) in the region of the middle third. After this, they were fixed in 10 % neutral formalin. Then, after fixation in formalin, the tissue of the small intestine was held in alcohol of increasing strength, followed by embedding in paraffin (3, 23).

Microtome sections of the small intestine with a thickness of 4 μm were cut, then stained with haematoxylin and eosin, staining was subsequently conducted on each slide. Interpretation of histological materials was carried out by two independent specialists-histologists. Each slide was scored from 0 to 5 according to the scale by Chiu et al.,where: Grade 0-normal villi; Grade 1- development of subepithelial space; Grade 2- moderate lifting of subepithelial layer; Grade 3-massive epithelial lifting with a few tips denuded; Grade 4-denuded villi with lamina propria and dilated capillaries; Grade 5, digestion and disintegration of lamina propria with ulceration (24).

Determination of changes in the architectonics of the intestine after clamping of the PT was made by filling the vascular basin of rats with a 0.5 % solution of silver nitrate through the aorta followed by preliminary washing the blood vessels with warm (37 ° C) 0.9 % sodium chloride solution according to previously indicated method (25, 26). The manipulations were carried out under the strict control of a manometer, from which filmy clarified preparations were prepared. The diameter of the lumen of micro-vessels was measured with an ocular ruler "MOV-1-15xU4.2.". The division value and calibration of the ocular micrometre were determined using an object micrometre (26).

Microscopic analysis of tissue sections from various experimental groups of animals was performed using a Leica DM 2000 binocular light microscope (Leica Microsystems, Wetzlar, Germany) and digital software (Image-Pro plus 6.0; Media Cybernetics, USA).

Statistical analysis

Statistical analysis of the data obtained was carried out using the SPSS software 22 (USA). The arithmetic mean (M) and standard deviation (SD) were calculated. Data were presented as M ± SD. One-way analysis of variance ANOVA was chosen as a statistical test to determine statistical differences within a group. Differences were considered statistically significant if $p < 0.05$.

RESULTS

Histological evaluation

All laboratory animals were withdrawn from the experiment in accordance with the established protocols of the experiment. In the CG group, data obtained from healthy tissues of the small intestine of laboratory animals were used as a control (to compare with the results of other experimental series). The diameters of arterioles of the small intestine were 34 ± 4 μm, pre-capillaries 15 ± 2 μm, capillaries 5.4 ± 1 μm, post-capillaries 18 ± 2 μm, and the size of the venule lumen was 40 ± 3 μm, respectively.

In the 6-IG group, dilation of capillaries, post-capillaries and venules was observed after 3 hours, which were assessed as statistically significant compared to control values ($p < 0.05$).

After 6 hours, we observed a significant venous plethora along with a pronounced increase in the calibre of venules ($p < 0.05$). In addition, we detected the expansion of arterioles and other links of the microvasculature of the small intestine, which was considered statistically significant in comparison with the control indicators. After 12 hours, all the parts of the microvasculature of the small intestine, except for arterioles ($p > 0.05$), were significantly expanded compared to the control. After 1st day, significant expansion of pre-capillaries, capillaries, post-capillaries and venules ($p < 0.05$) remains the same (compared to the control' values). After 3 days, the diameters of micro-vessels of the small intestine, except for arterioles, pre-capillaries and capillaries, tended to be significantly decreased ($p < 0.05$), compared with the previous observation period obtained 1 day after the intervention. After 7 days, there was a decrease in the diameters of all links of the vascular bed of the small intestine, and it was not statistically significant ($p > 0.05$).

In the 12-IG group, after clamping the PT for 12 minutes, morphological changes in the microvasculature were recorded at 3, 6, 12 hours, 1, 3, and 7 days after surgery (see Table 2). After 3 hours, the diameter of all chains of the microvasculature was significantly expanded ($p < 0.05$). After 6 and 12 hours, dilatation remained in all parts of the vascular system ($p < 0.05$) in comparison with the control group. However, in contrast to the measurements obtained 3 hours after the intervention, a decrease in the diameters of the vascular system was observed ($p < 0.05$). After 1 day, the expansion of the elements of the vascular bed was also preserved in comparison with the control ($p < 0.05$). After 3 days, in addition to arterioles and venules, the diameters of the remaining micro-vessels of the small intestine showed a tendency to decrease, which was regarded as statistically significant ($p < 0.05$). After 7 days, the diameter of all parts of the microcirculatory system of the small intestine decreased in comparison with the indicators obtained 3 days after clamping of the PT. Nevertheless, the decrease in the lumen values of the arterioles and pre-capillaries was without any statistical significance ($p > 0.05$). It must be noted that compared with 6-minute exposure of the PT clamping, the diameter of the micro-vessels remained expanded during the period of the study.

In the 24-IG group, after clamping the PT for 24 minutes, morphological changes in the micro-vasculature were recorded 3, 6, 12 hours, 1.3, 7 days after surgery (see Table 3). After 3 hours, a significant maximum expansion of all parts of the vascular bed is observed in comparison with the control values of the small intestine ($p <0.05$). After 6 and 12 hours, the diameter of the vessels remains significantly dilated ($p <0.05$) in comparison with the indices of intact animals. However, after 12 hours, a decrease in the measured values was observed (in contrast to the data obtained after 6 hours of clamping the PT). After 1 day, the diameters of the micro-vessels of the small intestine had a statistically significant tendency ($p < 0.05$) to decrease in comparison with the indicators recorded 12 hours after surgery. However, the values of the post-capillaries remained dilated without differences from the data recorded after 6 hours compared to the control ($p < 0.05$). After 3 days, the diameter of the microvessels of the small intestine remained significantly enlarged compared to the control values ($p < 0.05$). After 7 days, the diameter of all parts of the microvasculature of the small intestine was decreased compared with the data obtained after 3 days ($p <0.05$). Nonetheless, in contrast to 12-minute PT compression, during the same period, vessels remained relatively expanded. The indices of the vascular bed of the small intestine do not return to the initial values during the entire study.

In the 6-IG group, 3 hours after 6 minutes of PT clamping, narrowed areas of arterioles and pre-capillaries with a weakly expressed capillary network were encountered (Fig. 1, A). After 6 hours, significant venous congestion led to the persistence of excessive tortuosity of capillaries, post-capillaries, and venules (Fig. 1, B). Twelve hours later, tortuosity and dilation of arterioles and pre-capillaries were observed along with a sharp depletion of the capillary system (Fig. 1, C). After 1 day, hyper-chromicity and dilation of capillaries, post-capillaries and venules were persisted (Fig. 1, D). After 3 days, dilated pre-capillaries and capillaries were visible and detectable (Fig. 1, E). After 7 days, moderate visibility of the pre-capillaries and capillaries of the serous and muscular membranes was observed (Fig. 1, F),

In the 12-IG group, 3 hours after acute extrahepatic portal hypertension, the damaged sites showed segmental narrowing of the arterioles and vague capillary network (Fig. 1, G). After 6 hours, there were signs of impaired microvascular permeability (Fig. 1, H). After 12 hours, the post-capillaries and venules remained moderately dilated (Fig. 1, I). After 1 day, the expansion of capillaries, post-capillaries and venules was accompanied by a violation of their permeability with the release of the dyes into the perivascular space (Fig. 1, J). It was confirmed by signs of haemorrhages on the macro specimen. After 3 days, a sharp expansion of the arterioles and pre-capillaries was visible (Fig. 1, K). After 7 days, we observed the expansion of the pre-capillaries and capillaries (Fig. 1, L).

In the 12-IG group, micro-aneurysmal dilatation of the arterioles accompanied by areas of narrowing vessels was detected after 3 hours (Fig. 1, M). After 6 hours, pronounced constriction of arterioles in the area of their origin from the arteries (sphincters) was observed (Fig. 1, N). After 12 hours, there was the uneven expansion of arterioles and pre-capillaries (Fig. 1, O). After 1 day, the arterioles and pre-capillaries of the serous layer were unevenly dilated and arterio-venular anastomoses appeared (Fig. 1, P). After 3 days, the pre-capillaries and the capillary network of the serous and muscular membranes were unevenly stained with silver salts (Fig. 1, Q). Then, after 7 days, there were areas of uneven narrowing of arterioles and pre-capillaries with a defect in filling the capillary network (Fig. 1, R).

In the 12-IG group, 3 hours after 6 minutes of PT clamping, the small intestine was characterized by submucosal

dissection (due to oedema). The structure of the muscle and mucous layers was preserved. In the submucosal layer, there were separate solitary lymph vessel covered with columnar epithelium (Fig. 2, A1). After 6 hours: the swelling of the submucosa increased leading to the compression towards the mucous membrane (Fig. 2, B1). After 12 hours: there was a decrease in the oedema of the submucosa with the appearance of lymphoid infiltration of the mucous membrane (Fig. 2, C1). After 1 day: oedema of the parenchyma of the lymphatic follicle was observed (Fig. 2, D1). After 3 days: a slight swelling of the submucosa and dilatation of vessels remained (Fig. 2, E1). After 7 days: the lymphatic vessels near the follicles were dilated (Fig. 2, F1).

In the 12-IG group, 3 hours after acute extrahepatic portal hypertension, loosening of the muscular membrane and submucosa was detected as a result of interstitial oedema and vasodilatation of the villous lamina propria (Fig. 2, G1). After 6 hours: there was an increase in submucosal oedema with marked swelling of the lymphatic vessel (Fig. 2, H1). After 12 hours: we detected the increase of oedema of the muscular membrane and submucosa (Fig. 2, I1). After 1 day: the expansion of the lymphatic capillaries in the mucous layer and submucosa with round-cell infiltration of the mucous membrane of the small intestine was revealed (Fig. 2, J1). After 3 days: the venules of the submucosa of the small intestine remain dilated (Fig. 2, K1). After 7 days: a sharp oedema of the submucosa of the small intestine persists, the vessels remained dilated (Fig. 2, L1).

In the 24-IG group, after 3 hours, there was a sharp vasodilation in the submucosa and the mucous layer of the small intestine. In addition, we observed the oedema of the lymph node with the expansion of the lymphatic capillaries (Fig. 2, M1). After 6 hours: the oedema and vasodilation of the serous layer and submucosa of the small intestine does not undergo significant changes, there is abundant infiltration of the mucous layer (Fig. 2, N1). After 12 hours: there was swelling of the stroma of the Peyer's patch of the small intestine and dilation of the lymphatic vessels (Fig. 2, O1). After 1 day: pronounced oedema of the submucosa of the small intestine with cellular infiltration persisted. The blood vessels were full-blooded, and the lymphatic vessels were dilated. There was oedema of the lymph node parenchyma (Fig. 2, P1). After 3 days: there was a thickening of the muscular membrane due to diffuse oedema, expansion of lymphatic vessels in the submucosa and abundant lymphocytic infiltration of the latter (Fig. 2, Q1). After 7 days: the oedema of the muscular layer of the small intestine decreased. The oedema of the submucosa, on the contrary, remained pronounced with the expansion of the lymphatic vessels (Fig. 2, R1).

Table 1. Diameter of the links of the microvasculature of the small intestine of experimental rats with clamping of the PT for 6 minutes, depending on the observation time (M ± SD) (μm)

Parts of microcirculatory system	Control	Observation time					
		3 hours	6 hours	12 hours	24 hours (1 day)	72 hours (3 days)	168 hours (7 days)
Arterioles	34±4	39,4±3,2	40±3,1*	39,8±2,8	38,4±2,7	35±2,6	34,5±2,9
Pre-capillaries	15±2	18,7±1,7	19,5±2,5*	20±2,4*	20±2,2*	18±2,1	17±2,0
Capillaries	5,4±1	8,3±0,7*	8,7±0,6*	8,9±0,5*	8,8±0,6*	6,7±0,5	6±0,9
Post-capillaries	18±2	27±2,1*	28±1,7*	29±2,0*	28,5±1,8*	23±1,9*	21,5±1,8
Venules	40±3,5	60±3,9*	61±3,4*	60±3,6*	58±3,4*	50±3,2*	45±3,4
* - $p < 0.05$ - reliability of difference with control							

Table 2. Diameters of the links of the microvasculature of the small intestine of experimental rats with clamping of the PT for 12 minutes, depending on the observation time (M ± SD) (μm)

Parts of microcirculatory system	Control	Observation time					
		3 hours	6 hours	12 hours	24 hours (1 day)	72 hours (3 days)	168 hours (7 days)
Arterioles	34±	42±3,1	40,6±2,7	41±2,8	40±2,7	38,3±2,	36±2,7
Pre-capillaries	15±2	24,5±2,5*	20±2,2*	21±2,1*	22±1,9*	19,5±1,9*	17,6±1,6
Capillaries	5,4±1	10,7±0,7*	9,8±0,5*	9,9±0,6*	9,0±1,0*	8,6±0,7*	8,2±0,6*
Post-capillaries	18±2	32±1,8*	30±1,8*	32±1,7*	33±1,9*	27,6±1,7*	26,8±1,5*
Venules	40±3,5	66±3,3*	58±3,4*	62±3,2*	61±3,4*	56±3,2	54±3,1*
* - $p < 0.05$ - reliability of difference with control							

Table 3. Diameters of the links of the microvasculature of the small intestine of experimental rats with clamping of the PT for 24 minutes, depending on the observation time (M ± SD) (μm)

Parts of micro-circulatory system	Control	Observation time					
		3 hours	6 hours	12 hours	24 hours (1 day)	72 hours (3 days)	168 hours (7 days)
Arterioles	34±4	46±3,3*	44±2,9*	45±3,0*	41±2,6*	40±2,5*	39±2,4
Pre-capillaries	15±2	28±2,7*	24,4±2,6*	22±2,3*	21±2,0*	22±1,8*	21±1,9*
Capillaries	5,4±1	12,6±0,6*	11,2±0,8*	10±0,7*	9,5±0,5*	9,0±0,7*	8,8±0,6*
Post-capillaries	18±2	35±1,9*	32±1,7*	31±1,6*	31±1,8*	32±2,0*	30±1,8*
Venules	40±3,5	72±3,4*	68±3,5*	66±3,4*	62±3,3*	61±3,2*	59±2,9*
* - $p < 0.05$ - reliability of difference with control							

Table 4. Mortality of experimental animals in different study groups

Groups	Experimental model	Operated animals	Lethality of animals (L / N)						Total %
			3h	6 h	12 h	24 h	72 h	168 h	
I	Control	10/ 94	-	-	-	-	-	-	-
II	Clamping PT for 6 min	28/94	-/4	-/4	-/5	-/5	-/5	-/5	-
III	Clamping PT for 12 min	28/94	-/4	-/4	-/5	1/5	1/5	2/5	4/14.3
IV	Clamping PT for 24 min	28/94	1/4	1/4	2/5	2/5	3/5	3/5	12/42.8
L-lethality in each time interval; N is the number of animals participating in the experiment in a certain time interval									

Fig.1. Histological picture of small intestine microcirculation in experimental rats during PT clamping for 6, 12 and 24 minutes, depending on the observation time (A, G, M - after 3 hours; B, H, N - after 6 hours; C, I, O - after 12 hours; D, J, P-1 day; E, K, Q - 3 days; F, L, R-7 days). The silver nitrate staining protocol. Magnification ×200, scale bars (white) 100 μm.

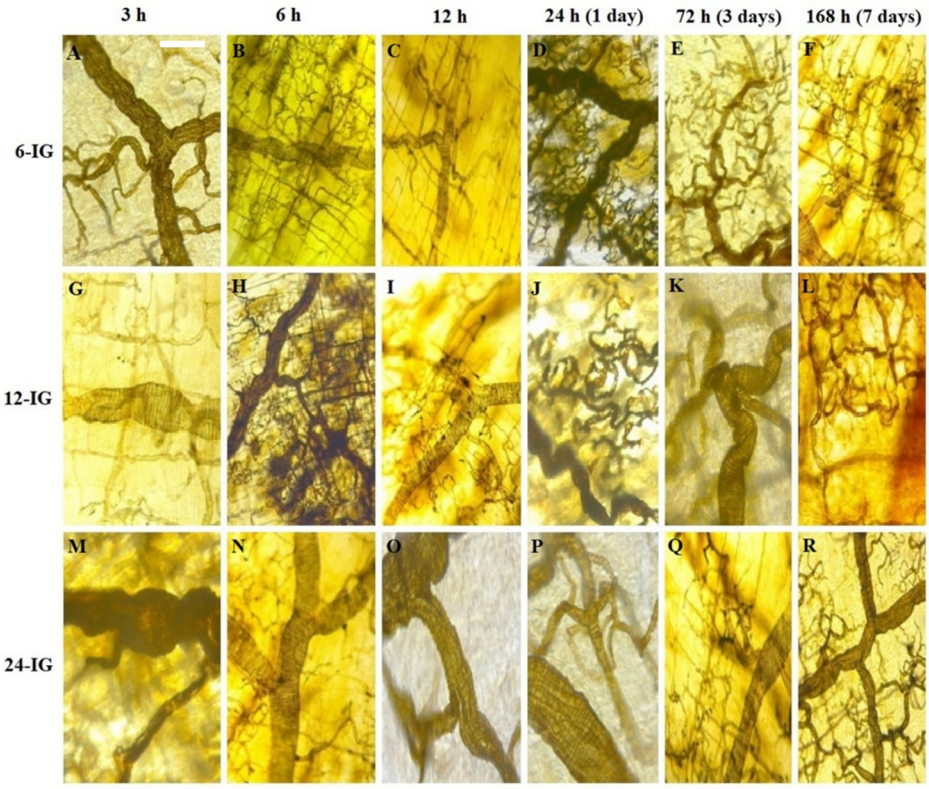
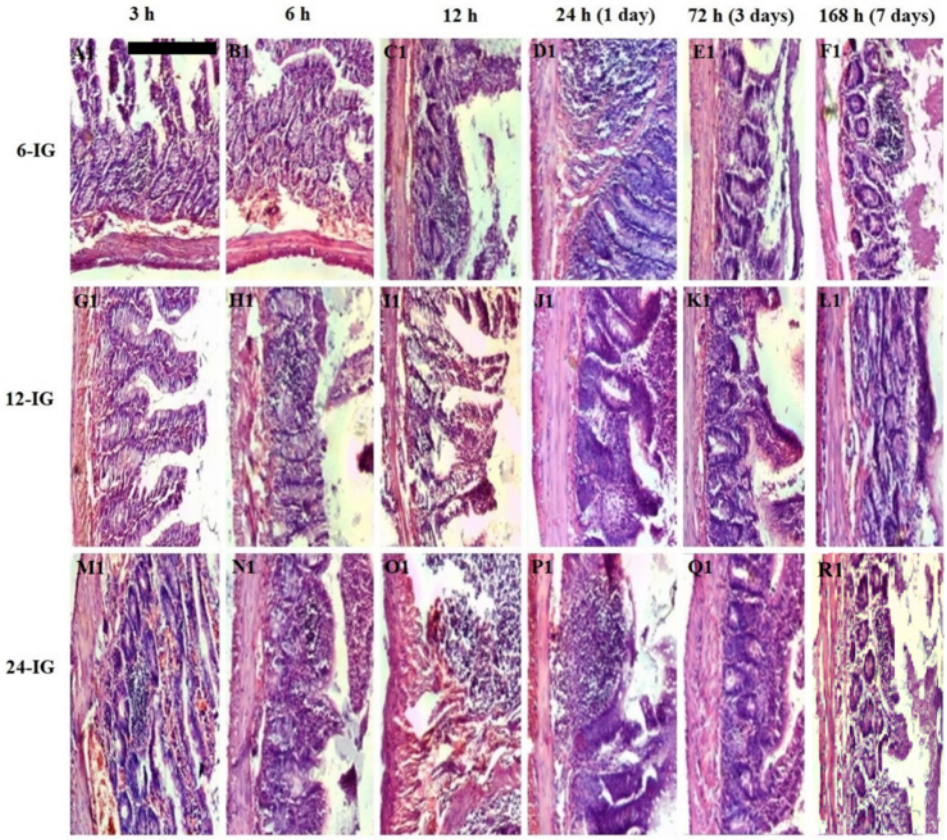


Fig. 2. Histological picture of small intestine microcirculation in experimental rats during PT clamping for 6,12 and 24 minutes, depending on the observation time (A1, G1, M1 - after 3 hours; B1, H1, N1 - after 6 hours; C1, I1, O1 - after 12 hours; D1, J1, P1 - 1 day; E1, K1, Q1 - 3 days; F1, L1, R1 - 7 days). Hematoxylin-eosin. Magnification ×100, scale bars (black) 100 μm.



DISCUSSION

The aim of this study was to elucidate the dynamics of morphological changes in the small intestine after clamping of the PT to determine the exact time of occurrence of irreversible disorders.

In fact, the PT clamping technique is an effective surgical method aimed at reducing bleeding during liver surgery (6). However, the reperfusion (the restoration of blood flow after a period of ischemia) may put ischemic organs at risk of further cell necrosis, and limit the recovery (27).

To reduce the undesirable consequences of this method, several variants of PT clamping were proposed, including ischemia preconditioning, intermittent clamping, and pharmacological preconditioning (4). However, such methods cannot guarantee undesirable complications. Hence, the determination of a safe interval of time clamping remains very important.

According to the results obtained, it can be assumed that the duration of the compression of the PT had a directly proportional effect on the outcome of the experiment. No fatalities were reported in the 6-IG and 12-IG studies. Mortality among rats of the 12-IG group was 14.3 %. The mortality of rats in the 24-IG group with 24 minutes of PT clamping was 42.8 % (Table 4). Previous studies have shown that 80 % of animals who underwent 120-minute PT clamping died from complications such as respiratory distress (34 %), intestinal complications (28 %), and cardiovascular disorders (18 %) (15).

As the matter of fact, disturbance of microcirculation plays a key role in the cascade of reactions associated with damage to intestinal structures. Microcirculatory damage manifests itself in disruption of the endothelium-dependent dilatation of arterioles, a decrease in blood flow due to the accumulation of leukocytes in capillaries and extravasation of leukocytes and plasma proteins in post-capillary venules (26, 27). In addition, one should remember the importance of oxidative stress and inflammatory reactions in the process of disruption and damage to extrahepatic structures (28).

An earlier study has argued that gastrointestinal perfusion disorders during cardiopulmonary bypass surgery can induce the loss of vascular integrity in the small intestine mucosa and an increase in permeability (with leukocyte infiltration). As a result, bacterial translocation and the entry of endotoxin into the systemic circulation triggers the manifestation of

toxic effects and systemic tissue damage (29). In the process of regeneration (after resection), the liver demonstrated a relatively low level of oxidative stress. At the same time, in the intestine, the oxidative damage of proteins is being increased, which contributes to the occurrence of intestinal dysfunction and endotoxin translocation (28). In our study, we observed the effect of hemodynamic disturbance after 6 hours as a result of 12 minutes of PT clamping. The histological evidence of vascular permeability disturbances was obtained. Such morphological changes persisted 1 day after the intervention. It was accompanied by the release of the dye to the perivascular space due to microvascular haemorrhage.

On the other hand, 24 minutes of PT clamping (after 3 days) led to abundant lymphocytic infiltration in the submucosa. These findings showed the development of irreversible morphological changes in the intestinal tissue, depending on the duration of the PT clamping.

In one described clinical case (30), it was indicated that after clamping of the PT in the intestine, there were changes in microcirculation in the serous layer. It was manifested in a decrease in vascular density and perfusion parameters between the initial level and recorded at the end of the operation. The results of video-monitoring demonstrated that dense capillary networks, visible at the initial level, dissipated after occlusion and reperfusion, revealing areas of incorrect perfusion (30). It indicates the appearance of pathological changes. However, according to these data, it is not possible to comprehend the reversibility of changes in the intestinal vessels. Moreover, there are no data on the duration of PT clamping.

In an experiment on rats with PT compression for 90 and 120 minutes, it was proved that necrotizing of mucosal cells manifests on the first day after surgery. Apart from that, the expansion of the spaces between smooth muscle cells in the damaged intestinal wall led to acute oedema of the gastrointestinal tract. In turn, it results in the accumulation of fluid and electrolytic or protein components in the interstitial tissue of the intestinal wall. However, the authors confirmed that on the third postoperative day, the microvilli structure of intestinal epithelial cells was restored (15).

Dello et al. studied the impact of PT clamping (5 and 30 minutes) on intestinal fatty acid binding protein (I-FABP) (a marker of damage to intestinal epithelial cells). It was revealed a significant increase in the concentration of this protein 8 hours after the procedure, which proves damage to the epithelial cells of the small intestines (5). In the context of apoptosis, apoptosis in intestinal crypts was increased significantly 30 min after performing PT clamping (18).

Mechanical stagnation in the hemodynamic of the viscera during the "shutdown" of the liver is known to be the main factor of the damage. Consequently, the pressure in the portal system can rise to 40-50 mm Hg. Art., and the capillaries in the intestinal tissue can undergo hypertensive damage during clamping of the PT (15).

In fact, ischemia with subsequent reperfusion provokes the failure of the mechanisms responsible for the regulation of the width of the lumen of blood vessels, as well as a significant change in the shape and size of the vessels. Since NO is one of the main agents regulating the myogenic tone of the vascular wall, damage to the small intestine can induce the deterioration of endothelial cells and loss of myogenic tone of the mesenteric blood vessels (as a result of NO activity). This feature is especially noticeable at the end of the reperfusion period (31).

We found out that the diameters of the microvasculature vessels tend to return to the initial parameters after clamping the PT for 6 minutes. However, longer periods of clamping of the PT (for 12 and 24 minutes) cannot guarantee the preservation of the restorative function of the vascular wall. Moreover, the expansion of the microvasculature arising in the lumens of the vessels with a sharp swelling of the submucosa becomes irreversible.

Guan et al. carried the study using the model of ischemia-reperfusion syndrome of the small intestine caused by temporary clamping of the mesenteric vessels in a rat. It was revealed that short ischemia with a duration of 15 minutes did not cause complications, while long-term ischemia with a duration of 45-50 minutes was unsafe in terms of preservation of small intestine tissue (32). In another study, a pig was chosen as an animal model to investigate the morphological changes as a result of ischemia. The results of the study showed that irreversible consequences of ischemia-reperfusion syndrome appeared after 4 hours of ischemia with further reperfusion (33). Moreover, the main pathological processes occur predominantly during the reperfusion process. The data obtained undoubtedly indicate the association between the time of ischemia-reperfusion and the duration of this manipulation.

Our experimental study on an animal model showed that changes in the microvasculature and morphological structure of the small intestine directly depend on the duration of acute extrahepatic portal hypertension induced by compression of the PT. We found out that 6-minute clamping of the PT led to the recovery of all control parameters, while a 24-minute clamping led to irreversible changes in the intestinal tissue.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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REFERENCES

1. Smyrniotis V, Farantos C, Kostopanagiotou G, Arkadopoulos N. Vascular control during hepatectomy: review of methods and results. World J Surg. 2005;29(11):1384-96.
2. Furka A, Németh N, Pető K, Szentkereszty Z, Tóth L, Furka I, et al. [Data on liver enzyme and histological changes caused by intermittent clampings of the hepatoduodenal ligament in an experimental model]. Magy Seb. 2013;66(3):166-70.
3. Akdogan RA, Kalkan Y, Tumkaya L, Alacam H, Erdivanli B, Aydin İ. Influence of infliximab pretreatment on ischemia/reperfusion injury in rat intestine. Folia Histochem Cytobiol. 2014;52(1):36-41.
4. Dimitroulis D, Moris D, Pikoulis E, Spartalis E, Kontadakis G, Vrugt B, et al. Variable Pringle Maneuvers and Effect on Intestinal Epithelium in Rats. A Pilot Experimental Study in Rats. PLoS One. 2015; 10(10):e0140707.
5. Dello SA, Reisinger KW, van Dam RM, Bemelmans MH, van Kuppevelt TH, van den Broek MA, et al. Total intermittent Pringle maneuver during liver resection can induce intestinal epithelial cell damage and endotoxemia. PLoS One. 2012;7(1):e30539.
6. Sheen-Chen SM, Su FI, Tang RP, Huang CC, Eng HL. Cellular changes in hepatocytes and intestinal endothelium after hepatoduodenal ligament occlusion and protective effects of caspase inhibition. Ann Surg. 2011;253(3):561-5.
7. Miyakawa S. [Experimental study of the effect of portal vein occlusion on the mesenteric microcirculation]. Nihon Geka Gakkai Zasshi. 1984;85(7):719-28.
8. Sarriá R, Latorre R, Henroteaux M, Henroteaux N, Soria F, Pérez-Cuadrado E, et al. Morphometric study of the layers of the canine small intestine at five sampling sites. Vet J. 2012;192(3):498-502.
9. Jansen-Winkeln B, Tagkalos E, Heimann A, Gaiser T, Hirsch D, Gockel I, et al. Pringle maneuver increases the risk of anastomotic leakage after colonic resection in rats. HPB (Oxford). 2018;20(5):392-7.
10. Miller BG, Woods RI, Bohlen HG, Evan AP. A new morphological procedure for viewing microvessels: a scanning electron microscopic study of the vasculature of small intestine. Anat Rec. 1982;203(4):493-503.
11. Vollmar B, Menger MD. Intestinal ischemia/reperfusion: microcirculatory pathology and functional consequences. Langenbecks Arch Surg. 2011;396(1):13-29.
12. Tralhão JG, Hoti E, Oliveiros B, Abrantes AM, Botelho MF, Castro-Sousa F. Intermittent pringle maneuver and hepatic function: perioperative monitoring by noninvasive ICG-clearance. World J Surg. 2009;33(12):2627-34.
13. van Riel WG, van Golen RF, Reiniers MJ, Heger M, van Gulik TM. How much ischemia can the liver tolerate during resection? Hepatobiliary Surg Nutr. 2016; 5(1):58-71.
14. Kolahdoozan M, Behdad A, Hosseinpour M, Behdad S, Rezaei MT. Evaluation of the Safe Ischemic Time of Clamping During Intermittent Pringles Maneuver in Rabbits. Arch Trauma Res. 2015;4(4):e30244.
15. Liu DL, Jeppsson B, Hakansson CH, Odselius R. Multiple-system organ damage resulting from prolonged hepatic inflow interruption. Arch Surg. 1996;131(4): 442-7.
16. Higuchi S, Wu R, Zhou M, Marini CP, Ravikumar TS, Wang P. Gut hyperpermiability after ischemia and reperfusion: attenuation with adrenomedullin and its binding protein treatment. Int J Clin Exp Pathol. 2008;1(5):409-18.
17. Unno N, Uchiyama T, Yamamoto N, Inuzuka K, Sagara D, Konno H. Portal triad occlusion induces endotoxin tolerance: role of portal congestion. J Surg Res. 2006;135(2):213-7.
18. Filos KS, Kirkilesis I, Spiliopoulou I, Scopa CD, Nikolopoulou V, Kouraklis G, et al. Bacterial translocation, endotoxaemia and apoptosis following Pringle manoeuvre in rats. Injury. 2004;35(1):35-43.
19. Su Y, Pan H, Guo Z, Zhou W, Zhang B. Bacterial translocation and endotoxemia after pringle maneuver in cirrhotic rats. Dig Dis Sci. 2015;60(2):414-9.
20. Sébe AA, Nigro AJT, Gomes PdO, Simões MdJ. Efeitos do clampeamento do pedículo hepático nos intestinos. Acta Cirurgica Brasileira. 2000;15:00-.
21. Donovan J, Brown P. Euthanasia. Current Protocols in Immunology. 2006;73(1):1.8.1-.8.4.
22. Nastos C, Kalimeris K, Papoutsidakis N, Tasoulis MK, Lykoudis PM, Theodoraki K, et al. Global consequences of liver ischemia/reperfusion injury. Oxid Med Cell Longev. 2014;2014:906965.
23. Gaifulina R, Maher AT, Kendall C, Nelson J, Rodriguez-Justo M, Lau K, et al. Label-free Raman spectroscopic imaging to extract morphological and chemical information from a formalin-fixed, paraffin-embedded rat colon tissue section. Int J Exp Pathol. 2016;97(4):337-50.
24. Chiu CJ, McArdle AH, Brown R, Scott HJ, Gurd FN. Intestinal mucosal lesion in low-flow states. I. A morphological, hemodynamic, and metabolic reappraisal. Arch Surg. 1970;101(4):478-83.
25. Haas T, Duling B. Morphology Favors an Endothelial Cell Pathway for Longitudinal Conduction within Arterioles. Microvascular research. 1997;53:113-20.
26. Vyrencov YE, Kataev SE. Microcirculatory bed of the liver according to scanning electron microscopy. Archive of Anatomy, Histology and Embryology. 1983; 84(4):61-70.
27. Carden DL, Granger DN. Pathophysiology of ischaemia-reperfusion injury. J Pathol. 2000;190(3):255-66.
28. Alexandris IH, Assimakopoulos SF, Vagianos CE, Patsoukis N, Georgiou C, Nikolopoulou V, et al. Oxidative state in intestine and liver after partial hepatectomy in rats. Effect of bombesin and neurotensin. Clin Biochem. 2004;37(5):350-6.

29. Dong GH, Wang CT, Li Y, Xu B, Qian JJ, Wu HW, et al. Cardiopulmonary bypass induced microcirculatory injury of the small bowel in rats. World J Gastroenterol. 2009;15(25):3166-72.
30. Shen L, Uz Z, Ince C, van Gulik T. Alterations in intestinal serosal microcirculation precipitated by the Pringle manoeuvre. BMJ Case Rep. 2019;12(1).
31. Gordeeva AE, Sharapov MG, Tikhonova IV, Chemeris NK, Fesenko EE, Novoselov VI, et al. Vascular Pathology of Ischemia/Reperfusion Injury of Rat Small Intestine. Cells Tissues Organs. 2017;203(6):353-64.
32. Guan Y, Worrell RT, Pritts TA, Montrose MH. Intestinal ischemia-reperfusion injury: reversible and irreversible damage imaged in vivo. Am J Physiol Gastrointest Liver Physiol. 2009;297(1):G187-96.
33. Strand-Amundsen RJ, Reims HM, Reinholt FP, Ruud TE, Yang R, Høgetveit JO, et al. Ischemia/reperfusion injury in porcine intestine - Viability assessment. World J Gastroenterol. 2018;24(18):2009-23.

THE PERCEIVED ASSESSMENT OF COVID-19 IMPACT ON MENTAL FUNCTIONING AND SUICIDALITY IN ADULT POPULATION OF SERBIA

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ABSTRACT

The aim of the study was to determine the extent to which people in Serbia in the post-pandemic period assess the impact of coronavirus pandemic on their mental/professional functioning, and whether this assessment is correlated with the occurrence of suicidal ideation and behaviours. The retrospective-prospective study was conducted online via Google Forms during January 2023. The sample included 341 respondents from the general population, 250 women (73.3%) and 91 men (26.7%), aged from 19 to 72 (M=36.41, SD=14.72). Sociodemographic questionnaire, a questionnaire on the pandemic effects, and the Risk Assessment Suicidality Scale were used to obtain data. The respondents assessed the pandemic not to have exerted a significant effect on their mental life (M=1.19±0.84) and professional functioning (M=1.55±1.02). The women, university students, pensioners and single respondents reported a greater impact of the pandemic on mental functioning, while university students and single respondents reported a greater impact on professional functioning. The respondents who were assessed to be at suicide risk (15.8%) reported a higher effect of the pandemic on mental (U=5385, p<0.001) and professional functioning (U=5799, p<0.01). Multivariant binary logistic regression showed that having a family history of mental disorders (odds ratio 2.73), younger age (OR, 1.1) and not being in a relationship (OR, 0.49) increased suicide risk in this sample. Results are in line with previous findings indicating that women, university students and pensioners reported a higher effect of the pandemic on the level of stress, depression and anxiety symptoms. The study also speaks in favour of a specific vulnerability of people suffering from mental difficulties after the pandemic.

Keywords: *COVID-19, pandemic, mental functioning, suicidality.*

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INTRODUCTION

The COVID-19 pandemic has been the most serious pandemic in the last hundred years since the Influenza Epidemic of 1918. First cases were detected in Wuhan, China, in December 2019, after which the virus started to spread rapidly worldwide. The World Health Organisation declared the outbreak of COVID-19 to be a public health emergency on 30 January 2020, and on 11 March 2020 the pandemic itself was declared (1). The first case of COVID-19 in Serbia was reported in March 2020, and over 2.5 million of cases have been reported so far, with over 17.000 deaths (2).

Psychological risk in times of crisis

Unpredictability, uncertainty and lack of control are basic characteristics of every crisis (herein: in the event of a pandemic), representing some of the key stressors that can worsen the pre-existing mental disorders in individuals and/or result in the development of the new ones (3). During pandemics, individuals experience the fear of the unknown, fear of diseases and death, as well as the feeling of helplessness, which is an additional risk to mental health (4-6).

As in most countries round the world, preventive health measures were introduced to stop the spread of virus in Serbia, including restrictions to human mobility, travel restrictions and lockdowns. The introduction of such measures caused an increase of mental tension at the community level, along with intensified fear, feeling of isolation, worries about one's own life, as well as life and health of the beloved ones. On the individual plane, there was deterioration of mental health and/or worsening of the pre-existing symptoms and the occurrence of the new ones (5). Research results in Serbia indicate that there was an elevated level of stress, anxiety and depression during lockdowns (7).

The pandemic also exerted a great influence on professional functioning of people.[1] Many people were left jobless, many of them experienced fear of losing their jobs. A great number of people started working from home, schools and universities were shut down, which lead to distance learning, adaptation and a different organisation of everyday activities and habits (6, 8). All the stated thus potentially impacted on human mental health.

Suicidality

Suicidality represents a complex phenomenon referring to the existence of some form of suicidal behaviour. The form of suicidal behaviour that leads to death – suicide, represents an active or passive self-destructive act in which an individual consciously and deliberately takes their own life (9-10). Suicide attempts also relate to self-harm with a specific degree of suicidal intent, not resulting in death. As a rule, suicide and/or suicide attempt is preceded by suicidal thoughts.

According to the World Health Organisation reports, every year more than 700.000 people commit suicide, with over 20 suicide attempts accompanying each (11). Suicide is a multifactorial phenomenon. A great number of adoption and twin studies, neurotransmission research, together with suicide completers' case history, point to the importance of genetic predispositions (12, 13). On the other hand, unfavourable environmental factors, such as stressful life events (14,15) and trauma histories (16,17) contribute to a great extent to the development of self-aggression and suicidal impulses in individuals. Although suicide is typically related to persons with some mental disorders (18-20), many suicides occur in persons with no history of mental illnesses in times of psychological crises and/or inability to cope with stressful life events (15). The coronavirus pandemic is definitely a large-scale crisis, and the mentioned risk factors during the pandemic have been acknowledged as risk factors for the occurrence of suicidality (3). Knox (21) states that traumatic experiences may have a large effect on mental health and lead to suicidal behaviour that can be manifested immediately after the pandemic, but also after many years. Research into human behaviour upon catastrophes and massive crises (cf. 22) show that a period of disappointment, injustice and reorganisation frequently occurs after a period of active struggle with the traumatic event (characterised by hope, optimism, commitment and cooperation), which may last for a few years following the crisis, with the possibility of triggering mental health issues in survivors.

Studies conducted in Serbia reported on an increasing number of suicide attempts, as well as other forms of suicidal behaviour in the previous two years, when the pandemic was at its peak and most threatening to people (23-25). Besides, an elevated level of stress was also recorded, together with the increased level of anxiety, depression, fear of losing someone, loneliness, among the general population, but also the population of people with pre-existing mental health issues (3,6).

Suicidal behaviour may occur even in a longer period after the initial crisis is over if an individual does not cope with the consequences of such a crisis. Accordingly, the subject matter of this study is the assessment of the pandemic impact and suicidality in the period *following the pandemic*. The aim of this research was to determine the extent to which people in Serbia in the post-pandemic period assess the impact of coronavirus pandemic on their mental and professional functioning, and whether this assessment is correlated with the occurrence of suicidal behaviours.

[1] In this paper, *professional functioning* entails work as well as study duties, as well as all forms of work engagement such as full- or part-time jobs, freelancing, and studying.

MATERIALS AND METHODS

This retrospective-prospective study was conducted online via Google Forms during January 2023, the method of collecting the data was snowball sampling. The research was anonymous, the participation in it voluntary in line with the Declaration of Helsinki. The study was approved by the Ethics Committee for Psychology Research, University of Kragujevac (Decision no. 7/2022). Informed consent was obtained from all respondents.

Sample

The sample recruited for the study involved 341 respondents from the general population, out of which 250 women (73.3%) and 91 men (26.7%), aged from 19 to 72 (M=36.41, SD=14.72). Out of the total number of respondents, 111 of them reported to be single (32.6%), 120 to be married (35.2), 83 to be in a relationship (24.34%), 16 to be divorced (4.69%), 6 to be a widow/widower (1.76%), and 5 did not belong in any of the listed categories – others (1.46%).

With regard to the employment status of the respondents, the majority of them were employed, i.e. 186 of them (54.54%), then 124 university students (36.36%), 17 unemployed (4.98%) and 14 pensioners (4.1%).

Out of the total number of the respondents, 234 reported to have been infected with COVID-19, out of which 208 had a mild clinical course (60.99%), 22 were asymptomatic cases (6.45%), and 9 underwent hospital treatment (2.64%).

Instruments

All the respondents filled in a sociodemographic questionnaire (to determine their sex, age, education, marital and employment status, chronic somatic illness(es), family history of mental disorders etc), a questionnaire on the pandemic (whether they have been infected with COVID-19, what their clinical course was like, whether they have been vaccinated against the coronavirus etc). The respondents also answered two questions on a four-point scale (ranging from 1 – not at all to 4 – very much), where they had to assess the impact of the pandemic on their mental and professional functioning, and they also completed the RASS scale.

Risk Assessment Suicidality Scale (RASS) is an instrument assessing suicide risk in the general population, and in population of patients with mental disorders (26). The scale includes twelve items which describe suicide-related behaviour on a scale ranging from 0 (not at all) to 3 (very much), grouped into three subscales: Intention (e.g. *Do you make plans concerning the method to use in order to finish your life*?), Life (e.g. *Have you felt that it's not worth living?*) and History (e.g. *Have you ever attempted suicide during your whole life so far?*). The study employed the standardised version for Serbian population, consisting of 9 items, which displayed good internal consistency and construct validity (α=.87), as well as discriminative power (27). In this study the RASS internal consistency coefficient was (α=.84). This version of the scale retained the three-factor model, but the factors were expanded as follows: Intention and planning (4 items), Life evaluation (3 items), and History of suicide attempts (2 items). These factors were in moderate-to-high intercorrelation, which supports a three-facet construct (27). The scale makes use of standardised scoring of items (items are differently weighted because not all items have equal predictive value in relation to suicide risk). Based on the cut-off scores, the respondents who had a score higher than 253 were placed in the group with suicide risk.

Statistical analyses

Since the distribution of the scores of the main variables deviated from the normal distribution, differences in frequencies were computed by means of the Chi-square test, and differences in scores via Mann–Whitney and Kruskal-Wallis tests. The correlation was examined by Spearman correlation coefficients. Multivariate binary regression analysis was performed to identify the predictors of suicide risk. SPSS22 was employed to perform statistical analyses.

RESULTS

The impact of the pandemic

The majority of the respondents assessed the pandemic not to have exerted a significant effect on their mental life, and only 15 respondents (4.4%) reported that the pandemic had a large impact on their mental functioning (M=1.19, SD=.84, Sk=.076, Ku=-.835), whereas 70 respondents (20.5%) stated that the pandemic had a large influence on their professional functioning (M=1.55, SD=1.02, Sk=-.081, Ku=-1.091).

Low negative correlations were detected between the age of the respondents and the perceived impact of the pandemic on mental ($r=-0.139$, $p=0.01$) and professional functioning ($r=-0.160$, $p<0.01$).

The women from the sample reported a higher influence of the pandemic on mental functioning (M=1.24) than men (M=1.04), but this was a borderline difference ($U(341)=9847.5$, $p=0.044$). No difference in sex was found concerning the perceived effect of the pandemic on professional duties ($U(341)=10912.5$, $p=0.551$).

The respondents who were single estimated a greater impact of the pandemic on mental ($U=11608$, $p<0.01$) and professional functioning ($U=12085$, $p<0.05$) than those engaged in some type of a relationship.

Regarding the employment status of the respondents, significant differences were detected between the groups in the perceived influence of the pandemic on mental functioning ($H(3)=12.631$, $p<0.01$), with the university students (M=1.4) and pensioners (M=1.29) being at the forefront. Likewise, significant differences were found in the degree of the perceived impact of the pandemic on professional functioning ($H(3)=13.917$, $p<0.01$), where the university students

reported the greatest effect (M= 1.74), while the pensioners reported the smallest (M= 0.71).

According to the level of education criterion, no significant differences were detected in the perceived impact of the pandemic on mental (H(2)=3.548, p=0.170), nor professional functioning (H(2)=3.665, p=0.160).

Suicidality

Out of the total number of the respondents, 54 of them (i.e. 15.8%) were under suicide risk (RASS score higher than 253). Moreover, 58 respondents (i.e. 17%) reported the existence of suicidal ideations to a certain extent (Intention and Planning factor), while 33 respondents (i.e. 9.7%) reported that they had harmed themselves intentionally, and 5 of them (1.5%) attempted suicide.

Basic descriptive data for the total score on RASS, as well as the scores on each of the three factors have been presented in Table 1.

Table 1. Descriptive statistics of RASS scores

	Min	Max	M	SD	Sk	Ku
RASS score	0	885	121.80	172.698	2.108	4.601
Intention and planning	0	400	29.09	79.134	3.305	11.001
Life evaluation	0	300	81.79	91.428	.894	-.466
History of suicide…	0	200	10.92	34.500	3.325	11.364

Significant differences by the sex criterion were detected only on the Life evaluation factor (U=9619, p=0.023), with females reporting higher scores. Single respondents reported significantly higher scores on all dimensions in comparison with the others (U_{ip}=11611.5, p<0.001; U_{le}=9978, p<0.001; U_{h}=13095, p<0.001) and the total score (U=9859, p<0.001). No significant differences were found in accordance with the level of education criterion.

Significant differences by the employment status criterion were detected in the total score (H(3)=28.198, p<0.001), Intention and Planning (H(3)=24.752, p<0.001), and Life Evaluation (H(3)=26.238, p<0.001). The lowest scores were found in the pensioners, the highest in the university students.

The following were found to be significantly correlated with the RASS score: age (r=-.35, p<.001), and family history of mental disorders (r=-.18, p<0.01). The respondents at suicide risk reported a greater influence of the pandemic on mental (U=5385, p<0.001) and professional functioning (U=5799, p<0.01) in comparison with the respondents not being at such risk.

Predicting suicide risk

After grouping all respondents into two groups (with or without suicide risk), multivariant binary logistic regression showed that having a family history of mental disorders (odds ratio [OR], 2.73), younger age (OR, 1.1) and not being in a relationship (OR, 0.49) increased suicide risk (cf. Table 2, Step 2).

Table 2. Multivariate binary logistic regression analyses of variables predicting suicide risk

							95% C.I. for EXP(B)	
		B	S.E.	df	Sig.	Exp(B)	Lower	Upper
Step 1	Age	-.095	.021	1	.001**	1.009	1.055	1.146
	Sex	.103	.392	1	.793	1.108	.515	2.387
	Work status	.740	.462	1	.109	2.096	.847	5.184
	Family history of mental disord.	.986	.421	1	.019*	1.332	.931	1.907
	Relationship status	-.754	.340	1	.026*	2.125	1.092	4.136
	Constant	19.066	4.269	1	.000	3.273		
Step 2	Age	-.094	.021	1	.001**	1.098	1.054	1.144
	Family history of mental disord.	1.004	.406	1	.013*	2.730	1.232	6.054
	Relationship status	-.723	.336	1	.031*	.485	.251	.937
	Constant	18.096	4.504	1	.000	3.603		

** p<.01, * p<.05

CONCLUSION

The effect of the pandemic on mental and professional functioning

Our study showed that women, single persons, university students and pensioners assessed the impact of pandemic on mental functioning as higher. The obtained results are in line with other studies whose findings indicate that symptoms of depression and anxiety were detected in women, and that women reported a higher effect of the pandemic on the level of stress and mental health in general (28-31). Recent research shows that a negative effect on mental health also reflects on university student population, which is visible in a growing number of depression and anxiety symptoms, a higher level of stress, together with more frequent alcohol and psychoactive substances abuse (28, 31, 32). A study conducted in Serbia points to the existence of a high number of symptoms of depression, anxiety and stress in university student population, who listed the fear of infecting the beloved ones and the fear of endangering their families as the most frequent fears (33). A special position of retired persons relates to the fact that in Serbia this population experienced the most restrictive measures against the spread of COVID-19. Large presence of chronic and acute illnesses in terms of percentage, a reduced level of social interaction and dependence on others, along with the previously stated special position of pensioners in Serbia, are concordant with the results of our research, as well as other studies in which this population reported on a great effect of the pandemic on their mental health (34). In further support of this is the fact that more than 25% of persons who sought psychosocial help via a helpline were aged from 71 to 80 (35). Finally, social problems caused by the COVID-19 pandemic and the level of social support are factors playing a significant role in the occurrence of psychiatric disorders during the coronavirus pandemic (3, 36, 37), which may account for a higher level of suicide risk and awareness of a negative effect of the pandemic on mental life in the respondents without a partner.

Beside its impact on mental functioning, the university students from this study assessed the effect of the pandemic on professional functioning to the greatest degree. Other studies also confirm this finding (38,39). Changes in modes of studies, reorganisation of learning activities, reduced motivation for the studies, increased pressures to learn independently, reduced contact with family and friends, among many, are the factors that made an effect on university students (8, 40). The abovementioned research conducted on university student population in Serbia shows that 14.6% of university students reported a great influence of the pandemic on their student duties, while 17.7% of them stated that they found it more stressful to complete their duties than in normal conditions prior to the pandemic (33). Apart from a lack of immediate contact, university students list technical problems in online classes, such as Internet accessibility and speed, not having adequate and reliable devices, and issues connected with practical classes and/or seminars, as well as inability to learn in face-to-face seminars (38, 39).

The pandemic and suicidality

The factors that singled out as being significantly associated with suicide risk in this study were the family history of mental disorders, relationship status and age. Furthermore, the persons assessed to be at suicide risk reported a larger effect of the pandemic on mental and professional functioning than the respondents not assessed to be at such risk.

Similar research during the pandemic shows that women, aged 18–29, student and unemployment status, prior psychiatric history, and those reporting a greater negative impact of pandemics on their quality of life, were at higher risk for increased anxiety and depression symptoms (28). The results of research conducted in Malaysia show a significant connection of suicidal behaviour with the relationship status, level of education, work status, depression, anxiety and sex (41). The findings of a study carried out in Portugal (42), indicate that the following are associated with suicide risk: the level of education, work status (the unemployed), existence of a psychiatric diagnose, existence of a chronic illness, as well as impact of the pandemic on mental functioning.

Studies investigating the rate of suicidal behaviours in the recent few years, suggest that the highest degree of suicidal ideations were in 2020 in comparison with the previous years, and that its number started declining slowly in 2021 and later (43). On the other hand, a meta-analysis that has synthesised international reports on suicidal behaviours in the recent years, speaks in favour of an upward trend of suicidal ideation and suicide attempts during the COVID-19 pandemic, with the suicide rate remaining stable (44). A study conducted in Serbia reports that the Psychiatric Clinic "Dr Laza Lazarević" had more suicide attempts registered in the period between March and July 2020 in comparison with the same period in the previous year (23). A similar study was carried out at the Psychiatric Clinic of the University Clinical Centre of Niš, which reported significantly more suicide attempts in 2020 than in prior years (24). The scale detecting suicidality in this research associated more that 40% of the respondents with some form of suicidal behaviour. By means of comparing the mean total scores on RASS from this study and the study conducted in 2016, upon validation of the scale (27), it has been established that the mean total scores, as well as scores on individual subscales are higher than in the previous period. With a hedge as regards the imbalance of these two samples, these data may point to an increase in suicide risk in the general population of Serbia in comparison with the period of seven years ago as well a potential connection of suicide risk with the outbreak of COVID-19.

Study limitations

One of the limitations of the current study is the nature of the sample (voluntary), where women outnumbered men, and university students made up more than a quarter of the sample. Another limitation refers to the study design (cross-sectional study). One more limitation relates to the fact that one question was used for the assessment of the pandemic

impact on mental and professional functioning, although the previous studies determined that subjective perception of pandemic-related distress is associated with the development of suicidality (3).

CONCLUSION

The results of the current study show that persons at suicide risk assessed the effect of the pandemic on mental and professional functioning to be higher than the respondents not being at such risk. The greatest impact was reported by the university students, who scored the highest on RASS at the same time. This speaks in favour of a specific vulnerability of this group. In addition, by comparing the results gathered in this study with the results obtained in the previous research conducted upon scale validation in 2016, what can be noticed is that the mean total scores as well as the mean scores on the individual factors are higher.

CONFLICTS OF INTEREST

The authors report no conflict of interest.

REFERENCES

1. World Health Organization. Coronavirus disease (COVID-19) pandemic. (2023, July 31). https://www.who.int/europe/emergencies/situations/covid-19
2. Ministry of Health, I*nstitute for Public Health "Dr Milan Jovanovic Batut" 2023*. Statistics on Corona virus – Covid 19. Belgrade: Ministry of Health. https://covid19.rs/homepage-english/
3. Kuljancic D, Cvjetkovic Bosnjak M, Siladji D, et al. The role of COVID-19-associated fear, stress and level of social support in development of suicidality in patients diagnosed with affective and stress-induced psychiatric disorders during the COVID-19 pandemic - A comparative analysis. Brain Sci. 2023;13:812.
4. Ho CS, Chee CY, Ho RC. Mental health strategies to combat the psychological impact of coronavirus disease 2019 (COVID-19) beyond paranoia and panic. Ann Acad Med. 2020;49(3):155-160.
5. Mendoza RNC, Lara TM, Rodríguez NMP, Guevara PYS. Harmful effects of COVID-19 on mental health and its social repercussions. Int J Ortho Res. 2021;4(2):3-7.
6. Ignjatović Ristić D, Hinić D, Banković D, et al. Levels of stress and resilience related to the COVID-19 pandemic among academic medical staff in Serbia. Psychiatry Clin Neurosci. 2020;74(11):604-5.
7. Vujčić I, Safiye T, Milikić B, et al. Coronavirus disease 2019 (COVID-19) epidemic and mental health status in the general adult population of Serbia: A cross-sectional study. Int J Environ Res Public Health. 2021; 8(4):1957.
8. Beara M, Rakić-Bajić G, Hinić D. Teachers' perceived stress and experience in online teaching during the early phase of the COVID-19 pandemic. Psihološka istraživanja, 2021;24(2):193-207.
9. Silverman MM, Berman AL, Sanddal ND, O'Carroll PW, Joiner TE. Rebuilding the tower of Babel: a revised nomenclature for the study of suicide and suicidal behaviors. Part 1: Background, rationale, and methodology. Suicide Life Threat Behav. 2007;37(3):248-63.
10. Silverman MM, Berman AL, Sanddal ND, O'Carroll PW, Joiner TE. Rebuilding the tower of Babel: a revised nomenclature for the study of suicide and suicidal behaviors. Part 2: Suicide-related ideations, communications, and behaviors. Suicide Life Threat Behav. 2007;37(3):264-77.
11. WHO 2023. Suicide. World Health Organization, Geneva. https://www.who.int/news-room/fact-sheets/detail/suicide
12. Voracek M, Loibl LM. Genetics of suicide: a systematic review of twin studies. Wien Klin Wochenschr. 2007;119(15-16):463-75.
13. Pompili M, Gentile G, Scassellati C, et al. Genetic association analysis of serotonin and signal transduction pathways in suicide attempters from an Italian sample of psychiatric patients. Neurosci Lett. 2017;656:94-102.
14. Buchman-Schmitt JM, Chu C, Michaels MS, et al. The role of stressful life events preceding death by suicide: Evidence from two samples of suicide decedents. Psychiatry Res. 2017;256:345-52.
15. Lečić-Toševski D, Vuković O, Pejušković B. Suicid u godinama stresa - srpsko iskustvo. Psihijatrija danas, 2015;47(1),19-27.
16. Xie P, Wu K, Zheng Y, et al. Prevalence of childhood trauma and correlations between childhood trauma, suicidal ideation, and social support in patients with depression, bipolar disorder, and schizophrenia in southern China. J Affect Disord. 2018;228:41-8.
17. Dedic G. Victimization in childhood as a suicide risk factor in adults. Eur Psychiatry. 2017; 41:S291.
18. Hawton K, Saunders K, Topiwala A, Haw C. Psychiatric disorders in patients presenting to hospital following self-harm: A systematic review. J Affect Disord. 2013;151(3):821-30.
19. Inder KJ, Handley TE, Johnston A, et al. Determinants of suicidal ideation and suicide attempts: parallel cross-sectional analyses examining geographical location. BMC Psychiatry. 2014;14:208.
20. Cavanagh JT, Carson AJ, Sharpe M, Lawrie SM. Psychological autopsy studies of suicide: a systematic review. Psychol Med. 2003;33(3):395-405.
21. Knox KL. Epidemiology of the relationship between traumatic experience and suicidal behaviors. PTSD Research Quarterly, 2008;19:1-7.
22. Hinić D 2023. Ekološka psihologija. Univerzitet u Kragujevcu, in press.
23. Stasevic-Karlicic I, Đorđević V, Dutina A, et al. The impact of COVID-19 pandemic on suicide attempts in the Republic of Serbia. Srp Arh Celok Lek. 2021;149(7–8):455–60.
24. Golubovic ST, Zikic O, Nikolic G, et al. Possible impact of COVID-19 pandemic and lockdown on suicide behavior among patients in Southeast Serbia. Open Med (Wars). 2022;17(1):1045-56.

25. Cvjetković-Bošnjak M. Suicidality in health crisis: Case reports. Sanamed, 2020;15(2):203-7.
26. Fountoulakis KN, Pantoula E, Siamouli M, et al. Development of the Risk Assessment Suicidality Scale (RASS): a population-based study. J Affect Disord. 2012;138(3):449-57.
27. Jovičić M, Hinić D, Drašković M, et al. Psychometric properties of the rass scale in the Serbian population. J Affect Disord. 2016;189:134-40.
28. Solomou I, Constantinidou F. Prevalence and predictors of anxiety and depression symptoms during the COVID-19 pandemic and compliance with precautionary measures: Age and sex matter. Int J Environ Res Public Health. 2020;17(14):4924.
29. Ulloa RE, Apiquian R, de la Peña FR, et al. Age and sex differences in the impact of the COVID-19 pandemic on mental health and coping mechanisms in Latin American youth. J Psychiatr Res. 2022;156:372-8.
30. Ye J, Ren Z. Examining the impact of sex differences and the COVID-19 pandemic on health and health care: findings from a national cross-sectional study. JAMIA Open. 2022;5(3):ooac076.
31. Prowse R, Sherratt F, Abizaid A, et al. Coping with the COVID-19 pandemic: Examining gender differences in stress and mental health among university students. Front Psychiatry. 2021;12:650759.
32. Zimmermann M, Bledsoe C, Papa A. Initial impact of the COVID-19 pandemic on college student mental health: A longitudinal examination of risk and protective factors. Psychiatry Res. 2021;305:114254.
33. Božović M, Radevic S, Janicijevic K, et al. Student population mental health during the COVID-19 pandemic. Glasnik javnog zdravlja, 2023;97(1):79-95.
34. Džamonja-Ignjatović T, Stankovic B, Klikovac T. Experiences and the quality of life of the elderly during the COVID-19 pandemic and the introduction of COVID-19 restrictive measures in Serbia. Psihološka istraživanja, 2020;23(2):201-31.
35. Stašević-Karličić I, Đorđević V, Stasevic M, et al. Perspectives on mental health services during the COVID-19 epidemic in Serbia. Srp Arh Celok Lek. 2020;148(5–6):379-82.
36. Pompili, M. A plea for the understanding of the suicidal mind. J. Psychopath. 2019;25:126-31.
37. Ciacchella C, Campedelli V, Veneziani G, Pellicano G, Sambucini D, Lai C. Impact of the coronavirus pandemic (COVID-19) on mood disorders and suicide. Front. Psychiatry. 2022;13:846112.
38. Mahdy MAA. The impact of COVID-19 pandemic on the academic performance of veterinary medical students. Front Vet Sci. 2020;7:594261.
39. Said GRE. (2021). How did the COVID-19 pandemic affect higher education learning experience? An empirical investigation of learners' academic performance at a university in a developing country. Adv Hum-Comput. Interact. 2021;6649524. https://doi.org/10.1155/2021/6649524
40. Grubic N, Badovinac S, Johri AM. Student mental health in the midst of the COVID-19 pandemic: A call for further research and immediate solutions. Int J Soc Psychiatry. 2020; 66(5): 517-8.
41. Razali S, Saw JA, Hashim NA, et al. Suicidal behaviour amid first wave of COVID-19 pandemic in Malaysia: Data from the COVID-19 mental health international (COMET-G) study. Front Psychiatry. 2022;13:998888.
42. Campos RC, Holden RR, Medinapereira A. Psychological impact of the COVID-19 pandemic and suicide ideation in community adults in Portugal: Preliminary data. Minerva Psychiatry, 2021;62(2):74-8.
43. Czeisler ME, Lane RI, Wiley JF, Czeisler CA, Howard ME, Rajaratnam SMW. Follow-up survey of US adult reports of mental health, substance use, and suicidal ideation during the COVID-19 pandemic, september 2020. JAMA Netw Open. 2021;4(2):e2037665.
44. Yan Y, Hou J, Li Q, Yu NX. Suicide before and during the COVID-19 pandemic: A systematic review with meta-analysis. Int J Environ Res Public Health. 2023;20(4):3346.

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THE ROLE OF GALECTIN 3 IN THE PATHOGENESIS OF DIABETES MELLITUS: FOCUS ON β-CELL FUNCTION AND SURVIVAL

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ABSTRACT

Galectin 3 is a lectin expressed in many tissues with a significant biological role in physiological and pathological processes. Our review aims to sublimate the effects of galectin 3 on the β-cells function and survival. Data about the effect of galectin 3 on β-cells are scarce and contradictory. Several studies have shown that reduced activity of the galectin 3 gene reduces the risk of developing type 1 diabetes in an experimental model of diabetes in galectin 3 deficient mice. On the other side, in an experimental model of type 1 diabetes with mice with selectively enhanced expression of galectin 3 in β-cells, was shown that increased expression of this lectin has a protective role. Unlike type 1 diabetes where the autoimmune process plays a dominant role in pathogenesis, the pathogenesis of type 2 diabetes is multifactorial. One of the main factors which contribute to type 2 diabetes, the insulin resistance, is related to the concentration of soluble galectin 3. The effect of galectin 3 is very important for β-cell function. When a harmful factor acts on a β-cell, its intracellular concentration increases to preserve the function of β-cells and prevent their apoptosis, by blocking the internal path of apoptosis. However, excessive accumulation of galectin 3 inside the cell leads to its secretion, which encourages tissue inflammation. Based on all the above, galectin 3 has a double effect on β-cells.

Keywords: *Galectin 3, type 1 diabetes mellitus, type 2 diabetes mellitus.*

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INTRODUCTION

Galectins are carbohydrate-binding proteins that are involved in many physiological functions (1). Galectin 3 is a monomer with two functional domains (2-5). So far, it is unique molecule in a lectins family with an extra-long and flexible N-terminal domain composed of 100 to 150 amino acid residues, while lacking a charged or large side chain of hydrophobic residues (3, 4). The N-terminal domain contains sites for phosphorylation of Ser 6, Ser 12, and other determinants important for lectin secretion by a nonclassical mechanism until the second C-terminus consists of about 135 amino acid residues (6-8). This lectin acts as a receptor that binds molecules - ligands that contain poly-N acetylactosamine sequences that consist of many disaccharide units. However, it appears to have an increased affinity for binding more complex oligosaccharides (9, 10). It is a unique galectin that can form pentamer units with other molecules. It has a specific pleiotropic biological function and plays an important role in many physiological and pathological processes.

Galectin 3 is the lectin that is ubiquitously expressed in many tissues. Experiments performed on mice during embryogenesis, have shown that its expression depends on the tissues and age. It is mainly expressed on epithelial cells and myeloid cells, and also in cells of the eye, renal tissue, pancreas, salivary gland ducts, as well as intrahepatic bile ducts (11). There are numerous data on the expression and effect of galectin 3 in immune system cells and cells involved in the immune response (12-14). In some cell types, galectin 3 is not expressed, but its expression can be induced through stimulation by various stimuli (15). In cells of a human exocrine pancreas, it is quite poorly expressed. Galectin 3 is mostly present in ductal cells (in about 50% expression is high) while some of the acinar cells shows low expression in nuclei (16).

Pancreatic β-cells

Pancreatic β-cells are very important for normal metabolism as the only cells that produce insulin. As such, many conditions in the body require their increased involvement. Apoptosis is the dominant type of β-cell damage in the development of type 1 (T1DM) and type 2 diabetes mellitus (T2DM) (17-18). When the damage of β-cells is caused by metabolic causes, the main place is occupied by activation of the intrinsic apoptosis pathway (19). On the other side, in the case of damage by immune mechanisms such as pro-inflammatory cytokines or cells, the external path of apoptosis occupies a significant place (17). Disruption of the number and function of β-cells, which is followed by a disorder of glucose metabolism, simultaneously leads to a disorder of the metabolism of other organic substances (20-22). Insulin, by inhibiting lipoprotein lipase, has a strong effect on fat breakdown (23, 24). In the absence of insulin effect in the body, a redirection of metabolism to fats represents the dominant process (25, 26).

Galectin 3 in type 1 diabetes mellitus

Data about the effect of galectin 3 on β-cells are scarce and contradictory. The onset of T1DM is associated with the development of an autoimmune process (27, 28). During this process, the immune system cells can directly cause β-cell damage or by secreting pro-inflammatory cytokines (29). The effect of galectin 3 in the β-cells pathophysiology on human cells was investigated *in vitro*. *Karlsen* et al. examined the survival of human cells after treatment with harmful immune factors. They showed that this molecule is a naturally up-regulated defense protein of β-cells, whose increase occurs upon stimulation by immune factors associated with T1DM. But, in the end, they concluded that although its enhanced production occurs, it is not sufficient to prevent β-cell damage from the effects of multiple pro-inflammatory cytokines (TNF-α, INF-γ, and IL-1β) (30). On the other side, *Saxida* et al. investigated the effects of complete galectin 3 deficiency in β-cells *in vitro,* by treating the pancreatic islets with the cocktail of pro-inflammatory cytokines (TNF-α, INF-γ, and IL-1β). They showed that galectin 3 deficiency promotes β-cell survival and function. Examining the expression of molecules associated with apoptosis showed that galectin 3 ablation affects the expression of the major components of the mitochondrial apoptotic pathway (internal) (31). *Mensah-Brown* et al. have shown that reduction in galectin 3 gene activity results in reduced susceptibility to T1DM development in the experimental model induced by the use of multiple low dose streptozotocin (MLD-STZ) in galectin 3 deficient mice (32). The results of our study, in mice with selective galectin 3 overexpression in β-cells, showed that enhanced galectin 3 expressions in β-cells in the MLD-STZ T1DM model had protective role. We have shown that increased expression of galectin 3 in β-cells leads to amelioration of metabolic parameters, accompanied by less severe inflammation of pancreatic islets and decreased number of pro-inflammatory cells in the islets and pancreatic lymph nodes (33). *Radosavljevic* et al. have summarized the effects of galectin 3 during autoimmunity. They showed that galectin 3 is involved in immune mediated β-cell damage and is required for diabetogenesis in the MLD-STZ model. This effect is achieved by promoting the expression of IFN-γ, TNF-α, IL-17 and iNOS in immune and accessory effectors cells (34). *Sano* et al. have shown that the presence of galectin 3 is very important for macrophage function. In the absence of this molecule, macrophages are not effective in eliminating intracellular and extracellular pathogens (35). *Radosavljevic* et al. have shown that galectin 3 can have different effects depending on the tissue in which it is located and its location (36).

Galectin 3 in type 2 diabetes mellitus

Unlike the T1DM pathogenesis where the autoimmune process plays a dominant role, the pathogenesis of T2DM is multifactorial (37). A couple of major factors in the development of this type of diabetes are associated with the

extracellular and soluble galectin 3 concentration. One of the major factors which contribute to β-cell damage and the development of T2DM is insulin resistance (IR). *Li* et al. showed the effect of soluble galectin 3 on the development of IR in a mouse model. They have shown that an increase in soluble galectin 3 leads to a disorder of the Glut 4 receptor expression on peripheral tissues. Decreased expression of this receptor due to interactions with soluble galectin 3 leads to increased IR. The same authors also showed that the administration of galectin 3 inhibitors improves insulin sensitivity (38). Contrary, *Pejnovic* et al. showed that galectin 3 deficient mice had a higher degree of IR compared to the control group (39). An interesting study showed that the application of piperine on C57BL/6 mice protects β-cells from dysfunction. This effect has been shown with significantly reduced levels of serum lipopolysaccharide, IL-1β, and galectin 3. Suppression of the presence of M1-like macrophages in epididymal adipose tissues and islets was also observed. In other words, piperine significantly reduces the pro-inflammatory polarization of macrophages and β-cell damage in the pancreatic islets (40). A several human studies showed that an increase in soluble galectin 3 is accompanied by an increase in IR while the results of few others say the opposite. Clinical studies have shown that patients with high serum galectin 3 values are at increased risk for obesity, prediabetes, T2DM, and chronic complications (41-43). On the other side, some clinical studies indicate that galectin 3 is negatively associated with insulinemia and insulin resistance index values and has a protective role in T2DM (44). At the same time, with the appearance of IR, metainflammation develops in adipose tissue and pancreatic islets (45). New condition is accompanied by increased production of pro-inflammatory cytokines, and also by the influx of pro-inflammatory cells. *Pejnovic* et al. showed that galectin 3 is a regulator of metaflammation in adipose tissue and pancreatic islets by examining the role of this molecule in galectin 3 deficient mice (46). Our previous results show that extracellular galectin 3 acts as an alarm. In conditions of increased concentration in the cell, galectin 3 secretion occurs. In secreted form, galectin 3 has paracrine effect on the surrounding cells, and increases the influx of inflammatory cells and inflammation (47). *Pejnovic* et al. examined the development of inflammation in adipose tissue and pancreatic islets in galectin 3 deficient mice on a high fat diet. Their results clearly showed protective roles in obesity induced inflammation and diabetes. They showed that galectin 3 deficiency accelerates the development of obesity and increases the degree of inflammation of adipose tissue and islets (39). A state of metainflammation is accompanied by increased production of pro-inflammatory cytokines, and increased influx of pro-inflammatory cells (48, 45). Some of the cytokines (IL-1β, IFN-γ, and TNF-α) can directly impair β-cell function, while at the same time, others (IL-1 and IL-6) can increase existing inflammation. A significant place in β-cell damage occupies an interaction of macrophages and β-cells, via galectin 3 and TLR-4 receptors on both types of cells (49, 46). *Caberoy* et al. have demonstrated that the expression of galectin 3 on apoptotic cells or cellular debris is an "eat-me" signal which stimulates phagocytosis of these cells. (50) *Cucak* et al. showed that pro-inflammatory M1-like macrophages invade diabetic islets in type 2 diabetes. They even shown that innate immunity has a significant place, with changes from the original pro-inflammatory phenotype to the profibrotic phenotype, which supports the concept that T2D represent an inflammatory disease (51).

An interesting study is coming from *Hu* et al. They showed that membrane and extracellular galectin 3 interaction with immune cells, lead to β-cell damage. By using pectin, galectin 3 binding molecule that prevents interacting of galectin 3 with other molecules/cells, they have shown the reduced ROS production in β-cells and reduced inflammation in islets (52). Increased concentration of pro-inflammatory cytokines in adipose tissue at the same time reduces the production of adipokines that promote β-cell function (53).

On the other side, the expression of galectin 3 in the β-cells has a major effect. Under conditions of IR and compensatory hyperinsulinemia, there is increased stress on β-cells. Increased insulin production is accompanied by the increased mitochondrial burden which is followed by increased production of reactive oxygen species (ROS) (54-56). In addition to increased β-cell engagement, hyperglycemia *per se* can lead to the accumulation of glycation end products (increased advanced glycation end products (AGE) generation) and redox imbalance. (57) In the state of increased stress and ROS formation, various mechanisms are activated in β-cells to prevent mitochondrial damage and cell death (58). Studies which examined the effect of galectin 3 on cell damage due to increased AGE production have indicated that galectin 3 is operating *in vivo* as an AGE receptor which protect against AGE dependent tissue injury (59, 60). Our *in vitro* results showed that enhanced intracellular galectin 3 expression was associated with the prooxidant state (47).

The function of the endoplasmic reticulum (ER) occupies a significant place in the normal functioning of β-cells (61). In addition to insulin synthesis, also plays an important role as a regulator of intracellular calcium concentration, which is especially important during the first phase of insulin secretion (62, 63). In chronic conditions of β-cell exposure to various nutritional factors or hyperinsulinemia, increased ROS production is accompanied by more pronounced ER stress (64-66). Simultaneously, high concentrations of ROS can induce DNA damage and alter mitochondrial membrane potential (67). In conditions of reduced ATP production, the influx of extracellular calcium into β-cells is also reduced (68). In order to maintain insulin secretion, in the new conditions, ER becomes a source of intracellular calcium, which is accompanied by a reduced concentration of calcium in the ER. Decreased calcium concentrations in the ER lead to disturbances in preproinsulin processing as well as transport to the Golgi complex (68). The net effect of the disorder in the ER will be strain on the ability of the β-cell to manufacture process and store sufficient insulin to cope with demand and to appropriately regulate insulin secretion. Experimental studies showed that impaired mitochondria dysfunction increased ER stress proteins and induced

apoptosis of mouse pancreatic β-cells (69). Increased concentration of intracellular galectin 3, in states of metabolic stress, stabilizes the mitochondrial membrane thereby allowing increased ATP production. Enough ATP allows the influx of extracellular calcium and prevents further increase of ER stress and ultimately allows longer survival of β-cells.

Finally, one of the last mechanisms that lead to β-cell damage is lipotoxicity (70-73). Lipotoxicity causes damage by two ways. First, in high-fat diets, blood free fatty acids act via TLR-4 receptor on β-cells (damage associated molecular patterns), which transmits signals inside the cell. On the other side, the accumulation of fat particles in β-cells can directly lead to their damage and disorders in insulin secretion (74, 71). Although most studies agree that galectin 3 is a marker of inflammation and fibrosis, many experimental studies indicate that increased expression of this molecule may be part of the adaptive response to tissue injury. The main goal of this response is to prevent the transition of the inflammatory process to a chronic course (75).

The effect of galectin 3 is very important for the function and survival of β-cells. In conditions when a harmful factor acts on a β-cell, an increased concentration of intracellular galectin 3 occurs. This increment occurs to preserve the function of β-cells and prevent apoptosis. The antiapoptotic effect of intracellular galectin 3 is achieved by suppressing the internal path of apoptosis. However, excessive accumulation of galectin 3 inside the cell leads to its secretion. Secretion starts when the intracellular calcium concentration increases and when the galectin 3 producing cell tends toward apoptosis due to significant ROS production. Secreted galectin 3 then attracts pro-inflammatory cells and promotes tissue inflammation. Immune cells effect on the β-cell is dominantly expressed via an external path of apoptosis in which galectin 3 does not play important role. When the concentration of galectin 3 rises in the blood, it further increases the severity of IR.

CONCLUSION

Based on all the above, we concluded that galectin 3 has a dual effect on β-cells. Intracellular galectin 3 protects β-cells by silencing the internal pathway of apoptosis while extracellular promotes insulin resistance and inflammation of the pancreatic islets.

Table 1. Summary effects of galectin 3 on β-cell

Effect of galectin 3 on β-cell function and survival	
Protective in T1 diabetes/T2 diabetes	
Study	**Type**
***Karlsen* et al. (2006)**	***in vitro***
***Jovicic* et al. (2021)**	**on galectin 3 OE mice**
***Pejnovic* et al. (2013)**	**on galectin 3 KO mice**
***Pejnovic* et al. (2013)**	**on galectin 3 KO mice**
Harmful in T1 diabetes/T2 diabetes	
Study	**Type**
***Saksida* et al. (2013)**	***in vitro***
***Mensah-Brown* et al. (2009)**	**on galectin 3 KO mice**
***Li* et al. (2016)**	**on Galectin 3 KO and WT C57BL/6 mice**
***Yuan* et al. (2021)**	**on C57BL/6 mice**
***Petrovic* et al. (2020)**	**on galectin 3 OE mice**
***Hu* et al. (2020)**	***in vitro***
***Caberoy* et al. (2012)**	***in vitro***
***Weigert* et al. (2010)**	**on humans**
***Yilmaz* et al. (2015)**	**on humans**
***Jin* et al. (2013)**	**on humans**

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CONFLICTS OF INTEREST

The authors declare no financial or commercial conflict of interest.

REFERENCES

1. Johannes L, Jacob R, Leffler H. Galectins at a glance. J Cell Sci. 2018; 131(9):jcs208884.
2. Hirabayashi J, Kasai K-i. The family of metazoan metal-independent β-galactoside-binding lectins: structure, function and molecular evolution. Glycobiology. 1993;3:297-304.
3. Birdsall B, Feeney J, Burdett ID, Bawumia S, Barboni EA, Hughes RC. NMR solution studies of hamster galectin-3 and electron microscopic visualization of surface-adsorbed complexes: evidence for interactions between the N-and C-terminal domains. Biochemistry. 2001;40:4859-66.
4. Hughes RC. Mac-2: a versatile galactose-binding protein of mammalian tissues. Glycobiology. 1994;4:5-12.
5. Wang JL, Laing JG, Anderson RL. Lectins in the cell nucleus. Glycobiology. 1991;1:243-52.
6. Huflejt M, Turck C, Lindstedt R, Barondes S, Leffler H. L-29, a soluble lactose-binding lectin, is phosphorylated on serine 6 and serine 12 in vivo and by casein kinase I. Journal of Biological Chemistry. 1993;268:26712-8.

7. Mazurek N, Conklin J, Byrd JC, Raz A, Bresalier RS. Phosphorylation of the β-galactoside-binding protein galectin-3 modulates binding to its ligands. Journal of Biological Chemistry. 2000;275:36311-5.
8. Menon RP, Hughes RC. Determinants in the N-terminal domains of galectin-3 for secretion by a novel pathway circumventing the endoplasmic reticulum–Golgi complex. European journal of biochemistry. 1999;264: 569-76.
9. Hirabayashi J, Hashidate T, Arata Y, Nishi N, Nakamura T, Hirashima M, et al. Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. Biochimica et Biophysica Acta (BBA)-General Subjects. 2002;1572:232-54
10. Collins PM, Bum-Erdene K, Yu X, Blanchard H. Galectin-3 interactions with glycosphingolipids. J Mol Biol. 2014; 426(7):1439-51
11. Dumic J, Dabelic S, Flögel M. Galectin-3: an open-ended story. Biochimica et Biophysica Acta (BBA)-General Subjects. 2006;1760:616-35.
12. Díaz-Alvarez L, Ortega E. The Many Roles of Galectin-3, a Multifaceted Molecule, in Innate Immune Responses against Pathogens. Mediators Inflamm. 2017; 2017:9247574
13. Chung AW, Sieling PA, Schenk M, Teles RM, Krutzik SR, Hsu DK, Liu FT, Sarno EN, Rea TH, Stenger S, Modlin RL, Lee DJ. Galectin-3 regulates the innate immune response of human monocytes. J Infect Dis. 2013; 207(6):947-56
14. Yang RY, Hsu DK, Liu FT. Expression of galectin-3 modulates T-cell growth and apoptosis. Proc Natl Acad Sci U S A. 1996; 93(13):6737-42. doi: 10.1073/pnas.93.13.6737
15. Hughes RC. Secretion of the galectin family of mammalian carbohydrate-binding proteins. Biochimica et Biophysica Acta (BBA)-General Subjects. 1999;1473: 172-85.
16. Schaffert C, Pour PM, Chaney WG. Localization of galectin-3 in normal and diseased pancreatic tissue. International journal of pancreatology. 1998; 23: 1-9.
17. Thomas HE, McKenzie MD, Angstetra E, Campbell PD, Kay TW. Beta cell apoptosis in diabetes. Apoptosis. 2009; 14(12):1389-404.
18. Hui H, Dotta F, Di Mario U, Perfetti R. Role of caspases in the regulation of apoptotic pancreatic islet beta-cells death. J Cell Physiol. 2004; 200(2):177-200.
19. Wali JA, Masters SL, Thomas HE. Linking metabolic abnormalities to apoptotic pathways in Beta cells in type 2 diabetes. Cells. 2013; 2(2):266-83.
20. Goldberg RB. Lipid disorders in diabetes. Diabetes care. 1981; 4: 561-572.
21. Abbate SL, Brunzell JD. Pathophysiology of hyperlipidemia in diabetes mellitus. J Cardiovasc Pharmacol. 1990;16 Suppl 9:S1-7.
22. Hirano T. Pathophysiology of Diabetic Dyslipidemia. J Atheroscler Thromb. 2018; 25(9):771-782.
23. Nielsen TS, Jessen N, Jørgensen JO, Møller N, Lund S. Dissecting adipose tissue lipolysis: molecular regulation and implications for metabolic disease. J Mol Endocrinol. 2014; 52(3):R199-222.
24. Santoro A, McGraw TE, Kahn BB. Insulin action in adipocytes, adipose remodeling, and systemic effects. Cell Metab. 2021; 33(4):748-757.
25. Bjornstad P, Eckel RH. Pathogenesis of Lipid Disorders in Insulin Resistance: a Brief Review. Curr Diab Rep. 2018; 18(12):127.
26. Goldberg RB. Lipid disorders in diabetes. Diabetes Care. 1981; 4(5):561-72.
27. Kawasaki E. Type 1 diabetes and autoimmunity. Clinical pediatric endocrinology. 2014; 23: 99-105
28. Chen J, Stimpson SE, Fernandez-Bueno GA, Mathews CE. Mitochondrial reactive oxygen species and type 1 diabetes. Antioxidants & redox signaling. 2018; 29(14): 1361-72.
29. Paschou SA, Papadopoulou-Marketou N, Chrousos GP, Kanaka-Gantenbein C. On type 1 diabetes mellitus pathogenesis. Endocrine connections. 2018; 7(1):R38-46.
30. Karlsen AE, Størling ZM, Sparre T, Larsen MR, Mahmood A, Størling J, et al. Immune-mediated β-cell destruction in vitro and in vivo—A pivotal role for galectin-3. Biochemical and biophysical research communications. 2006; 344(1):406-15.
31. Saksida T, Nikolic I, Vujicic M, Nilsson UJ, Leffler H, Lukic ML et al. Galectin-3 deficiency protects pancreatic islet cells from cytokine-triggered apoptosis in vitro. Journal of cellular physiology. 2013; 228(7):1568-76.
32. Mensah-Brown EP, Al Rabesi Z, Shahin A, Al Shamsi M, Arsenijevic N, Hsu DK. Targeted disruption of the galectin-3 gene results in decreased susceptibility to multiple low dose streptozotocin-induced diabetes in mice. Clinical Immunology. 2009; 130(1):83-8.
33. Jovicic N, Petrovic I, Pejnovic N, Ljujic B, Miletic Kovacevic M et al. Transgenic Overexpression of Galectin-3 in Pancreatic β Cells Attenuates Hyperglycemia in Mice: Synergistic Antidiabetic Effect With Exogenous IL-33. Frontiers in Pharmacology. 2021;12(2799).
34. Radosavljevic G, Volarevic V, Jovanovic I, Milovanovic M, Pejnovic N, Arsenijevic N et al. The roles of Galectin-3 in autoimmunity and tumor progression. Immunol Res. 2012; 52(1-2):100-10
35. Sano H, Hsu DK, Apgar JR, Yu L, Sharma BB, Kuwabara I et al. Critical role of galectin-3 in phagocytosis by macrophages. The Journal of clinical investigation. 2003; 112(3):389-97.
36. Radosavljevic GD, Pantic J, Jovanovic I, Lukic ML, Arsenijevic N. The two faces of galectin-3: roles in various pathological conditions. Serbian Journal of Experimental and Clinical Research. 2016; 17(3):187-98.).
37. DeFronzo RA, Ferrannini E, Groop L, Henry RR, Herman WH, Holst JJ et al. Type 2 diabetes mellitus. Nature reviews Disease primers. 2015 Jul; 1(1):1-22.
38. Li P, Liu S, Lu M, Bandyopadhyay G, Oh D, Imamura T et al. Hematopoietic-derived galectin-3 causes cellular and systemic insulin resistance. Cell. 2016; 167(4):973-84.

39. Pejnovic NN, Pantic JM, Jovanovic IP, Radosavljevic GD, Milovanovic MZ, Nikolic IG et al. Galectin-3 deficiency accelerates high-fat diet–induced obesity and amplifies inflammation in adipose tissue and pancreatic islets. Diabetes. 2013; 62(6):1932-44.
40. Yuan Y, Zhou J, Hu R, Zou L, Ji L, Jiang G. Piperine protects against pancreatic β-cell dysfunction by alleviating macrophage inflammation in obese mice. Life Sciences. 2021; 274:119312
41. Weigert J, Neumeier M, Wanninger J, Bauer S, Farkas S, Scherer MN et al. Serum galectin-3 is elevated in obesity and negatively correlates with glycosylated hemoglobin in type 2 diabetes. The Journal of Clinical Endocrinology & Metabolism. 2010; 95(3):1404-11.
42. Yilmaz H, Cakmak M, Inan O, Darcin T, Akcay A. Increased levels of galectin-3 were associated with prediabetes and diabetes: new risk factor? Journal of endocrinological investigation. 2015;38: 527-33
43. Jin Q-h, Lou Y-f, LI T-l, Chen H-h, Qiang L, HE X-j. Serum galectin-3: a risk factor for vascular complications in type 2 diabetes mellitus. Chinese medical journal. 2013;12: 2109-15.
44. Ohkura T, Fujioka Y, Nakanishi R, Shiochi H, Sumi K, Yamamoto N, et al. Low serum galectin-3 concentrations are associated with insulin resistance in patients with type 2 diabetes mellitus. Diabetology & metabolic syndrome. 2014;6: 106.
45. Zatterale F, Longo M, Naderi J, Raciti GA, Desiderio A, Miele C et al. Chronic adipose tissue inflammation linking obesity to insulin resistance and type 2 diabetes. Frontiers in physiology. 2020; 10:1607.
46. Pejnovic NN, Pantic JM, Jovanovic IP, Radosavljevic GD, Djukic AL, Arsenijevic NN et al. Galectin-3 is a regulator of metaflammation in adipose tissue and pancreatic islets. Adipocyte. 2013; 2(4):266-71.
47. Petrovic I, Pejnovic N, Ljujic B, Pavlovic S, Miletic Kovacevic M, Jeftic I et al. Overexpression of galectin 3 in pancreatic β cells amplifies β-Cell apoptosis and islet inflammation in Type-2 diabetes in mice. Frontiers in endocrinology. 2020;11:30.
48. Böni-Schnetzler M, Meier DT. Islet inflammation in type 2 diabetes. InSeminars in immunopathology 2019 Jul (Vol. 41, No. 4, pp. 501-513). Springer Berlin Heidelberg.
49. Burguillos MA, Svensson M, Schulte T, Boza-Serrano A, Garcia-Quintanilla A, Kavanagh E et al. Microglia-secreted galectin-3 acts as a toll-like receptor 4 ligand and contributes to microglial activation. Cell reports. 2015; 10(9):1626-38.
50. Caberoy NB, Alvarado G, Bigcas JL, Li W. Galectin-3 is a new MerTK-specific eat-me signal. J Cell Physiol. 2012; 227(2):401-7.
51. Cucak H, Grunnet LG, Rosendahl A. Accumulation of M1-like macrophages in type 2 diabetic islets is followed by a systemic shift in macrophage polarization. J Leukoc Biol. 2014; 95(1):149-60.
52. Hu S, Kuwabara R, Beukema M, Ferrari M, de Haan BJ, Walvoort MT et al. Low methyl-esterified pectin protects pancreatic β-cells against diabetes-induced oxidative and inflammatory stress via galectin-3. Carbohydrate Polymers. 2020; 249:116863.
53. Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. Nature reviews immunology. 2011; 11(2):85-97.
54. Maechler P, Wollheim CB. Mitochondrial function in normal and diabetic β-cells. Nature. 2001; 414: 807-812.
55. Wiederkehr A, Wollheim CB. Impact of mitochondrial calcium on the coupling of metabolism to insulin secretion in the pancreatic β-cell. Cell calcium. 2008; 44: 64-76.
56. Chen J, Stimpson SE, Fernandez-Bueno GA, Mathews CE. Mitochondrial reactive oxygen species and type 1 diabetes. Antioxidants & redox signaling. 2018; 29(14):1361-72.
57. Panigrahy SK, Bhatt R, Kumar A. Reactive oxygen species: sources, consequences and targeted therapy in type 2 diabetes. Journal of drug targeting. 2017; 25: 93-101.
58. Tajeddine N. How do reactive oxygen species and calcium trigger mitochondrial membrane permeabilisation? Biochimica et Biophysica Acta (BBA)-General Subjects. 2016; 1860: 1079-1088.
59. Iacobini C, Menini S, Oddi G, Ricci C, Amadio L, Pricci F, et al. Galectin-3/AGE-receptor 3 knockout mice show accelerated AGE-induced glomerular injury: evidence for a protective role of galectin-3 as an AGE receptor. The FASEB journal. 2004; 18: 1773-5.
60. Pugliese G, Pricci F, Iacobini C, Leto G, Amadio L, Barsotti P, et al. Accelerated diabetic glomerulopathy in galectin-3/AGE receptor 3 knockout mice. The FASEB Journal. 2001; 15: 2471-9.
61. D'Hertog W, Maris M, Ferreira GB, Verdrengh E, Lage K, Hansen DA et al. Novel insights into the global proteome responses of insulin-producing INS-1E cells to different degrees of endoplasmic reticulum stress. Journal of proteome research. 2010; 9(10):5142-52.
62. Berridge MJ. The endoplasmic reticulum: a multifunctional signaling organelle. Cell Calcium 2002;32(5–6):235–249.
63. Worley 3rd JF, McIntyre MS, Spencer B, Mertz RJ, Roe MW, Dukes ID. Endoplasmic reticulum calcium store regulates membrane potential in mouse islet beta-cells. Journal of Biological Chemistry. 1994;269(20):14359-62.
64. Zeeshan HM, Lee GH, Kim HR, Chae HJ. Endoplasmic reticulum stress and associated ROS. International journal of molecular sciences. 2016; 17(3):327.
65. Bhandary B, Marahatta A, Kim HR, Chae HJ. An involvement of oxidative stress in endoplasmic reticulum stress and its associated diseases. International journal of molecular sciences. 2013; 14(1):434-56.
66. Sabatini PV, Speckmann T, Lynn FC. Friend and foe: β-cell Ca^{2+} signaling and the development of diabetes. Mol Metab. 2019; 21:1-12.
67. Rocha M, Diaz-Morales N, Rovira-Llopis S, Escribano-Lopez I, Bañuls C, Hernandez-Mijares A et al. Mitochondrial dysfunction and endoplasmic reticulum stress in diabetes. Current Pharmaceutical Design. 2016; 22(18):2640-9.

68. Hasnain SZ, Prins JB, McGuckin MA. Oxidative and endoplasmic reticulum stress in b-cell dysfunction in diabetes. J Mol Endocrinol. 2016; 56(2):33-54.
69. Lee JW, Kim WH, Yeo J, Jung MH. ER stress is implicated in mitochondrial dysfunction-induced apoptosis of pancreatic beta cells. Molecules and cells. 2010; 30(6):545-9.
70. Weinberg JM. Lipotoxicity. Kidney international. 2006 Nov 1;70(9):1560-6.
71. Oh YS, Bae GD, Baek DJ, Park EY, Jun HS. Fatty acid-induced lipotoxicity in pancreatic beta-cells during development of type 2 diabetes. Frontiers in endocrinology. 2018; 9:384.
72. Yazıcı D, Sezer H. Insulin Resistance, Obesity and Lipotoxicity. Adv Exp Med Biol. 2017;960:277-304.
73. Engin AB. What Is Lipotoxicity? Adv Exp Med Biol. 2017; 960:197-220.
74. Suganami T, Tanaka M, Ogawa Y. Adipose tissue inflammation and ectopic lipid accumulation. Endocrine journal. 2012:EJ12-0271.
75. Pugliese G, Iacobini C, Ricci C, Fantauzzi CB, Menini S. Galectin-3 in diabetic patients. Clinical Chemistry and Laboratory Medicine (CCLM). 2014; 52: 1413-23.

A RARE CASE OF THE MALIGNANT PHYLLODES BREAST TUMOR - CASE REPORT

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ABBREVIATIONS

BIRADS - breast imaging reporting and data system
CNB - core needle biopsy
SI -signal intensity
Ki67 - protein, proliferation marker of the tumor cells
MRI - magnetic resonance imaging
p53 - cellular tumor antigen
EGFR - epidermal growth factor receptor
STIR, T1W, T2W- examination magnetic resonance sequences

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ABSTRACT

The phyllodes tumor (cystosarcoma phyllodes) is a rare fibroepithelial neoplasm presenting less than 1% of all breast tumors. Based on histologic features World Health Organization (WHO) classifies into benign, border line and malignant tumors (the rarest). Only around 5-15% of all malignant form cases metastasize hematogeneosly in the lung, bone and brain. Clinically are mostly presented the phyllodes tumor mostly presents as a rapid growth, palpabile, painless, elastic, oval or lobulated masses over 5 cm among women between 40 to 50 years old. On ultrasound and mammography these tumors usually resembles other round or oval benign lesions, mostly like fibroadenomas. These tumors on ultrasound and mammography usually resembles other round or oval benign tumors, mostly like fibroadenomas. Ultrasound guided core needle biopsiy (CNB) performed under ultrasound is a gold standard for palpabile breast masses. Surgical resection remains the gold standard of treatment. To date, there is no consensus regarding the recommendations for radiotherapy, hormonal therapy and systemic chemotherapy. We present the case of 30 years old woman, who noted a painless mass in her left breast, which was gradually increasing in size. Clinical, ultrasound and magnetic resonance examination confirmed the existence of tumor formation without the possibility of making an accurate diagnosis. Malignant phyllodes tumor was initially diagnosed by core biopsy. The patient underwent a radical modified mastectomy and postoperative radiotherapy. During the follow-up for the first 11 months, our patient has been feeling well without signs of local recurrence and metastasis. The aim of our case is to emphasize the importance of early diagnosis and treatment of this rare breast tumor.

Treatment is based on the size and the extent of the mass with surgical resection and adequate margins extremely important in successful outcome. We present the case of 30 old woman, who noted a painless mass in her left breast, which was gradually increasing in size. She was diagnosed with ultrasound and magnetic resonance imaging and the diagnosis of the malignant phyllodes tumor was confirmed by patohistological verification.

Keywords: *Malignant phyllodes tumor, fibroadenoma, core needle biopsy , mastectomy.*

INTRODUCTION

Phyllodes tumors are very rare breast neoplasms and account for less than 1% of all tumors. To date, no genetic predisposition has been identified for the development of this type of tumor except in individuals with Li-Fraumeni syndrome (1). Some studies cite trauma, pregnancy, and hormonal imbalance as possible risk factors, based on the stimulation of endothelin1 as a mediator of breast fibroblast growth (1). Biologically and histologically, they are classified as benign (most common), borderline and malignant types (very rare) (1). 5-15% of malignant cases metastasize hematogenously, most commonly to the lungs, bones, and brain (1). Clinically, they are presented as fast-growing, mobile, painless masses, over 5 cm in size, in women between 40-50 years of age. Ultrasound and mammography show sharp-edged, oval or round changes, with clear contours, which most often resemble fibroadenomas (1,2). Magnetic resonance imaging (MR) with basic and advanced sequences allows a more detailed evaluation of the phyllodes tumor. These are most often lobulated masses, clear contours, mixed structures, with internal septations and bleeding foci, hypointense on T1W, heterogeneous SI on T2W and STIR sequences, dynamic characteristics in the form of fast initial "wash in" and TIC curve by plateau type or "wash out ". MRI spectroscopy with choline quantification is very important as an indicator of malignant type (2). Ultrasound-guided core needle biopsy (CNB) is the next step in diagnosis and is a minimally invasive biopsy technique for palpable suspicious changes in the breast. Pathohistological analysis of the obtained samples shows the epithelial and stromal component, whose stroma determines the malignant potential of the tumor (2,3). Analysis of immunohistochemical markers p53, Ki67, EGFR has not been shown to be of the clinical significance for this type of neoplasm in previous studies (3).

Because of the rarity of this tumor there is a lack of sufficient prospective study data, especially for border and malignant tumors . 5-year disease-free survival rates of 96% is for benign and 66% for malignant types, while median survival after metastatic disease is poor, ranging from 4 to 17 months (4). The malignant type most often metastasizes to the lungs (66%), bones (28%) and brain (9%) (4,5).

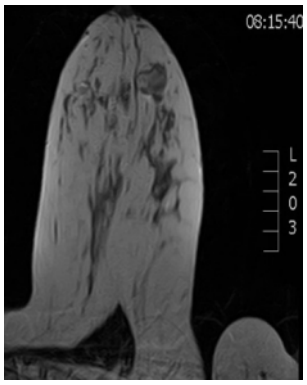
According to the National Comprehensive Cancer Network (NCCN) guidelines for breast cancer, the management of phyllodes tumor dimension over 3.0 cm is a wide surgical excision with clean margins (≥ 1.0 cm) in the form of tumorectomy or mastectomy without axillary staging (6).The surgical approach is the method of choice in the treatment of phyllodes tumors in the form of tumorectomy or radical mastectomy with axillary dissection. The use of radiotherapy and chemotherapy depends exclusively on the extensiveness and biological nature of the tumor itself. Malignant phyllodes tumors do not respond to hormone therapy and chemotherapy, which are standard in the treatment of breast cancer. After postoperative treatment these patients should be closely followed up with regular clinical and ultrasound examinations (7,8).

CASE REPORT

We present the case of a 30-year-old patient in good general condition who noted a fast-growing, painless, clearly limited mass in her left breast. She had no other symptoms, no comorbidities and no data on trauma. Also, she had regular menstrual cycles, did not give birth and no family history of breast cancer. Anamnestic without other ailments and comorbidities, she did not give birth, regular menstrual cycles, no data on trauma, negative family history of breast cancer. The previous ultrasound examination peformed a year ago was unremarkable. Clinical examination revealed a palpabile formation measuring about 3 cm without significant axillary lymphadenopathy. Ultrasound examination detected a lobulated tumor mass dimension 35x28x11mm, heteroechoic structure, horizontally oriented with few oval shaped axillary lymph nodes up to 9 mm, mostly like reactive type, which is classified as BIRADS 4a (Figure 1).

Figure 1. Ultrasound axial plane showing lobulated, heteroechoic tumor mass

Figure 2. Axial MRI T2W section showing heterointense tumor mass



The review was supplemented by MRI examination, which showed retro and paramamilar tumor mass, in the lower outer quadrant, mixed signal intensity (SI) in T2W / STIR sequences, decreased SI in T1W sequence, postcontrast elevated SI, dynamic characteristics of rapid "wash in", with a curve of plateau type with few oval shaped lymph up to 10 mm, classified as BIRADS 4 (Figures 2,3,4). Differential diagnostic could correspond to fibroadenoma or fibroadenoma like lesions (FA like lesion). CNB was performed under

ultrasound control under local anesthesia and three samples were taken.

Figure 3. Axial poscontrast T1W section showing hiperintense, lobulated tumor mass

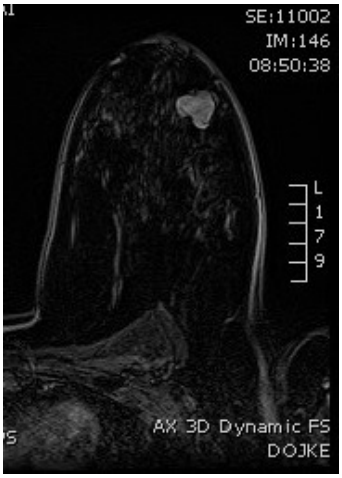


Figure 4. MIP poscontrast reconstruction axial plane showing tumor mass neovascularisation

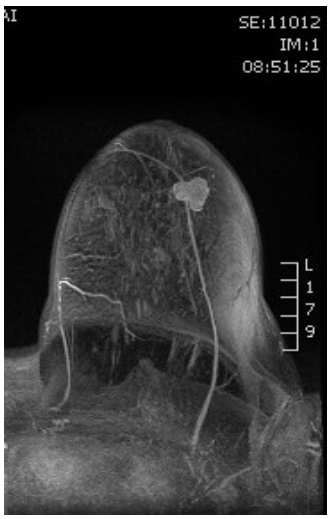


Figure 5, 6. Pathohistological findings showing extensive stromal component, moderate to pronounced cellularity, focally high degree of atypia, with visible mitosis and minor foci of necrosis

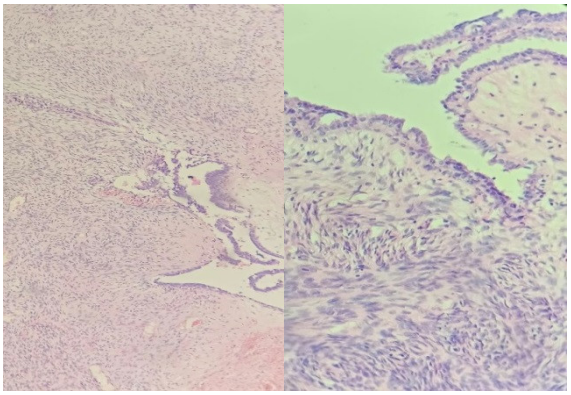
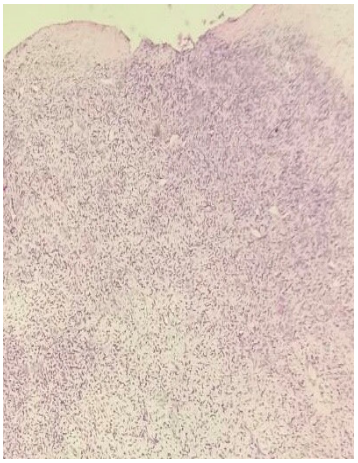


Figure 7. Pathohistological findings showing moderate cellularity, focal moderate atypia, stromal component dominance



A pathohistological finding of malignant phyllodes tumor low grade was obtained, where two component tumor proliferation is observed in the analyzed tissue biopsy samples, in which the stromal component dominates in relation to the epithelial one. The epithelial component is sparse, leaf-like type of growth without signs of atypia. The stromal component is extensive, moderate to pronounced cellularity, moderate, focally high degree of atypia, with visible mitosis and minor foci of necrosis (Figure 5,6).

The case was presented in the oncologic consilium and taking into consideration the ultrasound, MRI examination and the pathohistological findings the surgical treatment was indicated. Retro and paramamilar tumor localization, and required wide excision margins could have limited the surgeon to achieve a good cosmetic result. Also, patient preference resulted in decision for radical mastectomy instead of breast sparing surgery as a preferable treatment of choice. Patient underwent a left radical modified mastectomy (Madden). Postoperative pathohistological findings confirmed the diagnosis of malignant phyllodes tumor low grade in which cellularity was moderate, atypia focal moderate, epithelial/mesenchymal component not monitored, dominance of stromal component, mitotic index focal about 5/10 HPF, necrosis: presents in about 10% of tumor tissue, growth mode mostly expansive, focally infiltrative, with negative resection margins and no significant morphological changes of the surrounding breast tissue. An immunohistochemical analysis was not performed. The presence of secondary tumor deposits (0 + / 9) were not observed in any of the nine histologically verified, reactively altered lymph nodes (figure 7).

The patient postoperative recovery was uneventful with well healed wound. She was referred to the oncologic consilium, where postoperative adjuvant radiotherapy was indicated. During her follow up for the first 11 months, the first clinical and ultrasound examination in 3 month and the second in 6 month showed no signs of local recurrence and metastasis.

DISCUSSION

Phyllodes tumors are very rarely mentioned in daily routine work. The great ultrasound resemblance to benign changes, primarily fibroadenomas, can lead a radiologist to misdiagnosis. An important anamnestic data on the rapid growth of changes in the breast, usually within a few months, as in the case of our patient, should lead an experienced clinician to suspect a phyllodes tumor with the need for further diagnostic procedures (4,5).

Ultrasound is the first diagnostic modality of evaluation of pathological changes, high sensitivity and specificity with the possibility of distinguishing cystic and solid lesions, but as a method it is very subjective and dependent on the experience of clinicians (3). Ultrasound characteristics that may indicate a malignant form are: dominant hypoechoic structure, irregular contours, and detectable vascularization. In our patient, based on the heteroechoic structure and pronounced vascularization of the pathological change, a suspicion of a phyllodes tumor was established, with an indication for MRI examination (4). Mammography was not performed in our case, due to the pronounced glandular structure of the breast and reduced diagnostic sensitivity.

MRI represents a unique combination of non-invasiveness and the possibility of precise visualization of anatomy, localization, characterization of pathological changes, assessment of disease extent, planning of adequate surgical treatment, as well as regular monitoring (4,5). In the case of our patient, there is a change in lobular contours, mixed structures with foci of necrosis, markedly vascularized without axillary lymphadenopathy, postcontrast dynamically rapid wash in and plateau-type curve. In previous studies, such dynamic characteristics indicate the possibility of benign change in 32% and malignancy in 68% of cases (5). Minimally invasive CNB under ultrasound control is indicated.

Ultrasound guided CNB under local anesthesia is the gold standard in the diagnosis of palpable breast masses . The method is fast, painless, minimally invasive and enables obtaining quality pathohistological samples for the purpose of efficient diagnosis. In the case of our patient, the obtained samples were sufficiently representative to diagnose a malignant phyllodes tumor.

According to the National Comprehensive Cancer Network (NCCN) guidelines for breast cancer, the management of phyllodes tumor dimension over 3.0 cm is a surgical wide excision with clean margins ($\geq$ 1.0 cm) in the form of tumorectomy or mastectomy without axillary staging (6). Our patient underwent a radical mastectomy with axillary dissection, with postoperative radiotherapy. In terms of comparision breast conserving surgery and mastectomy for the treatment of borderline and malignant phyllodes tumors previous studies have shown conflicting results (9,10,11). Local recurrence appears to be related to the extent of the initial surgery. Providing clear margins are maintained than breast conservation is preferable, but even with wide surgical resection, the local recurrence rate remains high 8 to 36% (11,12). Some studies showed no significant difference between breast conserving surgery and mastectomy regarding overall survival, although the patients who underwent breast conserving surgery had higher recurrence rate. Phyllodes tumors grow radially, compress the adjacent breast parenchyma and create a pseudo-capsule through which tongues of phyllodes stroma may protrude and grow into adjacent breast tissue. For this reason, many authors support that mastectomy may be the best surgical procedure for the borderline or malignant phyllodes tumor. In our case localization, required wide excision margins, pathohistological findings and patient preference resulted in decision for radical modified mastectomy instead of breast conserving surgery as a treatment of choice (10,12).

The rate of local recurrence in the first 2 years is high ranging from 10 to 40% in all three histological types and directly depends on the size and the hystological grade of the primary operated tumor (8,11,13). Compared to benign and borderline, malignant phyllodes types regardless tumor size have a higher rate of disease recurrence, decreased overall survival and distant metastasis. It is well documented that recurrent phyllodes tumors can progress to a more malignant phenotype comparing to the the initial tumor. Also, it was showed that stromal overgrowth, high stromal cellularity, large tumor size and mitotic rate are histological features associated with high risk of recurrence. Specifically, data support that stromal overgrowth is a key predictor of disease relapse, while stromal cellularity and high mitotic rate are less consistently (8,13). To date, no single immunomarker can reliably distinguish between benign and borderline phyllodes and also to accurately predict clinical behavior in all cases. Further studies are required to establish the correct combination of immunomarkers (14). In our case pathohistological findings showed dominance of tumor stromal component,stromal cellularity, atypia and mitotic rate, whereby this tumor could be classified as a tumor with high reccurence risk.

The incidence of axillary lymph node involvement in malignant form is low, ranging from 1.1% to 3.8% (8). In spite of the lack of supporting data for lymph node dissection review of current surgical practice reveals that axillary staging continues to be performed for many cases of malignant phyllodes tumors (12,16). Data regarding sentinel lymph node biopsy in phyllodes tumors are lacking. In our case ultrasound and MRI examination revealed insignificant lymphadeno-pathy, most likely of the reactive type, but given the pathohistological findings it was decided to perform axillary dissection.

Previous studies have showed that young age (<35 years) is an adverse prognostic factor for reccurence free survival in patients with malignant phyllodes tumor, while older age (>50 years) was correlated with advanced tumor extension and poor cancer-specific survival (8,15). These findings may help to distinguish the patients at high risk of developing local recurrence and imply that surgery such as wide local

excision and mastectomy, should be performed on younger patients, especially with malignant phyllodes tumors.

Still, there is no consensus regarding the recommendations for radiotherapy, hormonal therapy and systemic chemotherapy. Because of the rarity of this tumor there is a lack of sufficient prospective study data, especially for border and malignant tumors (9,16,17). Currently, the use of adjuvant therapy remains controversial, but it has been more frequently utilized. According to a study in the National Cancer Database from the American College of Surgeons'Commission on Cancer involving 3120 patients in 2008-2009 year, adjuvant radiotherapy was used in 19.5% of cases, more than doubled compared to the rate of 9.5% in 1998-1999 year. Many authors support that in patients. Many authors support that in patients with malignant phyllodes tumor adjuvant radiotherapy may be more effective in younger patient and should be used without consideration of the surgery type. It is associated with an increased time to local recurrence compared to women who had surgery alone (10,16,17). In our case taking into account patients age and pathohistological findings the oncologic consilium recommended the adjuvant radiotherapy.

To date, there has been only one prospective study involving 28 patients, which has showed that chemotherapy has little effect on survival (9). The sample sizes of few retrospective studies were too small to prove the efficacy of chemotherapy (9,18). For this reason, most clinicians avoid chemotherapy as a first-line treatment due to lack of evidence. Our patient did not received chemotherapy.

After operative treatment, this group of patients should be followed up with regular physical and ultrasound examinations (8,9,19). In our case during postoperative follow up for the first 11 months, patient has been feeling well, with good quality of life and no current signs of local recurrence and metastasis.

Malignant phyllodes tumors do not respond to hormonal and chemotherapy, which is standardly used in the treatment of the breast cancer. The malignant type most often metastasizes to the lungs (66%), bones (28%) and brain (9%). The incidence of axillary lymph node involvement in malignant form is low, ranging from 1.1% to 3.8% In the case of hematogenous dissemination, this type of tumor is treated according to the sarcoma treatment protocol. Because, there are not many reports of phyllodes tumors, past case reports and studies have reported that the rate of local recurrence in all three histological types or in the form of distant metastases in the first 2 years is up to 27% and directly depends on the size of the primary operated tumor .Also, 5-year disease-free survival rates of 96% is for benign and 66% for malignant .types., while median survival after metastatic disease is poor, ranging from 4 to 17 months. Compared to benign and borderline, malignant phyllodes types have a higher rate of disease recurrence, decreased overall survival, and distant metastasis. After surgical treatment, this group of patients requires regular monitoring in the form of ultrasound examinations. (8,9) Our patient has been feeling well since the time of surgery with no current signs of metastasis or local recurrence.

Phyllodes breast tumors, especially malignant cases are very rarely referred to by foreign and domestic authors. One of the work is a review of a patient who was treated for a giant exulcerated borderline philodes breast tumor (10,20).

CONCLUSION

We conclude that phyllodes tumors, especially malignant forms are very rarely thought of in routine daily work and there is a high likelihood of missing these tumors. Important anamnestic data on the rapid growth of the breast palpable mass, regardless of the age of the patient with the initial ultrasound examination is should be the main indication for further diagnostic evaluation. It is necessary to be vigilant in the prompt diagnosis and management of this rare tumors. The early diagnosis is crucial for improving the overall outcome of the disease after treatment, as well as for promoting the good quality of life of the patient. With a precise choice of diagnostic multimodalities this tumor can be successfully treated. Still, there are various types of treatment modalities for phyllodes tumors. The surgical treatment is the gold standard, but should be better tailored in the management of malignant or recurrent tumors. The role of adjuvant radiotherapy and chemotherapy remains uncertain, but encouraging. It is certain, that future prospective studies with more patients are required.

REFERENCES

1. Chu JS. Prognostic factors in phyllodes tumor of the breast: are immunohistochemical biomarkers useful? J Chin Med Assoc 2004; 67: 1-2.
2. Mylvaganam S, Toro C, Frank L, Vesty S, Thrush S. Phyllodes tumors of the breast: the best practice for follow-up. Updat Surg 2015; 67: 91-5.
3. Wang H, Wang X, Wang CF. Comparison of clinical characteristics between benign borderline and malignant phyllodes tumors of the breast. Asian Pac J Cancer Prev 2014; 15: 10791-5.
4. Yom CK, Han W, Kim SW, Park SY, A. Park IA, Noh DY. Reappraisal of conventional risk stratification for local recurrence based on clinical outcomes in 285 resected phyllodes tumors of the breast. Ann Surg Oncol 2015; 22: 2912-8.
5. Rodrigues MF, Truong PT, McKevitt EC, Weir LM, Knowling MA, Wai ES. Phyllodes tumors of the breast: the British Columbia cancer agency experience. Cancer Radiother 2018; 22: 112-9.
6. National Comprehensive Cancer Network *Breast Cancer. Clinical Practice Guidelines in Oncology (NCCN Guidelines).* Plymouth Meeting, PA: NCCN; 2019.
7. Chang J, Denham L, Dong EK, Malek K, Lum SS. Trends in the diagnosis of phyllodes tumors and fibroadenomas before and after release of WHO classification

standards. Annals of Surgical Oncology 2018; 25: 3088-95.
8. Wei J, Tan Y, Cai Y, et al. Predictive factors for the local recurrence and distant metastasis of phyllodes tumors of the breast : a retrospective analysis of 192 cases at a single center .China J Cancer.2014 ;33: 492-500
9. Onkendi EO, Jimenez RE, Spears GM, Harmsen WS, Ballman KV, Hieken TJ. Surgical treatment of borderline and malignant phyllodes tumors: the effect of the extent of resection and tumor characteristics on patient outcome. Ann Surg Oncol 2014; 21(10): 3304-9.
10. Abe H, Teramoto A, Takei Y, Tanaka Y, Yoneda G. Malignant phyllodes of the breast with rapid progression: a case report. Surg Case Rep 2020; 6: 308.
11. Spanheimer PM, Murray MP, Zabor EC, Stempel M, Morrow M, Van Zee KJ, et al. Long-term outcomes after surgical treatment of malignant/borderline phyllodes tumors of the breast. Ann Surg Oncol 2019; 26(7): 2136-43.
12. Thind A, Patel B, Thind K, Isherwood J, Phillips B, Dhaliwal K, et al. Surgical margins for borderline and malignant phyllodes tumours. Ann R Coll Surg Engl 2020; 102(3): 165-73.
13. Sawalhi S, Al-Shatti M. Phyllodes tumor of the breast: a retrospective study of the impact of histopathological factors in local recurrence and distant metastasis. *Ann Saudi Med* 2013; 33(2): 162-8.
14. Shubham S., Ahuja A, Bhardwaj M. Immunohistochemical expression of Ki-67, p53, and CD10 in phyllodes tumor and their correlation with its histological grade. J Lab Physicians 2019; 11(4): 330-4.
15. Spitaleri G, Toesca A, Botteri E, Bottiglieri L, Rotmensz N, Boselli S, et al. Breast phyllodes tumor: a review of literature and a single center retrospective series analysis. Crit Rev Oncol Hematol 2013; 88(2): 427-36
16. Kim YJ, Kim K. Radiation therapy for malignant phyllodes tumor of the breast: an analysis of SEER data. Breast 2017; 32: 26-32.
17. Gnerlich JL, Williams RT, Yao K, Jaskowiak N, Kulkarni SA. Utilization of radiotherapy for malignant Phyllodes tumors: analysis of the National Cancer Data Base, 1998-2009. *Ann Surg Oncol.* 2014;21(4):1222-1230.
18. Varghese SS, Sasidharan B, Manipadam MT, Paul MJ, Backianathan S. Radiotherapy in phyllodes tumour. *J Clin Diagn Res* 2017; 11(1): XC01-XC03.
19. Morales-Vásquez F, Gonzalez-Angulo AM, Broglio K, Lopez-Basave HN, Gallardo D, Hortobagyi GN, et al. Adjuvant chemotherapy with doxorubicin and dacarbazine has no effect in recurrence-free survival of malignant phyllodes tumors of the breast. Breast J. 2007; 13(6): 551-6.
20. Spasić M, Milošević B, Mitrović S, Marković N, Pavlović M, Jevdjić J, et al. Giant exulcerated phylloid breast tumor - a case report. Serbian Journal of Experimental and Clinical Research 2020; 21(1): 83-6.

COMBINED ORTHODONTIC AND PROSTHETIC TREATMENT OF A PATIENT WITH ANGLE CLASS II DIVISION 1 MALOCCLUSION: A CASE REPORT

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ABSTRACT

Skeletal class II division 1 malocclusion is primarily characterized by maxillary protrusion, mandibular retrusion or a combination of both. Treatment modalities for these patients are different depending on the age and the severity of the case. In adult patients with skeletal class II division 1, where the discrepancy is very severe, orthodontic and orthognathic surgery combined are often necessary to ensure an appropriate treatment, while prosthodontic treatment may be a challenge due to the skeletal discrepancies of the maxilla and the mandible. This clinical report presents a 45-year-old patient with a diagnosis of Angle Class II division 1 malocclusion. After clinical and radiographic observation and consultation with an orthodontist and maxillofacial surgeon, a combination of surgical and orthodontic therapy was proposed. Considering that the patient refused the surgical procedure due to fear, orthodontic therapy was carried out first, which improved the aesthetic and functional characteristics, but did not give results in increasing the vertical dimension of the occlusion. Therefore, further therapy was prosthetic, and was carried out by making all-ceramic maxillary and mandibular fixed dental prostheses; the patient was successfully rehabilitated and the anteroposterior discrepancy between the dental arches was corrected. Combined orthodontic and prosthetic treatment can be a good option in the complex treatment of patients with skeletal class II division 1 malocclusion providing functional rehabilitation of the stomatognathic system and improving facial appearance and the quality of life of the patient.

Keywords: *Angle class II, malocclusion, prosthetic treatment, orthodontic treatment.*

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INTRODUCTION

Skeletal class II division 1 malocclusion is primarily characterized by maxillary protrusion, mandibular retrusion or a combination of both (1,2). Some studies found that the maxilla in Class II division 1 patients was in a protrusive position in relation to the cranial base, and the mandible was normal in size and position (3). Other studies demonstrated a normal position of the maxilla while the mandible was retrusive (4-6). On the other hand, a few previous studies reported that the skeletal pattern in Class II/1 patients is due to both maxillary protrusion and mandibular retrusion (7,8). Also, the common characteristics of this malocclusion include the normal or proclined maxillary incisors and the normal, proclined or even retroinclined mandibular incisors (9,10). Furthermore, some studies (11,12) found an increase in lower facial height in most of the subjects, while other studies reported that the lower facial height was significantly reduced (7,13). These typical features of Class II/1 may affect facial appearance causing a convex profile, non-aesthetic facial proportions and occlusal disharmonies and may result in negative psychological impacts and functional problems in adult patients (14).

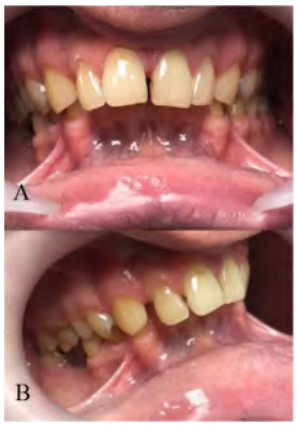
Treatment modalities for this group of patients are different depending on the age and the severity of the case. In adult patients with skeletal class II division 1, where the discrepancy is very severe, orthodontic and orthognathic surgery combined are often necessary to ensure an appropriate treatment (15-17). Prosthodontic treatment for these patients may be a challenge due to the skeletal discrepancies of the maxilla and the mandible (18). In these cases, orthognathic surgical procedures could correct severe skeletal problems and simplify the prosthodontic phase of treatment without damaging the tooth structure (19). For these reasons, a comprehensive multidisciplinary approach including orthodontists, oral and maxillofacial surgeons and prosthodontists is needed to reestablish facial and dental harmony, occlusal function as well as health and stability of the orofacial structures (20).

The objective of this case report was to present multidisciplinary cooperation in treating a patient with Angle class II division 1 in order to improve his facial appearance, self-confidence and provide functionally acceptable result.

CASE REPORT

A 45-year-old male patient was referred to the Department of Prosthodontics, Faculty of Medical Sciences, University of Kragujevac with a chief complaint of protruded maxillary anterior teeth, teeth malalignment, unpleasant aesthetics and difficulty in chewing. The patient had no history of any medical problems. Extraoral examination revealed a convex profile with mild mandibular retrusion, an obtuse nasolabial angle and an everted lower lip. There was no masticatory and facial muscle hyperactivity and no signs of temporomandibular disfunction (TMD). On intraoral examination a severe deep-bite and an increased overjet with anterior maxillary protrusion were detected (Figure 1).

Figure 1. Initial oral examination.

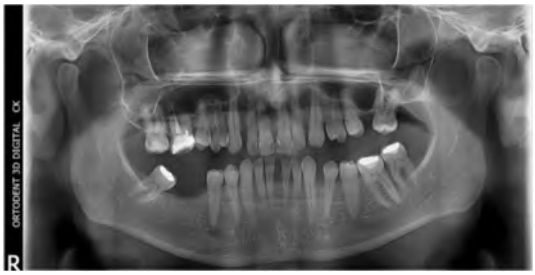


A) Frontal view; B) Lateral view T

he anteroposterior discrepancy of the anterior teeth was very severe and the mandibular incisal edges were occluded with the palatal tissues in the opposing arch. An overbite of 9 mm and an overjet of 12 mm were observed. There was clinical evaluation of reduced VDO. The patient's interocclusal space that was measured between nose and chin tips was 8-9 mm (the difference between physiologic rest position of the mandible and VDO). Furthermore, facial analysis revealed that the maxillary midline was coincident with the facial midline but there was slight deviation of the mandibular midline to the right. Steiner cephalometric analysis was done. The angle of the ANB is greater than 4, by measuring it is 6. The angle of I/Spp is less than 70, by measuring it is determined to be 63. With this analysis, we determined that it is a skeletal irregularity of the II skeletal class with protrusion of the upper incisors. The patient was diagnosed as an Angle Class II division 1 malocclusion.

Intraoral and radiograph examination verified that 26 and 46 were missing and the endodontic treatment of 16 was no adequately performed. Caries lesions were found on the mesial surfaces of 11, 21 and 12. There was no pocket depth of over 2 mm or mobility around any of the remaining teeth. Panoramic radiograph confirmed that all the maxillary and mandibular teeth had favorable crown/root ratio of 2:3 (Figure 2). The patient's oral hygiene was good.

Figure 2. Panoramic radiograph before treatment



The treatment plan was formulated in consultation with an orthodontist and a maxillo-facial surgeon. The first

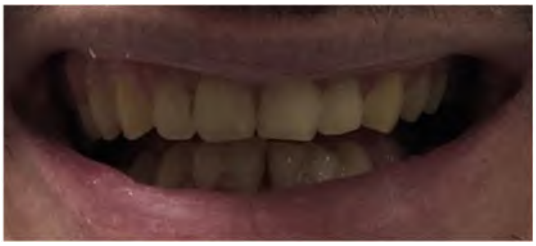
treatment plan offered to the patient was the osteotomy in the upper jaw to move the whole anterior maxillary segment upward and backward with surgical mandibular advancement in the lower jaw. However, the patient was scared of the surgical procedure, so this option was excluded. The other option was the retraction of the upper anterior segment and dental alignment using orthodontic fixed appliances in the upper and lower jaws. Furthermore, full mouth rehabilitation with all-ceramic fixed dental prostheses (FDPs) was planned after the orthodontic treatment in order to provide the desired esthetic and functional result. Also, in this case with the reduced VDO, we considered increasing the VDO since it provides space for the restorative material and enhances the esthetic tooth display, allows the correction of anterior teeth relationships, improves horizontal and vertical overlap, improve lip support and allows the re-establishment of physiologic occlusion (21). As there was clinical evaluation of reduced VDO, full mouth rehabilitation with all-ceramic restorations was planned after the orthodontic treatment.

The patient gave written informed consent before the treatment. Fifteen months after the orthodontic treatment (Figure 3), the maxillary incisors were retruded by 2 mm, the mandibular incisors were protruded by 1 mm, leveling of the occlusal plane of the upper and lower dental arch by 0.5mm according to cephalometric analysis. Facial convexity was also reduced with the retraction of upper lip and marked improvement in aesthetics and function was obtained. Although, there was improvement in the antero-posterior dimension with a decreased overjet, the VDO was reduced. The patient was provided with removable vacuum-formed retainers in the maxilla and mandible. The thickness of the retainers was 2 mm. Retainers have been used for 2 months. The patient's muscle sensitivity and temporomandibular pain were evaluated in this period. No muscle hyperactivity or TMD was found.

Figure 3. Initial preoperative view after the orthodontic treatment

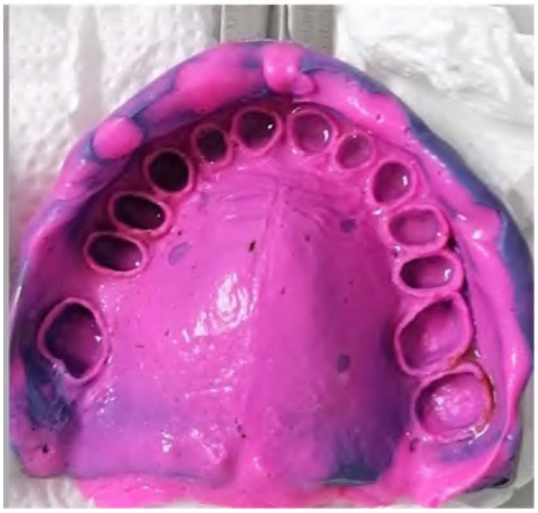
At the first stage of prosthetic rehabilitation, the proper VDO was determined by the Niswonger method (22). The distance between nose and chin tips was measured at the physiologic rest position and compared to the VDO at the centric relation position. The patient's freeway space was 8-9 mm (difference between the physologic rest position and VDO). This method was verified with the closest speaking space method (23). Furthermore, the upper and lower impressions were made in alginate (irreversible hydrocolloid) and the diagnostic casts were poured in dental stone (type IV). The casts were mounted on semi-adjustable articulator (Bio-Art A7plus) using a standard face-bow record and an inter-occlusal record with occlusal registration material (Occlufast rock, Zhermack, Germany). The new VDO was set by approximately 4 mm increase in the incisal guidance pin of the articulator. We followed the rule called a 1:2:3 relationship (24). According to this rule, for each 1 mm that the VDO is increased vertically in the posterior region, the incisal pin of the articulator should be increased vertically by 3 mm. For this reason, the VDO was increased posteriorly by 1.3 mm and the incisal pin of the articulator was increased by 4 mm. Diagnostic wax-up was done at increased VDO and used for fabricating the provisional restorations. Then, the maxillary and mandibular teeth were prepared following principles of tooth preparation (25) with the shoulder of 1 mm wide as a gingival finish line. Provisional restorations were made using the over-impressions that were produced from the diagnostic wax-up. The provisional restorations were cemented with temporary cement (Temp-Bond, Kerr, Switzerland) and the patient's adaptation has been monitored for 2 months (Figure 4).

Figure 4. Provisional restorations fabricated at the increased VDO



During this period, the patient's mastication, muscle sensitivity, temporomandibular pain and phonation were evaluated. No muscle hyperactivity or TMD was detected. Furthermore, the final impressions of the maxillary and mandibular arches were taken with polyvinil-siloxane impression material (I SiL-A-Silicon, Spident, Korea) using one-step technique (Figure 5).

Figure 5. Final impression made in polyvinyl-siloxane using one-step technique



The relationship of the maxillary teeth to the transverse horizontal (hinge) axis was recorded using the arbitrary face-bow. The diagnostic casts (Figure 6 and 7) were mounted on a semi-adjustable articulator using a face-bow record and a centric relation record replicating the relationship between the maxillary and mandibular arches. Furthermore, the

frameworks milled from solid zirconia blocks (Vita In-Ceram) were tried-in.

Figure 6. Working maxillary (right) and mandibular (left) casts

Figure 7. Working maxillary (left) and mandibular (right) casts

The fixed dentures were made as 6 units all-ceramic FDP (13-23), 4 units all-ceramic FDP (24-27), 3 units all-ceramic FDP (14-16) and 1 all-ceramic crown on 17 in the maxilla. In the lower jaw, the units were connected as 7 units all-ceramic FDP (33-44), 3 units all-ceramic FDP (45-47) and 4 units all-ceramic FDP (34-37).

Then, the appropriate shade was selected using 3D-Master VITA guide (Vita Easyshade Compact, Vita Germany). Porcelain layering was made with porcelain VITA VM (9) (Vita Zahnfabric, Germany) (Figure 8 and 9).

Figure 8. 6 units all-ceramic FDP (13-23), 4 units all-ceramic FDP (24-27), 3 units all-ceramic FDP (14-16) and 1 all-ceramic crown on 17 in the maxilla

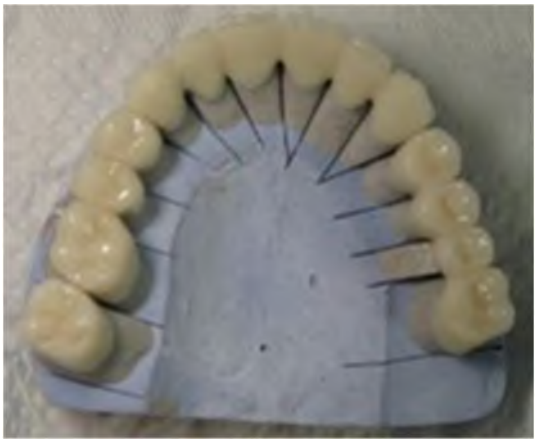
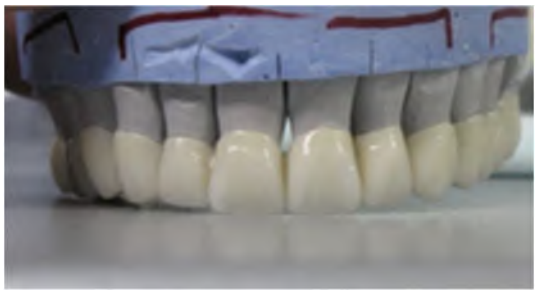


Figure 9. All-ceramic FDP on dental cast model - frontal view



At the final step, porcelain try-in of the maxillary and mandibular fixed dental prostheses (FDPs) were done in the patient's mouth. Interocclusal adjustments were performed in the intercuspal position, protrusive and lateral movements. FDPs were cemented with a dual cure luting composite resin (Multilink Speed, Ivoclar) (Figure 10 and 11). The mutually protected occlusion was established as the concept of occlusion (Figure 12). The patient was provided with a night guard. Oral hygiene instruction and regular check-up were administered. Panoramic X ray was taken after two years follow-up period (Figure 13). Analyzing the radiograph images before and after treatment, bone rarefaction zones in the area of the lower incisors were found on the post-treatment panoramic X-ray. Although the described changes were observed on the radiograph image, there were no clinical signs of insufficient periodontium in the lower anterior region.

Figure 10. Final result of 6 units all-ceramic FDP (13-23), 4 units all-ceramic FDP (24-27), 3 units all-ceramic FDP (14-16) and 1 all-ceramic crown on 17 after cementation

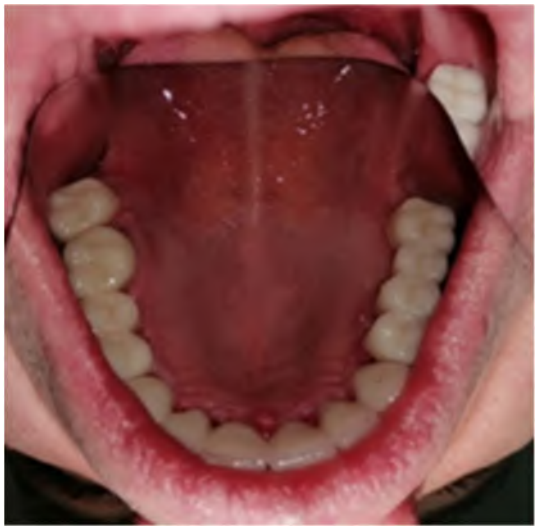


Figure 11. Final result of 7 units all-ceramic FDP (33-44), 3 units all-ceramic FDP (45-47) and 4 units all-ceramic FDP (34-37) after cementation

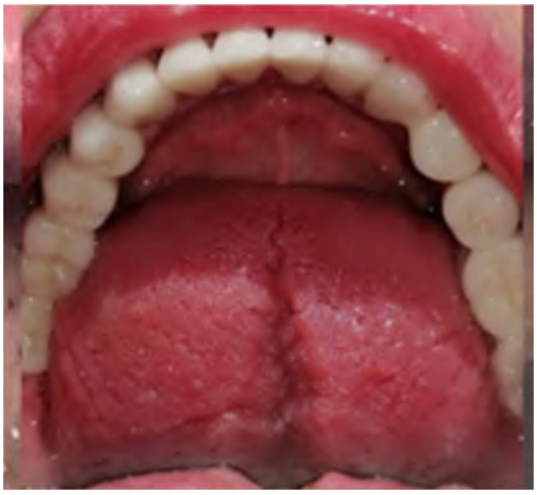


Figure 12. Final result after cementation all-ceramic FDPs in the maxillary and mandibular arches

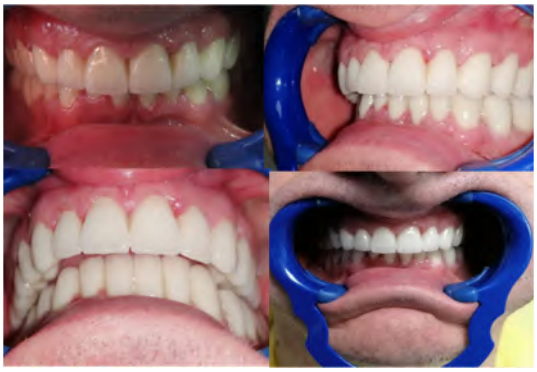
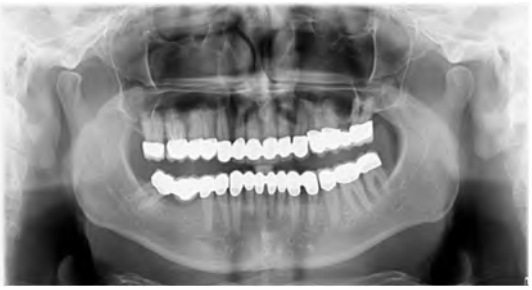


Figure 13. Panoramic radiograph after treatment



DISCUSSION

An interdisciplinary approach during the planning of the treatment in patients with Angle class II division 1 is very important for establishing normal facial profile, good occlusion and function. In the present case, surgical-orthodontic treatment could be very good option producing harmonious facial, skeletal and soft tissue relationships and good occlusal function. However, orthognathic reconstruction might have disadvantages including the costs, a wide variety of intraoperative and postoperative complications and length of recovery. Also, the patient was young and preferred to have a non-surgical treatment. For this reason, to restore dentition and improve the facial appearance, the combined orthodontic and prosthetic treatment was suggested to the patient.

In the present clinical case, comparing the initial and post orthodontic result, it was noticed that there was an improvement in the skeletal and dental aspects with a greater reduction of overjet. However, the VDO was reduced and the patient's freeway space was 8-9 mm. With an orthodontic treatment, it is possible to extrude the posterior teeth and increase the VDO and different orthodontic appliances can be used for this purpose (26, 27). However, some of them showed serious mechanical and biological side effects (28) and some were of questionable effectiveness (27). In this case, in order to reduce the orthodontic treatment time (the treatment time was only 15 months), we initially made two stages treatment plan. Firstly, we wanted to retract the upper anterior segment and align the teeth using orthodontic fixed appliances in the upper and lower jaws. Secondly, we wanted to restore the reduced VDO by full mouth rehabilitation with all-ceramic FDPs.

Previous studies showed that increasing VDO by restorative procedures should be performed cautiously, because any alteration of the VDO will subsequently interfere with the physiology of the masticatory system and the patient's ability to adapt (29, 30). Increasing the VDO may result in hyperactivity of the masticatory muscles, elevation in occlusal forces, bruxism and TMDs (29-31). On the other hand, some authors have reported that such symptoms are transitory (31-33). In the present case, increased VDO was tested first with removable vacuum-formed retainers worn for 2 months as a diagnostic tool to evaluate patient's adaptation to the altered VDO. TMD or muscle pain were not observed during this period. Secondly, at the first stage of prosthetic rehabilitation, the patient was provided with temporaries at the increased VDO. Also, the patient's adaptation has been tested for 2 months in accordance with previous studies indicated that the testing period of increased VDO with provisional restorations could be 2–6 months (34, 35) and no muscle hyperactivity or TMD was detected. Eventually, full mouth rehabilitation including the fabrication of all-ceramic FDPSs in the maxilla and mandible was done and both the facial appearance and the occlusion were significantly improved at the end of the prosthetic rehabilitation. This treatment option was chosen because of a possible shifting of the teeth after the orthodontic treatment and orthodontic relapse that can occur as a result of forces from the periodontal fibers around the teeth which tend to pull the teeth back towards their pretreatment positions as it has been discussed widely in the literature (36, 37). The units of fixed dentures were connected to maintain the teeth in the position they were in at the end of orthodontic treatment. On the other hand, despite extensive materials and technique developments, the chipping of the veneering layer in zirconia-based crowns is still not fully solved. There are many factors that could be associated with chipping fracture of layered zirconia, but residual and transient thermal stresses seem to be the most significant factors, which cannot be clinically evaluated (38). Some studies

indicated the importance of the design of the FDPs and postulated this to modulate the risk of veneering chipping, so more teeth that are in block construction and individualized design of the framework according to each clinical has been proposed in an attempt to give good support to the veneering ceramic layer (39, 40). Layered zirconium compared to monolithic has better aesthetic characteristics, and also the fact that in this case, for orthodontic reasons, more teeth are connected to the block, this type of FDP was chosen.

In this case, after 2-years follow-up period, bone rarefication zones were found in the area of the lower incisors on the post-treatment X-ray. It can be explained by orthodontic movement of teeth.

Similar prosthetic rehabilitation with all-ceramic FDPs was done in case report conducted by Escalante (41). In the mentioned case, a patient had Class II division 2 and decreased VDO, as well. Increase of VDO was 2 mm with full-mouth open bite splint, that patient has worn for a total of 6 weeks. After the patient`s well toleration and adaptation to new VDO, final prosthetic rehabilitation was done in both dental arches with monolithic zirconia crowns. For the presented case, we chose all-ceramic system (zirconia) as a material for definitive prosthetic rehabilitation of the upper and lower arches due to its excellent aesthetic characteristics, ability to mimic natural tooth color, which is the primary advantage, and good mechanical properties, as well. Also, zirconia was used in other similar studies (42, 43).

Although, orthognathic surgery can play an important role in the correction of maxillary and mandibular protrusion or retrusion to achieve improved occlusion and facial profile, when patients cannot accept optimal dental treatment, compromises in the treatment plan are necessary. However, specific prosthodontic principles should be observed. In the present clinical report, as a result of complex treatment, the patient was successfully rehabilitated and the anteroposterior discrepancy between the dental arches was corrected. The patient was very happy and satisfied with the treatment done for him.

CONCLUSION

Combined orthodontic and prosthetic treatment can be a good option in the complex treatment of patients with skeletal class II division 1 malocclusion providing functional rehabilitation of the stomatognathic system and improving facial appearance and the quality of life of the patient.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. Voluntary written and informed consent was obtained from the patient prior to enrollment in the study.

CONFLICT OF INTEREST

There are no conflicts of interest.

FUNDING

None.

REFERENCES

1. Mossey P. The heritability of malocclusion: part 2. The influence of genetics in malocclusion. Br J Orthod. 1999;26:195-203.
2. Chou S, Tseng Y, Pan C, Chang J, Chang H. Craniofacial skeletal dysplasia of opposite-sex dizygotic twins. J Formos Med Assoc. 2011;110:342-6.
3. Rosenblum, R. E.Class II malocclusion: mandibular retrusion or maxillary protrusion? Angle Orthod. 1995;65:49-62.
4. Craig, CE.The skeletal patterns characteristic of Class I and Class II, Division 1 malocclusions in norma lateralis. Angle Orthod. 1951;21:44-56.
5. Hitchcock, H. P. A cephalometric description of Class II division 1 malocclusion. Am J Orthod. 1973;63:414-23.
6. McNamara JA Jr. Components of Class II malocclusion in children 8–10 years of age. Angle Orthod. 1981;51:177-202.
7. Pancherz H, Zieber K, Hoyer B. Cephalometric characteristics of Class II division 1 and Class II division 2 malocclusions: a comparative study in children. Angle Orthod. 1997;67:111-20.
8. Henry RG. A classification of Class II division 1 malocclusion. Angle Orthod. 1957;27:83-92.
9. Proffit W, Fields H, Sarver D. 2013. Contemporary orthodontics (5 th ed). St. Louis, MO: Mosby Elsevier.
10. Moreno Uribe LM, Howe SC, Kummet C, Vela KC, Dawson DV, Southard TE. Phenotypic diversity in white adults with moderate to severe Class II malocclusion. Am J Orthod Dentofacial Orthop. 2014;145:305-16.
11. Wallis SF. Integration of certain variants of the facial skeleton in Class II division 2 malocclusion. Angle Orthod. 1963;33:60-7.
12. Isik F, Nalbantgil D, Sazinsu K, Arun T. A comparative study of cephalometric and arch width characteristics of Class II division 1 and division 2 malocclusions. Eur J Orthod. 2006;28:179-83.
13. Al-Khateeb EA, Al-Khateeb SN. Anteroposterior and vertical components of class II division 1 and division 2 malocclusion. *Angle Orthod.* 2009;79(5):859-66.
14. Olsen JA, Inglehart MR. Malocclusions and perceptions of attractiveness, intelligence, and personality, and behavioral intentions. Am J Orthod Dentofacial Orthop. 2011;140(5):669-79.
15. Hsieh TJ, Pinskaya Y, Roberts WE. Assessment of Orthodontic Treatment Outcomes: Early Treatment versus Late Treatment. Angle Orthod. 2005;75:162-70.
16. Dugoni SA. Comprehensive mixed dentition treatment. Am J Orthod Dentofacial Orthop. 1998;113:75-84.

17. Tulloch JF, Phillips C, Proffit WR. Benefit of early Class II treatment: Progress report of a two-phase randomized clinical trial. Am J Orthod Dentofacial Orthop. 1998;113:62-72.
18. Sakar O, Beyli M, Marsan G. Combined prosthodontic and orthodontic treatment of a patient with a Class III skeletal malocclusion: a clinical report. J Prosthet Dent. 2004;92:224-8.
19. Basa S, Varol A, Sener ID, Sertgoz A. Posterior maxillary segmental osteotomy for restoring the mandible with dental implants: A clinical report. J Prosthet Dent. 2008;99:340-3.
20. Devita R, Pinho S, Ustrell J, et al. Multidisciplinary oral rehabilitation in partially edentulous adult patients with malocclusion: a cross-sectional survey study. J Clin Exp Dent. 2018;10(12):1177-83.
21. Fabbri G, Sorrentino R, Cannistraro G, Mintrone F, Bacherini L, Turrini R, Bombardelli T, Nieri M, Fradeani M. Increasing the Vertical Dimension of Occlusion: A Multicenter Retrospective Clinical Comparative Study on 100 Patients with Fixed Tooth-Supported, Mixed, and Implant-Supported Full-Arch Rehabilitations. Int J Periodontics Restorative Dent. 2018;38(3):323-35.
22. Sudhir N, Chittaranjan B, Kumar BA, Taruna M, Kumar MP, Reddy MR. Digital Cephalometric Tracings by PRO-CEPH V3 Software for Comparative Analyses of Vertical Dimension in Edentulous Patients. J Clin Diagn Res. 2015;9(5):ZC01-5.
23. Sakar O, Bural C, Sülün T, Öztaş E, Marşan G. Evaluation of the closest speaking space in different dental and skeletal occlusions. J Prosthet Dent. 2013;109(4):222-6.
24. Rebibo M, Darmouni L, Jouvin J, Orthlieb JD. Vertical dimension of occlusion: the keys to decision we may play with the VDO if we know some game's rules. J Stomat Occ Med 2009;2:147–59.
25. Goodacre CJ, Campagni WV, Aquilino SA. Tooth preparations for complete crowns: an art form based on scientific principles. J Prosthet Dent. 2001;85(4):363-76.
26. Horliana RF, Horliana AC, Wuo Ado V, Perez FE, Abrão J. Dental extrusion with orthodontic miniscrew anchorage: a case report describing a modified method. Case Rep Dent. 2015;2015:909314.
27. Jia-Yuan Zhao, Cheng-Si Hu, Yu Jin, Ling-Ling Zhang, Fang Yi, Yan-Qin Lu, Cheng-Ri Li. Efficiency of clear aligners in extrusion of posterior teeth evaluated by 3-dimensional model superimposition. Shanghai Kou Qiang Yi Xue. 2022;31(5):556-60.
28. Han G, Huang S, Von den Hoff JW, Zeng X, Kuijpers-Jagtman AM. Root resorption after orthodontic intrusion and extrusion: an intraindividual study. Angle Orthod. 2005;75(6):912-8.
29. D'Arcangelo C, Vadini M, Buonvivere M, De Angelis F. Safe clinical technique for increasing the occlusal vertical dimension in case of erosive wear and missing teeth. Clin Case Rep. 2021;9(12):e04747.
30. Abduo J. Safety of increasing vertical dimension of occlusion: a systematic review. Quintessence Int. 2012;43(5):369-80.
31. Ormianer Z, Gross M. A 2-year follow-up of mandibular posture following an increase in occlusal vertical dimension beyond the clinical rest position with fixed restorations. J Oral Rehabil. 1998;25:877-83.
32. Ormianer Z, Palty A. Altered vertical dimension of occlusion:a comparative retrospective pilot study of tooth- and implant-supported restorations. Int J Oral Maxillofac Implants. 2009;24:497-501.
33. Gross MD, Ormianer Z. A preliminary study on the effect of occlusal vertical dimension increase on mandibular postural rest position. Int J Prosthodont. 1994;7:216-26.
34. Cekic-Nagas I, Ergun G. Implant-supported prosthetic rehabilitation of a patient with localized severe attrition: A clinical report. J Prosthodont. 2015;24:322-8.
35. Song MY, Park JM, Park EJ. Full mouth rehabilitation of the patient with severely worn dentition: A case report. J Adv Prosthodont. 2010;2:106-10.
36. Johnston CD, Littlewood SJ. Retention in orthodontics. Br Dent J. 2015;218(3):119-22.
37. Abdulraheem S, Schütz-Fransson U, Bjerklin K. Teeth movement 12 years after orthodontic treatment with and without retainer: relapse or usual changes? Eur J Orthod. 2020;42(1):52-9.
38. Roscoe MG, Cavalero JG, Barboza MST, Ballester RY,Meira JBC. Current Scientific Evidence of Clinical Performance of Veneered Zirconia Crowns and Fixed Dental Prostheses. Curr Oral Health Rep. 2020;7:352–60.
39. Lohbauer U, Amberger G, Quinn GD, Scherrer SS. Fractographic analysis of a dental zirconia framework: a case study on design issues. J Mech Behav Biomed Mater. 2010;3(8):623–9.
40. Bonfante EA, Coelho PG, Navarro JM Jr, Pegoraro LF, Bonfante G, Thompson VP, Silva NR. Reliability and failure modes of implant-supported Y-TZP and MCR three-unit bridges. Clin Implant Dent Relat Res. 2010;12(3):235-43.
41. Escalante F. Raising the Vertical Dimension of Occlusion. Inside Dental Technology. 2017;8(7):52-3.
42. Agrawal M, Sankeshwari B, Pattanshetti CV. Use of zirconia to restore severely worn dentition: a case report. Case Rep Dent. 2012;2012:324597.
43. Ashish RJ, Sindhu P. Full Mouth Rehabilitation of a Bruxer with Severely Worn Dentition Using All Ceramic Zirconia Crowns – A Case Report. J Pharm Sci Res. 2017;9(11):2001-3.

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