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IN SILICO ASSESSMENT OF BIOAVAILABILITY, PHARMACOKINETIC AND TOXICOLOGICAL PROPERTIES OF NEUROTRANSMISSION MODULATORS OF 5-HT$_7$ RECEPTORS

Predrag Džodić[1], Stefan Stojanović[2]

Serotonin transmission is important for psychiatric disorders (depression, anxiety, schizophrenia and epilepsy). 5-HT$_7$ receptors are new target receptors for the development of drugs as a therapeutic alternative in the treatment of psychiatric diseases, therefore there is a need to discover 5-HT$_7$ receptor agonists and antagonists. For 38 selected compounds, 5-HT$_7$ receptor neurotransmission modulators, bioavailability, pharmacokinetics and toxicological properties were assessed. Based on the calculations, 37 compounds (except compound 28) do not show more than one deviation from the Lipinski rule, and good absorption and permeability are possible after oral administration. Good blood-brain permeability was predicted for 29 tested compounds, while poor blood-brain permeability was predicted for 9 compounds. Moreover, 30 compounds exhibited inhibition of the CYP 450 3A4 isoenzyme, while 16 compounds were not a substrate for P-glycoprotein. The risk of mutagenicity is not shown by any of the tested compounds (except compounds 5, 22, 23). Thirty-five tested compounds do not show the risk of carcinogenicity. Most of the 5-HT$_7$ receptor modulators tested do not pose a risk for reproductive toxicity and irritant effect. Based on the obtained results for drug similarity parameters and pharmacokinetic properties, the tested 5-HT$_7$ receptor modulators (compounds 1-38) should have good bioavailability after oral administration, as well as good blood-brain permeability (29 tested compounds). *In vitro* and *in vivo* studies of the tested 5-HT$_7$ receptor modulators, except for compounds 5, 11, 17, 22, 23, 34, could be performed to verify the results obtained because the tested compounds have the potential to be new drugs in the treatment of psychiatric diseases in the future.

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Key words: *5-HT$_7$ neurotransmission modulators, in silico, bioavailability, pharmacokinetic properties, toxicological properties*

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Introduction

Serotonin or 5-hydroxytryptamine (5-HT) is a neurotransmitter that participates in many physiological functions. Serotonin transmission is important in psychiatric illnesses such as depression, anxiety, schizophrenia, and epilepsy. Abnormal cognition disorder is observed in Alzheimer's disease, which is associated with impaired serotonin transmission (1).

There are 7 types of serotonin receptors (5-HT$_1$, 5-HT$_2$, 5-HT$_3$, 5-HT$_4$, 5-HT$_5$, 5-HT$_6$ and 5-HT$_7$) and 14 subtypes that have been identified and described in detail. Receptors are ionotropic (5-HT$_3$) or metabotropic (associated with G-protein in 5-HT1, 2, 4-7) and are located in somatodendritic cells (as presynaptic and postsynaptic receptors). G-protein-associated 5-HT receptors are membrane proteins with 7 transmembrane alpha-helixes (1). 5-HT$_7$ receptors are located in the periphery (intestines and blood vessels) and the central nervous system (thalamus, hypothalamus, cerebral cortex, hippocampus and amygdala) (2).

Knowing 5-HT$_7$ receptors is important as a new target receptors for development of new drugs in the treatment of various psychiatric diseases, therefore there is a need to discover agonists and antagonists of these receptors that will be effective, highly selective and exhibit a good safety profile. For newly synthesized compounds, it is possible to assess bioavailability based on the fact that the compounds have appropriate chemical structure. Namely, the chemical structure of the compound

enables the assessment of bioavailability based on drug similarity parameters as well as absorption and metabolic properties.

The primary goal is to assess the bioavailability of selected compounds, 5-HT_7 neurotransmission modulators, based on the calculation of drug similarity parameters. The secondary goal is to evaluate the toxicological and pharmacokinetic properties of selected compounds as potential drugs in the treatment of psychiatric diseases.

Materials and methods

Topological polar surface area (TPSA)

The polar surface of molecules (polar surface area, PSA) is the surface occupied by polar atoms (usually nitrogen, oxygen and bound hydrogen atoms). It is a parameter that affects the transport of the drug through the membranes. This parameter correlates with good intestinal absorption and passage through the blood-brain barrier. The classic way of calculating PSA takes a lot of time because it is necessary to generate a 3D structure of molecules to determine the surface. For easier and faster calculation, a topological polar surface area (*TPSA*) is used. In short, the procedure is based on the summation of the tabular values of the surfaces occupied by the polar fragments of molecules (atoms and their surroundings). *TPSA* gives results of approximately the same quality as the classic 3D PSA, but the calculation is 2-3 times faster (3).

Lipinski's rule

Lipinski's rule ("Rule of 5") predicts poor absorption and permeation in compounds with more than 5 hydrogen bond donors, more than 10 hydrogen bond acceptors, molecular weight greater than 500 D and where the calculated log P is greater than 5. Molecules that meet 2 or more of the above criteria have poor bioavailability. Lipinski's rules apply to drugs that pass through the cell membrane by passive diffusion. Exceptions to this rule are drugs that pass through the cell membrane by active transport (4).

The decimal logarithm of the octanol-water partition coefficient (*log P*) is used as a measure of the hydrophobicity of molecules. Hydrophobicity affects the absorption, bioavailability of the drug, hydrophobic drug-receptor interactions, metabolism as well as drug toxicity (4). The *log P* value prediction method (*miLogP*) was developed by *Molinspiration Cheminformatics* software (Bratislava, Slovakia) and is based on group contributions. Group contributions were obtained by fitting the calculated *log P* values with the experimental *log P* values for about 12,000 molecules. Therefore, the hydrophobic values of 35 smaller "basic" fragments were determined, as well as the values for 185 larger fragments, which determines the contribution of intermolecular hydrogen bonds and charge interactions to log P value. *Molinspiration Cheminformatics* methodology for calculating *log P* is very robust and can be applied to compounds of organic and organometallic origin (5).

Number of rotatable bonds (nrotb)

Molecular parameters, such as the number of bonds that can rotate, the topological polar surface area (*TPSA*) and the total number of hydrogen bonds (sum of hydrogen-bond acceptors and donors), are important indicators of good oral bioavailability and are independent of molecule volume. However, the number of bonds that can rotate and the number of hydrogen bonds increase with the increasing volume of molecules, which can be considered a valid parameter for assessing oral bioavailability (6). A rotatable bond is defined as a simple bond to which a ring is not attached and is attached to a non-terminal heavy atom (not hydrogen-bonded) (5).

Volume of molecules (Volume)

The volume of molecules affects the transport of molecules, during intestinal absorption and when passing through the blood-brain barrier. The method for calculating the volume of molecules, developed by *Molinspiration Cheminformatics* is based on group contributions to the 3D volume of molecules. Group contributions were obtained by taking into account the fragmentary contributions to the "real" 3D volume for about 12,000 molecules. The calculated volume of molecules is expressed in units of cubic Angstroms (Å3) (5).

Calculation of drug-likeness parameters

For 38 selected compounds, $5HT_7$ receptor neurotransmission modulators, drug-likeness parameters were calculated, which may indicate their oral bioavailability. *Molinspiration property engine v2018.10* was used to calculate the following drug-likeness parameters: *miLogP* (P is the octanol-water partition coefficient), topological polar surface area (*TPSA*), number of non-hydrogen atoms (*natoms*), molecular weight (*MW*), the number of hydrogen bond acceptors (*nON*), the number of hydrogen bond donors (*nOHNH*), the number of deviations from the Lipinski rule (*nviolations*), the number of rotatable bonds (*nrotb*) and the volume of molecules (*Volume*) (5).

Assessment of pharmacokinetic properties

Assessment of the pharmacokinetic properties for compounds 1-38, $5HT_7$ receptor neurotransmission modulators, was performed by using:

The SwissADME web tool (available at website: http://www.swissadme.ch). Predictions of gastrointestinal absorption and permeability across the blood-brain barrier are based on the graphical BOILED-Egg model (7). Databases of known substrates for P-glycoprotein and inhibitors for CYP 450 isoenzymes have been used to predict other pharmacokinetic properties (such as a substrate for

P-glycoprotein and inhibitor for CYP 450 isoenzymes) (8).

Assessment of toxicological properties

Assessment of toxicological properties for compounds 1-38, 5-HT_7 receptor neurotransmission modulators, was performed by using computer program *OSIRIS Datawarrior v.5.5.0* (9). The division was made into four main classes of toxicity: mutagenicity, carcinogenicity, irritant effect and reproductive toxicity. Toxicity risk assessment is based on the identification of a fragment within the structure of molecules that indicates the risk of toxicity. A list of fragments for each toxicity class was obtained from an RTECS database of compounds known to be active in a particular toxicity class (for example, mutagenicity). A set of toxic compounds (RTECS database) and a set of non-toxic compounds (commercially available drugs) are used for prediction (10).

Results

Table 1 shows the calculated values of the drug-likeness parameters for compounds 1-38. Compounds 1-23 and 38 are antagonists of 5-HT_7 receptors, even though compounds 24-37 are agonists of 5-HT_7 receptors (1).

Tables 2 and 3 display the pharmacokinetic properties of compounds 1-38. Table 2 shows the absorption properties. Moreover, Table 3 shows the metabolic properties of compounds 1-38 obtained by using The SwissADME web tool.

Table 4 displays the toxicological properties of compounds 1-38 obtained by using the OSIRIS Datawarrior program.

Figure 1 shows chemical structure of 5-HT_7 antagonists (compounds 1-18).

Figure 2 displays chemical structure of 5-HT_7 antagonists (compounds 19-23 and 38).

Figure 3 displays chemical structure of 5-HT_7 antagonists (compounds 24-37).

Table 1. The calculated values of the drug-likeness parameters for compounds 1-38

No.	miLogP[a]	TPSA[b] (Å^2)	natoms[c]	Mw[d] (g/mol)	nON[e]	nOHNH[f]	nviolations[g]	nrotb[h]	Volume[i] (Å^3)
1	4.82	32.34	29	386.54	3	1	0	6	379.04
2	6.12	32.34	31	455.43	3	1	1	6	406.11
3	3.83	66.37	35	470.62	6	2	0	7	445.73
4	5.33	35.57	30	429.97	4	1	1	7	393.47
5	6.20	20.64	34	447.58	4	0	1	5	424.49
6	3.83	23.55	26	346.47	3	0	0	6	340.15
7	4.50	79.70	41	557.67	8	1	1	11	515.65
8	2.35	85.34	33	456.52	8	2	0	7	411.69
9	4.26	58.64	29	420.55	5	1	0	8	382.73
10	4.24	58.64	29	420.55	5	1	0	8	382.73
11	4.62	58.64	31	446.59	5	1	0	8	405.55
12	4.26	67.68	33	474.67	7	0	0	10	448.58
13	4.86	78.87	34	501.05	6	2	1	9	437.64
14	3.46	40.62	23	338.52	4	0	0	6	330.91
15	2.59	60.85	24	352.50	5	1	0	5	328.81
16	3.52	40.62	24	350.53	4	0	0	5	337.35
17	4.16	65.64	33	488.05	6	1	0	7	427.14
18	2.96	71.11	29	419.55	7	1	0	9	382.72
19	4.32	3.24	18	239.36	1	0	0	4	251.52
20	5.12	24.94	29	388.51	4	0	1	6	376.69
21	4.15	29.85	24	337.85	3	1	0	3	307.18
22	3.94	97.99	26	350.43	7	4	0	8	326.46
23	4.27	97.99	28	386.41	7	4	0	8	336.32
24	5.65	35.57	37	495.71	4	1	1	11	497.38
25	4.28	35.57	33	465.71	4	1	0	11	460.66
26	5.35	35.57	36	479.67	4	1	1	12	480.05
27	3.75	70.96	31	420.51	7	1	0	8	393.46
28	5.69	44.81	38	513.66	5	1	2	11	493.73
29	3.21	21.06	21	283.42	3	0	0	2	288.64
30	3.25	33.62	17	337.16	3	1	0	2	222.99

31	3.34	33.62	18	355.15	3	1	0	2	227.92
32	3.56	16.13	18	258.39	2	0	0	5	248.93
33	3.14	21.06	20	271.41	3	0	0	4	282.42
34	2.68	41.29	20	273.38	4	1	0	4	273.87
35	3.94	21.71	21	285.39	3	0	0	6	286.05
36	3.14	24.50	20	268.36	3	1	0	3	262.55
37	4.47	37.38	25	363.57	3	0	0	6	357.87
38	2.49	40.71	17	247.73	3	2	0	1	218.59

[a]calculated *logP* values;
[b]topological polar surface area;
[c]number of non-hydrogen atoms;
[d]molecular weight;
[e]number of hydrogen bond acceptors (*O* and *N* atoms);
[f]number of hydrogen bond donors (*OH* and *NH* groups);
[g]number of Lipinski's rule violations;
[h]number of rotatable bonds; [i]volume of molecules

Table 2. Predicted absorption properties for the tested compounds (1-38)

Absorption properties	The tested compounds
Good GIT[a] absorption	1-38
Poor GIT absorption	/
Good blood-brain permeability	1-6, 9, 10, 14-16, 19-21, 24-38
Poor blood-brain permeability	7, 8, 11-13, 17, 18, 22, 23
Substrate for P-gp[b]	1-8, 11, 13, 15, 17, 20, 21, 24-28, 30, 31, 38
Not substrate for P-gp	9, 10, 12, 14, 16, 18, 19, 22, 23, 29, 32-37

[a]gastrointestinal tract
[b]P-glycoprotein

Table 3. Predicted metabolic properties for the tested compounds (1-38)

Metabolic properties	The tested compounds
CYP 450 1A2 inhibitor	5, 19-23, 26, 30-32, 34-36, 38
CYP 450 2C19 inhibitor	1, 3, 5, 6, 7, 9, 10, 11-13, 17, 18, 22, 24, 26, 28, 30, 32, 35, 37
CYP 450 2C9 inhibitor	2, 3, 7, 9, 10, 12, 13, 17, 18, 22, 23
CYP 450 2D6 inhibitor	1-38
CYP 450 3A4 inhibitor	1-4, 6-14, 16-18, 20-28, 30, 31, 36-38

Table 4. Predicted toxicological properties for the tested compounds (1-38)

Toxicological properties	The obtained results
Mutagenicity	5, 22, 23 (high level)
Carcinogenicity	5 (high level), 22, 23 (low level)
Reproductive toxicity	11 (low level), 22, 23, 34 (high level)
Irritating effect	17, 22, 23 (high level)

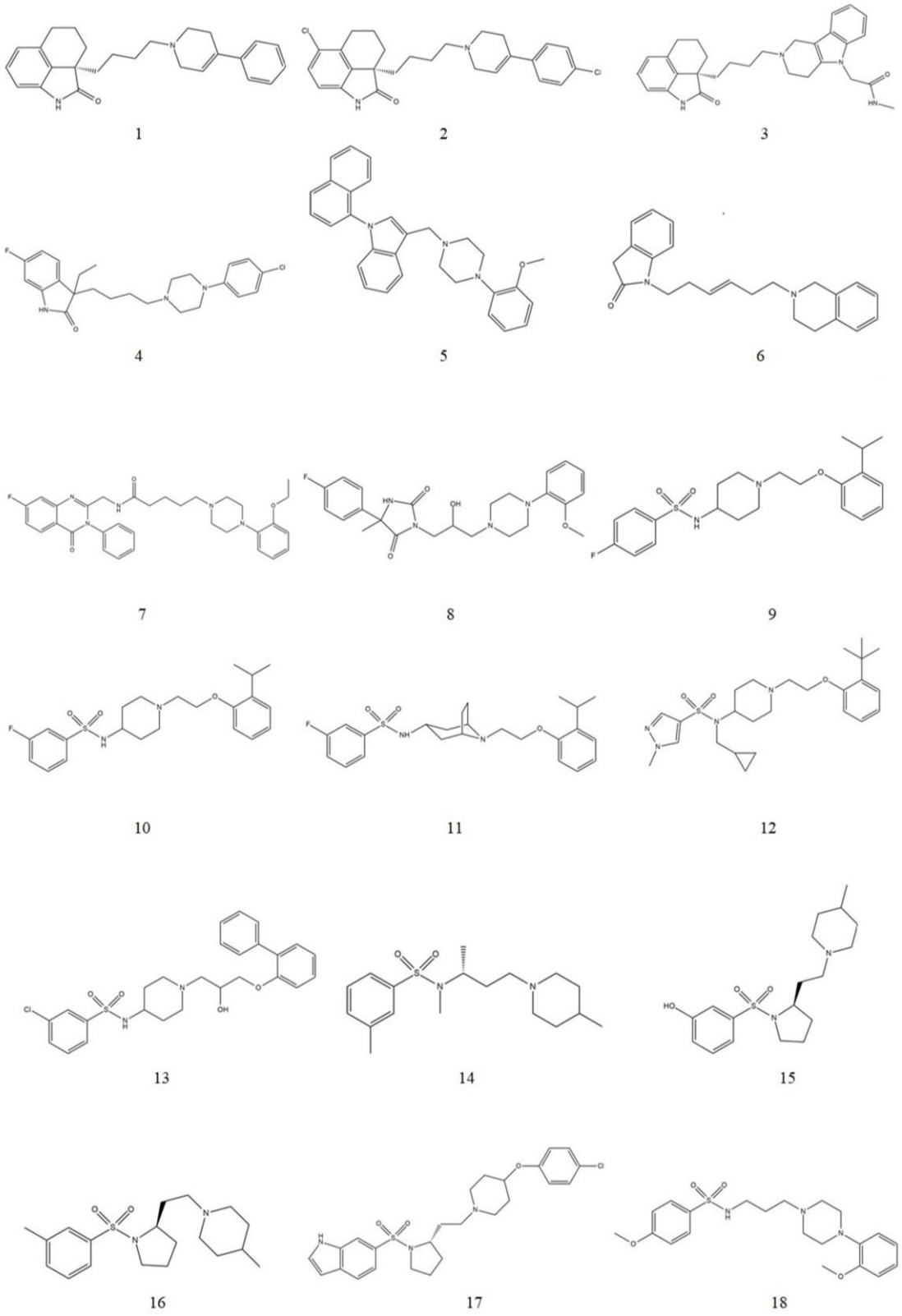

Figure 1. Chemical structure of 5-HT$_7$ antagonists (compounds 1-18)

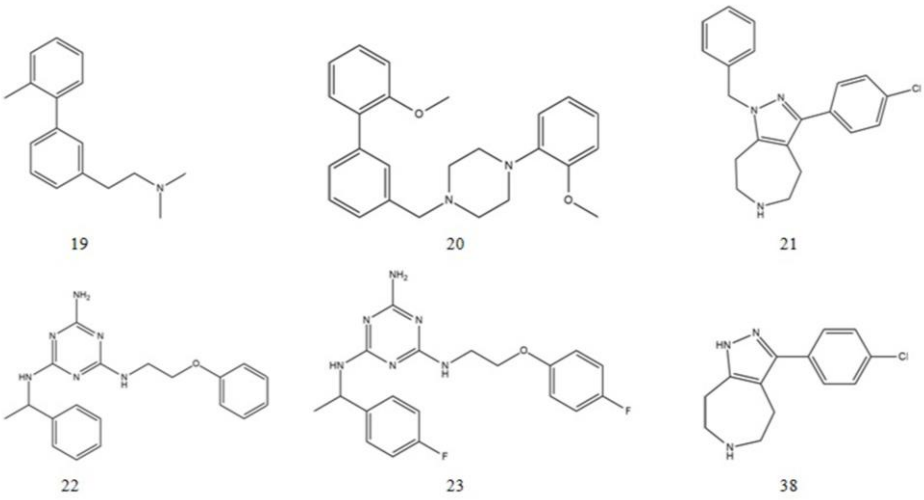

Figure 2. Chemical structure of 5-HT_7 antagonists (compounds 19-23 and 38)

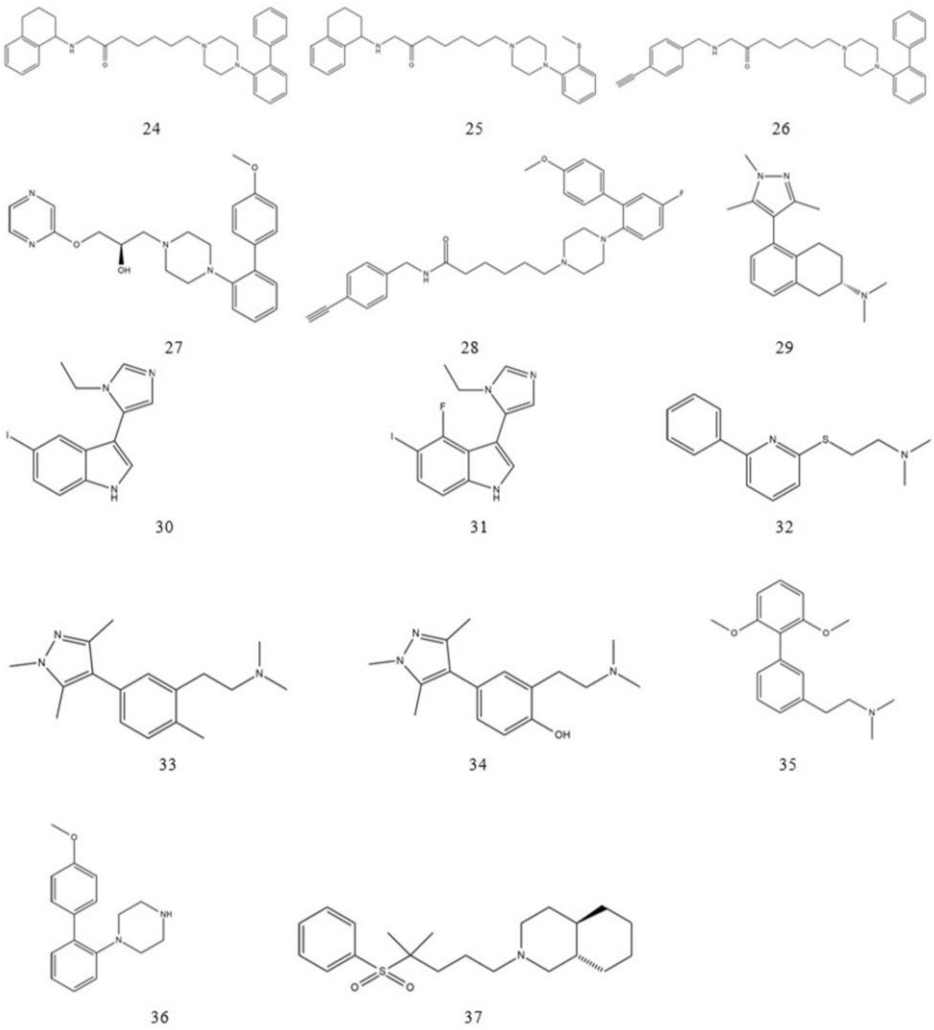

Figure 3. Chemical structure of 5-HT_7 agonists (compounds 24-37)

Discussion

The tested compounds, 5-HT_7 receptor modulators (except for compounds 2, 4, 5, 7, 13, 20, 24, 26, 28) have less than 5 H-bond donors, have less than 10 H-bond acceptors, molecular weight is less than 500 D and the value of *miLogP* is less than 5, so there is no deviation from Lipinski's rule, which indicates good absorption and permeability. Compound 28 has a molecular weight greater than 500 D and *miLogP* value greater than 5, so there are two deviations from Lipinski's rule, indicating poor absorption and permeability.

All tested compounds have *TPSA* value of less than 140 $Å^2$, which may indicate good intestinal absorption and permeability. Moreover, compounds 1, 2, 4-6, 9-11, 14, 16, 19-21, 24-26, 28-38 have *TPSA* value of less than 60 $Å^2$, which may be an indicator of good penetration through the blood-brain barrier. All tested compounds (except for 7, 12, 24-26, 28) have *nrotb* ≤ 10 which means that the compounds may have adequate bioavailability after oral administration. In addition to this, the molecular volume of all tested compounds (except for compound 7) is less than 500 $Å^3$, which may indicate good oral bioavailability. The obtained results are presented in Table 1.

The *SwissADME* web tool predicts good gastrointestinal absorption of all test compounds. Good blood-brain permeability was predicted for 29 tested compounds and poor blood-brain permeability for only 9 compounds. Most of the tested compounds (22 compounds) can be a substrate for P-glycoprotein, while 16 compounds will not be able to be a substrate for P-glycoprotein. Details on the absorption properties of the test compounds are shown in Table 2.

Considering metabolic properties of the tested compounds, 14 compounds may be inhibitors of the CYP 450 1A2 isoenzyme. Moreover, 20 compounds may exhibit inhibition of CYP 450 2C19 isoenzymes, as well as 11 compounds, which may be inhibitors of CYP 450 2C9 isoenzymes. A large number of the tested compounds (30 compounds) are inhibitors of CYP 450 3A4 isoenzymes. Furthermore, all tested compounds are inhibitors of the CYP 450 2D6 isoenzyme. Details of the metabolic properties of the test compounds are shown in Table 3.

The risk for mutagenicity is not shown by any of the tested compounds except for compounds 5, 22, 23 which exhibit a high mutagenic risk. The test compounds do not show a risk of carcinogenicity (35 tested compounds) except for compound 5 with high carcinogenic risk and compounds 22 and 23 with low carcinogenic risk. Compound 11 shows low reproductive toxicity, while compounds 22, 23, 34 show high reproductive toxicity. However, most of the tested 5-HT_7 receptor modulators do not pose a risk for reproductive toxicity. Compounds 17, 22, 23 show a high risk of irritant effect, while most of the tested compounds do not show irritant effect. Data on the toxicological properties of the tested compounds are shown in Table 4.

Conclusion

Based on the obtained results for drug-likeness parameters and pharmacokinetic properties, the tested 5-HT_7 receptor modulators (compounds 1-38) should have good bioavailability after oral administration, as well as good blood-brain permeability (29 tested compounds). Tested 5-HT_7 receptor modulators do not show a risk for a mutagenic effect and a risk for a carcinogenic effect (except for compounds 5, 22, 23). Compounds 17, 22, 23 have a high risk of irritant effects. Compound 11 also shows low reproductive toxicity, while compounds 22, 23, 34 show high reproductive toxicity. However, most of the tested 5-HT_7 receptor modulators does not exhibit irritant effect and reproductive toxicity. Therefore, in order to experimentally verify the obtained results, *in vitro* and *in vivo* studies of the tested 5-HT_7 receptor modulators could be performed, except for compounds 5, 11, 17, 22, 23, 34, because these compounds show the potential to be new drugs in the treatment of psychiatric illnesses in the future.

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Serotoninska transmisija značajna je za psihijatrijska oboljenja poput depresije, anksioznosti, šizofrenije i epilepsije. 5-HT$_7$ receptori su nova terapijska alternativa u lečenju psihijatrijskih oboljenja, pa stoga postoji potreba za otkrićem agonista i antagonista 5-HT$_7$ receptora. Za 38 odabranih jedinjenja, modulatora neurotransmisije 5-HT$_7$ receptora izvršena je procena bioraspoloživosti i farmakokinetičkih i toksikoloških osobina. Na osnovu izvršenih kalkulacija, 38 jedinjenja (osim jedinjenja 28) ne pokazuju više od jednog odstupanja od pravila Lipinskog, te su nakon oralne primene moguće dobra apsorpcija i permeabilnost. Za 29 ispitivanih jedinjenja predviđa se dobra krvno-moždana permeabilnost, dok se za 9 jedinjenja predviđa loša krvno-moždana permeabilnost. Štaviše, 30 jedinjenja ispoljavaju inhibiciju izoenzima CYP 450 3A4, dok 16 jedinjenja nisu supstrat za P-glikoprotein. Rizik od mutagenosti ne ispoljava ni jedno od ispitivanih jedinjenja (osim jedinjenja 5, 22, 23). 35 ispitivanih jedinjenja ne ispoljavaju rizik od kancerogenosti. Većina ispitivanih modulatora 5-HT$_7$ receptora ne ispoljavaju rizik od reproduktivnih toksičnosti i iritantnih efekata. Na osnovu dobijenih rezultata za parametre sličnosti sa lekom i farmakokinetičkih osobina, ispitivani modulatori 5-HT$_7$ receptora (jedinjenja 1 – 38) trebalo bi da imaju dobru bio-raspoloživost nakon peroralne primene, kao i dobru krvno-moždanu permeabilnost (29 ispitanih jedinjenja). *In vitro* i *in vivo* studije ispitivanih modulatora 5-HT$_7$ receptora, izuzev jedinjenja 5, 11, 17, 22, 23, 34, mogle bi da budu izvedene u cilju provere dobijenih rezultata, jer ispitivana jedinjenja imaju potencijal da budu novi lekovi u terapiji psihijatrijskih oboljenja u budućnosti.

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Ključne reči: *modulatori 5-HT$_7$ neurotransmisije, in silico, bioraspoloživost, farmakokinetičke osobine, toksikološke osobine*

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ANTIMICROBIAL ACTIVITY OF FRACTIONS AND THE EXTRACT FROM *GENTIANA ASCLEPIADEA* L. UNDERGROUND PARTS WITH MOLECULAR DOCKING ANALYSIS

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The willow gentian (*Gentiana asclepiadea* L.) is a valuable source of secoiridoids, *C*-glycosylated flavones and xanthones used empirically in the treatment of liver and gastrointestinal disorders. Guided by ethnopharmacological data on the use of *G. asclepiadea* underground parts in the treatment of diarrhea, antimicrobial activity against selected pathogens of gastrointestinal significance was examined. Presented study was aimed to evaluate antimicrobial activity of the aqueous-ethanolic extract of *G. asclepiadea* underground parts and its petroleum ether, ethyl acetate, butanol and water fractions. A molecular docking analysis was performed as well. The antimicrobial activity against pathogens related to gastrointestinal disorders was tested by a microdilution method. The ethyl acetate fraction showed the greatest antimicrobial activity. The lowest MIC of 0.78 mg/ml was observed against *Bacillus cereus* and *Staphylococcus aureus*, achieved by the petroleum ether and ethyl acetate fractions, respectively. The greatest bactericidal activity (MBC of 0.78 mg/ml), achieved by the ethyl acetate fractions, was recorded against *Enterococcus faecalis*. The yeast *Candida albicans* was the most resistant against the fractions and the extract. *C*-glycosylated flavones isoorientin and isovitexin showed the best binding affinity on *Enterococcus faecalis* lipoate-protein ligase A as determined by a molecular docking analysis. Considering the results of our study, underground parts of *G. asclepiadea* could be used as a valuable natural source of secondary metabolites with promising antimicrobial activity.

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Key words: *Gentiana asclepiadea, antimicrobial activity, extracts, fractions, molecular docking*

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Introduction

Infectious diseases are still a major cause of morbidity and mortality and a leading public health problem worldwide, especially in developing countries. According to the World Health Organization (WHO), there are approximately 1.7 billion cases of diarrhoeal disease among child populations every year, of which over half a million with a lethal outcome. Diarrhea most often appears with symptoms of gastrointestinal infection caused by various pathogens (1). Although many of these infections are treatable, the need for research and development in this area remains due to the existence of side effects of the drugs, its irrational use and the consequent emergence of resistant strains of pathogens (2).

A widely accepted approach in pharmaceutical research of drug candidates or new indications for already existing drugs is the harvesting of ethnopharmacological data on indigenous drugs and their experimental valorization (3). One of the ethnopharmacologically valuable plant sources, which is traditionally used among the population of south-eastern Serbia for the treatment of diarrhea, is the underground part of the willow gentian (*Gentiana asclepiadea* L., *Gentianaceae*). For that purpose, tea or macerate from the pulverized underground parts of willow gentian is used orally (4). Sarić (1989) noticed that the roots and rhizomes of this plant are used empirically in the treatment of infectious

hepatitis type A, while Milojević and Mihajlović (1966) reported its use among the local population of Prokletije Mountain in the treatment of cough and tuberculosis (5, 6). Recent studies have confirmed the hepatoprotective effect of this plant species (7).

The *G. asclepiadea* L. belongs to a large cosmopolitan genus *Gentiana* L. The genus *Gentiana* comprises about 400 plant species, of which 11 species occur in the central part of the Balkan Peninsula (9). Plants of this genus are chemotaxonomically characterized by the biosynthesis of bitter secoiridoid compounds gentiopicroside, sweroside and/or swertiamarin as secologanin derivatives (8, 9). Due to their bitterness, plants of this genus are traditionally used as appetite stimulants and tonics (*amara pura*) (10). In addition to secoiridoids, the main secondary metabolites of *G. asclepiadea* are *C*-glycosylated flavones (isoorientin and isovitexin) and *C*-glycosylated xanthones (isogentisin, mangiferin). Mangiferin is present only in the aboveground part of this plant (9).

Some recent studies (11, 12) confirmed an antimicrobial activity of underground parts of *G. asclepiadea*. Ballast materials such as biopolymers and organic acids can be co-extracted simultaneously with the target compounds, therefore it is necessary to fractionate the primary (crude) extract (13). The aim of this study was to evaluate the *in vitro* antimicrobial properties of the primary aqueous-ethanolic extract of the underground parts of *G. asclepiadea* and its fractions using a set of pathogens with gastrointestinal significance. Additionally, an *in silico* molecular docking analysis of selected secondary metabolites on the target protein of the most sensitive bacterial strain (*Enterococcus faecalis* lipoate-protein ligase A) was carried out to examine their binding affinities and interaction patterns.

Materials and methods

Plant material

Pulverised underground parts (rhizomes with roots) of willow gentian (*G. asclepiadea* L., Gentianaceae) were donated from the Institute for Medicinal Plants Research "Dr. Josif Pančić", batch number 26841019. Until the experiment, the plant material was stored in a dark and dry place at room temperature.

Extraction and fractionation procedure

The primary extract was obtained by a maceration method at room temperature. Powdered underground parts (100 g) were measured in an Erlenmeyer flask, inside of which 70% aqueous ethanol (1 L) was added and was left on a laboratory shaker (100 rpm) for 14h. After filtration, the collected liquid extract was evaporated to dryness on a laboratory Buchi rotavapor R-114. A portion of the obtained primary extract was separated for further antimicrobial testing, while the residue was dissolved in 100 ml of distilled water and further used for fractionation. Fractionation was performed in a funnel by successive liquid-liquid re-extraction using organic solvents of increasing polarity (petroleum ether, ethyl acetate and n-butanol). The liquid fractions were evaporated to dryness on a vacuum evaporator yielding 1.42, 2.01, 10.01, and 70.42% of primary extract for petrolether, ethyl acetate, n-butanol, and water fraction, respectively (14).

Antimicrobial activity

Samples were tested against pathogens related with foodborne poisoning and gastrointestinal disorders. A set of eight microbial strains were used:

- three Gram-positive bacterial strains - *Staphylococcus aureus* (ATCC 6538), *Enterococcus faecalis* (ATCC 19433), and *Bacillus cereus* (ATCC 11778);
- four Gram-negative bacterial strains - *Escherichia coli* (ATCC 25922), *Salmonella enteritidis* (ATCC 13076), *Enterobacter aerogenes* (ATCC 13048), and *Pseudomonas aeruginosa* (ATCC 9027); and
- one yeast strain - *Candida albicans* (ATCC 24433). Overnight cultures (18h) were prepared on a Mueller Hinton Agar (MHA) for bacterial strains or a Sabouraud Dextrose Agar (SDA) for yeast strain.

The antimicrobial activities of the crude extract and its fractions were examined *in vitro* by microdilution method according to CLSI (2012), with slight modification (15). Overnight cultures of selected pathogens were used to prepare the suspensions of 0.5 McFarland turbidity which corresponds to a density of 10^8 CFU/mL. Stock solutions of the crude extract and the collected fractions were prepared in 10% dimethylsulfoxide (DMSO) at a concentration of 400 mg/ml. Testing samples in concentrations ranging from 0.01 to 200.00 mg/ml were prepared by a series of two-fold dilutions of stock solutions. Inoculated Mueller Hinton broth and prepared testing samples were poured in 96-well microtiter plates to a final volume of 100 µL and a microorganism density of 10^6 CFU/mL. Microtiter plates were incubated at 37 ^{O}C for 18 hours. Antimicrobial activity is expressed as the values of minimum inhibitory concentration (MIC) and a minimum bactericidal concentration (MBC). The MIC was defined as the concentration of the sample in which there is no visible growth of microorganisms. Visualization of cell growth was determined using a 0.5% aqueous solution of triphenyltetrazolium chloride (TTC). MMC is the sample concentration at which 99.9% of the cells of the microorganisms are killed. MMC was determined by transferring the contents of the wells without visible growth of microorganisms onto Petri dishes with MHA for bacteria, or SDA for yeasts and counting the grown colonies. The experiment was conducted in the Microbiological Laboratory of the Department of Biology, Faculty of Science and Mathematics - University of Niš. All of the tests were performed three times.

Molecular docking

Molecular binding simulation of gentiopicroside, swertiamarin, sweroside, isovitexin, isoorientin and isogentisin, as main ingredients of underground parts of *G. asclepiadea* and potential antimicrobial ligands, was conducted on the *Enterococcus faecalis* lipoate-protein ligase A (lplA-1) as the target. A molecular docking analysis was performed using an AutoDock Vina 1.1.2 and AutoDock Tools 1.5.6 software. The three-dimensional structures of gentiopicroside, swertiamarin, sweroside, isovitexin, isoorientin and isogentisin were acquired from PubChem (CID number: 88708, 442435, 161036, 162350, 114776, 5281640, respectively), while the lplA-1 was obtained from the Protein Data Bank (PDB ID: 5IJ6). The binding pocket of lplA-1 was prepared based on the reference complexed ligand (lipoic acid). After removing all water molecules and the ligand, as well as adding polar hydrogen, the PDBQT format of the target protein and the selected compounds were prepared by AutoDock Tools. The dimension of the grid box was 20 × 20 × 20 Å with the coordinates of the center being x = 63.38, y = 72.83, z = 127.86. The amino acid residues involved in the interaction and binding affinity, expressed as binding free energy, were determined by the AutoDock Vina analysis. The visualization of the compound-receptor docked complex was performed using the Discovery Studio 2020 Client (Biovia Corp. of San Diego, USA).

Results

In vitro antimicrobial activity

The antimicrobial activity of the primary 70% ethanol extract and its petroleum ether, ethyl acetate, butanol and water fractions against a set of eight selected pathogens (three Gram-positive bacteria, four Gram-negative bacteria and one strain of yeast) are summarized in Table 1. Doxycycline and nystatin were used as a positive control to compare the antibacterial and antifungal activity, respectively, with the examined extract and fractions.

Table 1. Antimicrobial activity of extract of *Gentiana asclepiadea* underground parts and its fractions

Pathogen	70% ethanolic extract	Petrolether fraction	Ethyl acetate fraction	Butanol fraction	Water fraction	Positive control†
	MIC / MMC* (mg/ml)					MIC / MMC (μg/ml)
Gram (+) bacteria						
Staphylococcus aureus (ATCC 6538)	25.00 / 50.00	25.00 / 50.00	0.78 / 25.00	6.25 / 12.50	6.25 / 25.00	7.81 / 15.61
Bacillus cereus (ATCC 11778)	3.13 / 25.00	0.78 / 12.50	1.56 / 12.50	3.13 / 25.00	25.00 / 50.00	0.90 / 15.61
Enterococcus faecalis (ATCC 19433)	3.13 / 3.13	6.25 / 6.25	1.56 / 0.78	3.13 / 1.56	6.25 / 6.25	0.90 / 1.90
Gram (-) bacteria						
Escherichia coli (ATCC 25922)	6.25 / 12.50	12.50 / 25.00	6.25 / 25.00	6.25 / 12.50	6.25 / 25.00	15.61 / 15.61
Salmonella enteritidis (ATCC 13076)	12.50 / 25.00	25.00 / 25.00	6.25 / 25.00	12.50 / 12.50	12.50 / 25.00	0.90 / 1.90
Enterobacter aerogenes (ATCC 13048)	50.00 / 50.00	50.00 / > 200.00	3.13 / 12.50	12.50 / 50.00	50.00 / > 200.00	7.81 / 15.61
Pseudomonas aeruginosa (ATCC 9027)	25.00 / 50.00	25.00 / 50.00	6.25 / 25.00	12.50 / 25.00	25.00 / 50.00	15.61 / 15.61
Yeast						
Candida albicans (ATCC 24433)	50.00 / > 200.00	50.00 / 50.00	12.50 / 12.50	12.50 / 25.00	25.00 / 50.00	3.91 / 7.81

*MIC - minimum inhibitory concentration,
MMC - minimum microbicidal concentration;
†Positive control - doxycycline for bacteria or nystatin for yeast

The examined extract of *G. asclepiadea* underground parts and its fraction inhibited growth of all tested pathogen strains. The values of minimum inhibitory concentrations (MIC) for Gram-positive strains varied from 0.78 to 25 mg/ml. These values were slightly higher (ranged from 3.13 to 50 mg/ml) for Gram-negative strains, clearly indicating the selectivity of antimicrobial activity depending on

the structure of the bacterial cell wall. This has also been confirmed by the values of minimum bactericidal activity (MBC) which were mostly lower for Gram-positive strains (in the range of 0.78 - 50 mg/ml compared to 3.13 - > 200 mg/ml for Gram-negative strain). The lowest MIC values in all of the tested strains were related to the ethyl acetate fraction, with the exception of *B. cereus* strain where the most prominent antimicrobial activity (MIC of 0.78 mg/ml) was achieved by the petrolether fraction.

The obtained results indicate the separation of the basic 70% EtOH extract by liquid-liquid re-extraction can strongly affect antimicrobial activities. This effect was emphasized in the case of *S. aureus* where the MIC of the primary extract was about 32-fold higher than that of the MIC of its ethyl acetate fraction. This confirms that fractionation could improve the inhibitory activity of the extract. On the other hand, this discrepancy was mitigated in the case of *E. coli* where the MIC of the extract and its fractions were almost equal. *B. cereus* and *E. faecalis* showed the highest sensitivity to the extract and fractions in relation to all investigated strains. The greatest bactericidal activity (MBC of 0.78 mg/ml) was achieved by the ethyl acetate fraction against *E. faecalis*. Interestingly, the increase in polarity of the solvents used to prepare the extract fractions was associated with reducing *B. cereus* sensitivity.

Regarding yeast, the tested extract and fractions of *C. albicans* showed a moderate antifungal activity with a MIC ranging from 12.5 to 50 mg/ml, and a minimum fungicide concentration (MFC) ranging from 12.5 to > 200 mg/ml.

Molecular docking

A molecular docking analysis on lplA-1 was performed in order to predict extract ingredients with potential antimicrobial activity and to elucidate the possible ligand-protein interaction. The docking results of six dominant compounds of *G. asclepiadea* underground parts (representatives of the three main groups of compounds) and lipoic acid as the referent ligand are listed in Table 2. All examined compounds showed a significant binding affinity with the binding free energy in the range from 6.0 to -7.4 kcal/mol (lower free energy corresponds to higher binding affinity). The greatest binding affinity was observed in the case of isoorientin and isovitexin with values of -7.4 and -7.3 kcal/mol, respectively. The 2D structures of the lplA-1 active site complexed with potentially active ingredients are illustrated in Figure 1. The active site of the lplA-1 enzyme with isoorientin (3D) as the compound with the best docking score is illustrated in Figure 2.

Table 2. Binding affinity and amino acid residues involved in interaction of examined molecules as ligands and *Enterococcus faecalis* lipoate-protein ligase A (lp1A-1)

Ligand		Binding free energy (kcal/mol)	Residues involved in **H-bond** interactions		Residues involved in **hydrophobic** interactions	
			Amino acid	Distance (Å)	Amino acid	Distance (Å)
Reference ligand	Lipoic acid	-6.0	Asn135 Gly73 Asn123	2.11 2.04 2.70	Gly72 Tyr35 Ile42 His147	3.69 5.08 5.19 4.18
Secoiridoids	Gentiopicroside	-6.3	Asn135 Gly73 Lys131 Gly134	2.71 2.21 2.67 2.76	Asp124 Gly72	3.37 3.54
	Swertiamarin	-6.0	Ala74 Lys131	3.03 2.26	Ala74	3.43
	Sweroside	-6.0	Ala74 Lys131	3.03 2.29	Ala74 Lys131	3.39 3.50
C-glycosylated flavones	Isovitexin	-7.3	Asn123 Tyr35 Asn123 Asn123	2.79 3.04 2.91 2.07	Gly72 Gly72 Arg68 Gly73 Thr149 His147 Gly134 Asn135 Gly134 Asn135 Val75 Ala136 Val75	3.25 3.62 3.65 2.97 3.90 4.56 4.23 4.23 5.29 5.29 5.00 4.52 4.56

Ligand		Binding free energy (kcal/mol)	Residues involved in **H-bond** interactions		Residues involved in **hydrophobic** interactions	
			Amino acid	Distance (Å)	Amino acid	Distance (Å)
C-glycosylated flavones	Isoorientin	-7.4	Asp286 Asn123 Asn123 Asn123	1.86 2.88 2.94 2.23	Gly72 Arg68 His147 Gly134 Asn135 Val75 Ala136 Val75 Val75 Ala136	3.16 3.75 4.50 4.28 4.28 4.97 4.71 5.42 4.66 5.05
C-glycosylated xanthone	Isogentisin	-6.8	Lys131 Lys131	2.48 2.65	Asp286 Gly72 Asp124 Asp286 Gly73 Gly73 Asn123 Gly134 Asn135 Ala136	3.53 3.13 4.95 4.22 2.43 3.02 2.31 4.40 4.40 5.23

Discussion

The results of this study indicate that the extract of *G. asclepiadea* underground parts shows the potential to inhibit the growth of pathogens related with foodborne poisoning and gastrointestinal disorders.

A high sensitivity of *Bacillus* species (*B. subtilis*, *B. cereus*, and *B. subtilis* ATCC 6633) and the selectivity against Gram-positive bacterial strains, were similarly noted by Stefanović et al. (2018) and Mihajlović et al. (2011) (10, 11). In a comparative study of aqueous, ethanolic, acetone and ethyl acetate extracts from the root of *G. asclepiadea*, ethyl acetate extract showed the greatest antimicrobial activity (11). These findings were also in agreement with ours, indicating that the antimicrobial active ingredients have an affinity for distribution in an ethyl acetate solvent. Therefore, ethyl acetate can be efficiently used for extraction, purification and separation of active compounds from examined plant drug that have a potential antimicrobial activity.

The improvement in antimicrobial activity using extract fractionation can be explained by the different concentrations of the active ingredients. The ballast compounds of the extract contribute to the increase of the mass of the dry extract and cause dilution of the active ingredients, thus interfering with the antimicrobial activity. Using the re-extraction process, ballast compounds can be removed from extract. The drastic difference of activities between the primary extract and its fraction (as in the case of *S. aureus*) may be due to the presence of antagonistic compounds separated by fractionation. Such discrepancy in activities, points out the ability of the used extract fractionation procedure to elucidate the potential biological activity of the trace compounds and/or compounds whose activity is influenced by other ingredients of the mixture.

The antimicrobial potential of other plant species of the genus *Gentiana* showed a close pattern of antimicrobial activity. Olennikov et al. (2015) reported about potent antimicrobial activity of bitter herb tea decoctions of four *Gentian* species (*G. algida*, *G. triflora*, *G. macrophylla* and *G. decumbens*), as well as gentiopicroside and loganic acid-6-O-β-D-glucoside as major bitterness compounds against six gastrointestinal pathogens (16). Gentiopicroside, a major secondary metabolite of *G. asclepiadea* underground parts (17), showed an antimicrobial activity with a MIC ranging from 0.1 to 0.4 mg/ml. In accordance with our results, in all tested samples the yeast *C. albicans* was the most resistant. This is not unexpected, given that plants of genus *Gentiana*, especially the underground parts, are rich sources of carbohydrates (18, 19). The presence of carbohydrates as source of carbon in the microenvironment of *C. albicans*, significantly affects the level of yeast growth, survival and tolerance to antifungal drugs (20).

Root extract of *G. kuroo* showed a similar antimicrobial spectrum directed towards Gram-positive bacteria (21). Yin et al. (2017) determined an antibacterial activity of *G. macrophylla* roots extract against bacteria isolated from infected burn wounds that are predominantly Gram-positive strains (22). On the other hand, a broad-spectrum (non-selective) antimicrobial activity of *G. lutea* leaves and flowers extracts was observed (23). This non-selectivity of antimicrobial activity is probably due to differences in the phytochemical profile of the underground and aboveground organs. Namely, in the aboveground part of the *G. lutea*, the major compounds are flavonoids and xanthones, unlike

roots where secoiridoids are dominant (9). A similar antimicrobial spectrum of the root extracts of plants belonging to the genus *Gentiana* that target Gram-positive strains may indicate that ingredients with antimicrobial activity are secondary metabolites that are universally distributed in all or most plant species of this genus, such as secoiridoids, xanthones, and/or flavonoids. In order to explore the possible interactions of the six main compounds from underground parts of *G. asclepiadea* and the selected target protein, the research was further extended by a molecular docking analysis.

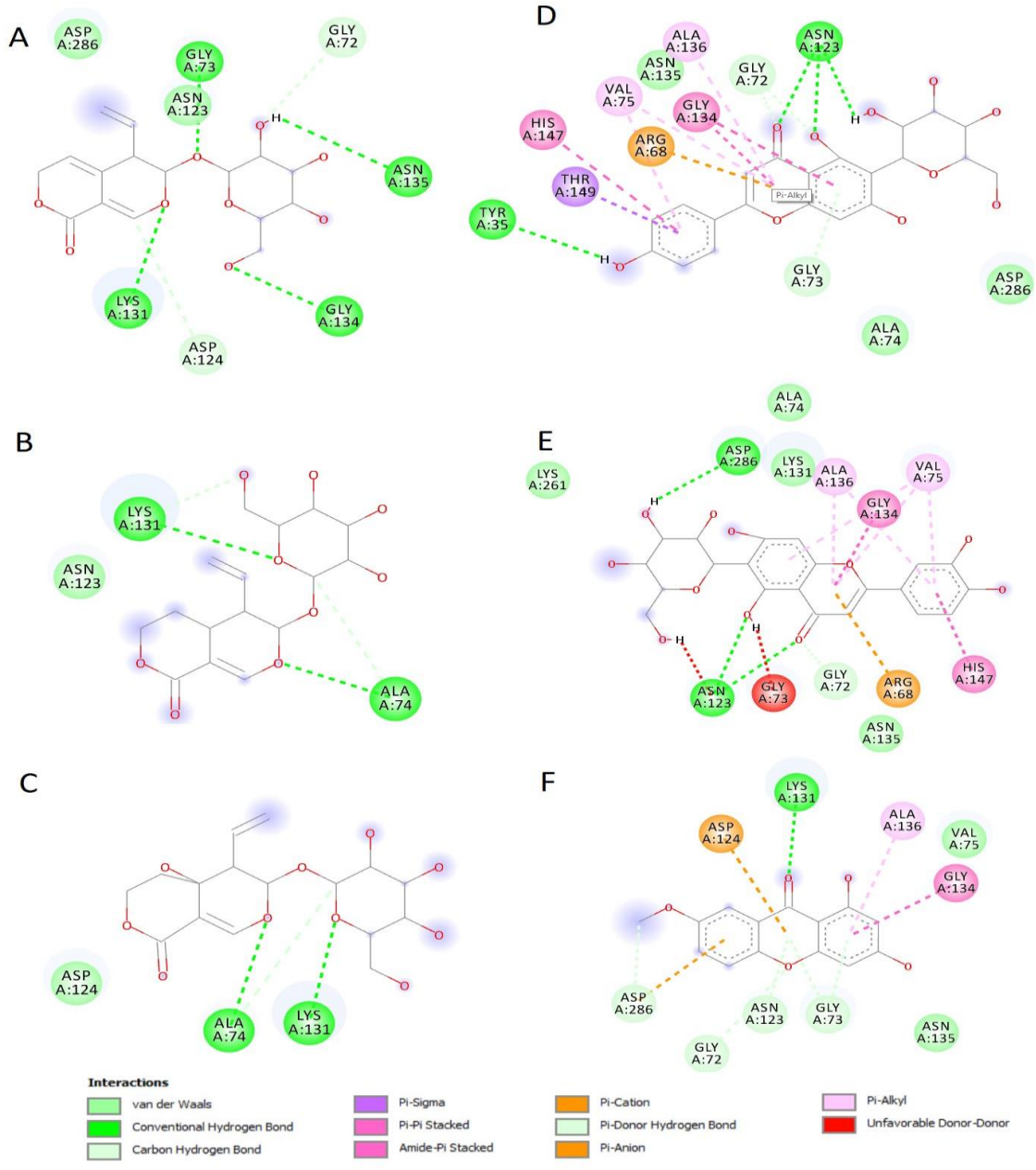

Figure 1. 2D structures of the lipoate-protein ligase A (lp1A-1) complexed with gentiopicroside (A), swertiamarin (B), sweroside (C), isovitexin (D), isoorientin (E), and isogentisin (F).

Enterococcus faecalis lipoate-protein ligase A (lp1A-1) was chosen as the target enzyme, because the lowest MBC (0.78 mg/ml) was recorded on this bacterial strain, which indicates its sensitivity to the constituents of the examined extract and fractions. lp1A-1, as one of the pivotal enzymes involved in

lipoic acid metabolism (24), is proved to be a potential target of *E. faecalis* directed antimicrobial compounds (25). The lowest negative value of the binding free energy of isoorientin (-7.4 kcal/mol) indicates that this ligand can bind more strongly and establish a more stable complex with the catalytic part of the enzyme compared to other compounds.

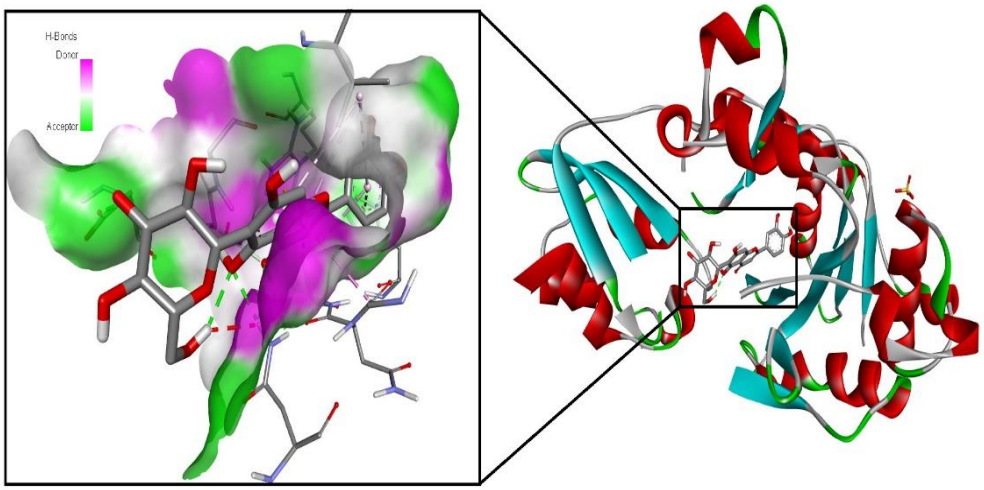

Figure 2. 3D structures of the lipoate-protein ligase A (lp1A-1) with an emphased binding pocket in interaction with isoorientin

An analysis of the interaction profile (Figure 1E) showed that isoorientin formed four conventional hydrogen bonds with Arg123 and Asp286 amino acid residues. It is well known that the ability to form hydrogen bonds between ligands and binding pockets is the primary factor influencing binding affinity (26). Isovitexin also formed four conventional hydrogen bonds via the two amino acid residues Asn123 and Tyr35 (Figure 1D). Although the number of hydrogen bonds is the same, the slightly lower binding free energy of isoorientin (-7.4 kcal/mol) compared to isovitexin (-7.3 kcal/mol) can be explained by the shorter bond length (distance, Å). Studies have shown that a higher hydrogen bond strength and a consequently higher complex stability is associated with a shorter bond length (26). Both mentioned compounds form a multitude of hydrophobic bonds, which are characterized by lower strength compared to the interactions via hydrogen bonds. The contribution of the hydrophobic binding to the docking score was highlighted in the case of genciopiroside. Although it formed four conventional hydrogen bonds (Figure 1A), a significantly lower binding affinity (-6.3 kcal/mol) was observed compared to isovitexin and isoorientin, which is a consequence of weaker hydrophobic binding. Amino acid residues involved in the interactions (hydrogen and hydrophobic) and the interatomic distance between selected compounds and lp1A-1 are listed in Table 2.

Conclusion

The results of the conducted study confirm that the underground parts of *Gentiana asclepiadea* are a valuable plant drugs with a significant antimicrobial potential against pathogens related with gastrointestinal diseases. Fractionation of the primary extract was a suitable method to improve the antimicrobial activity. The ethyl acetate fraction was particularly active, indicating that the active ingredients of the extract have an affinity for distribution in this solvent. The activity of the extract and the fractions was selective, mostly towards Gram-positive bacterial strains. The molecular docking and interaction profile analysis of the six major secondary metabolites of the examined drug on lipoate-protein ligase A (lp1A-1) show that the *C*-glycosylated flavones isoorientin and isovitexin have the best binding affinity. Further *in vivo* studies and detailed phytochemical analysis of extract and fractions are required to determine their active compounds and evaluate possible uses in the treatment of gastrointestinal disorders.

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ANTIMIKROBNA AKTIVNOST FRAKCIJA I EKSTRAKTA PODZEMNIH DELOVA BILJNE VRSTE *GENTIANA ASCLEPIADEA* L. SA ANALIZOM MOLEKULARNOG DOKINGA

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Trava od žutice (*Gentiana asclepiadea* L.) vredan je izvor sekoiridoida, *C*-glikoziliranih flavona i ksantona, koja se u tradicionalnoj medicini upotrebljava za lečenje bolesti jetre i gastrointestinalnog trakta. Cilj ovog istraživanja bio je ispitati antimikrobnu aktivnost vodeno-etanolnog ekstrakta podzemnih delova trave od žutice i njegovih frakcija (petrol-etarska, etil-acetatna, butanolna i vodena frakcija). Takođe, sprovedena je analiza molekularnog vezivanja. Antimikrobna aktivnost na patogene gastrointestinalnog trakta testirana je mikrodilucionom metodom. Generalno, najbolju aktivnost ispoljila je etil-acetatna frakcija. Najniža minimalna inhibitorna koncentracija od 0,78 mg/ml zabeležena je kod soja *Bacillus cereus* pomoću petrol-etarske frakcije, odnosno *Staphylococcus aureus* pomoću etil-acetatne frakcije. Najbolja baktericidna aktivnost (minimalna baktericidna koncentracija od 0,78 mg/ml) ostvarena je etil-acetatnom frakcijom za soj *Enterococcus faecalis*. *Candida albicans* bila je najotpornija na dejstvo ispitivanog ekstrakta i njegovih frakcija. Analizom molekulskog vezivanja, utvrđeno je to da *C*-glikozilirani flavoni izoorijentin i izoviteksin pokazuju najveći afinitet vezivanja prema *Enterococcus faecalis* lipoat-protein ligazi A. Na osnovu rezultata sprovedenog istraživanja, može se zaključiti da bi podzemni delovi trave od žutice mogli biti vredan izvor sekundarnih metabolita sa obećavajućom antimikrobnom aktivnošću.

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Ključne reči: *Gentiana asclepiadea, antimikrobna aktivnost, ekstrakti, frakcije, molekularni doking*

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MANTLE CELL LYMPHOMA – A KILLER WITH A CHILD'S FACE

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Mantle cell lymphoma (MCL) is a distinct type of non-Hodgkin lymphoma with very aggressive clinical behavior. Despite its bland morphology, MCL remains incurable and deadly disease, although several variants with more indolent clinical course have been recognized. This study aimed to comprehensively analyze pathological features of MCL in patients from Southeastern Serbia and to determine the frequency of this devastating disease in our population. During the five-year period, the diagnosis of MCL was established in 47 cases, which constitutes 10.3% of all newly diagnosed lymphomas in our Center for Pathology, University Clinical Center Niš. The majority of the patients were men, 72.3%, and the average patients' age at the time of diagnosis was 66.1 years. Extranodal presentation was observed in 61.7%. Every fourth case of MCL was diagnosed on bone marrow biopsy. The oral cavity and the gastrointestinal tract were equally represented as extranodal diagnostic location with 17% each. MCL encompasses large spectrum of architectural patterns and cytological variants thus its diagnosis requires immunohistochemical analysis of CyclinD1 and SOX11 for correct diagnosis and distinction from other lymphoid neoplasms and reactive and hyperplastic conditions. Variant morphology of MCL may be easily confused with potentially curable or indolent lymphomas. Accurate and precise diagnosis of MCL may improve patients' outcome through timely application of new and promising treatment strategies. Pathologist role in proper recognition and rapid diagnosis of MCL and its subtype, especially in biopsies from extranodal locations, including endoscopic biopsies, may contribute significantly to longer survival and better clinical outcome of the disease.

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Key words: *mantle cell lymphoma, diagnosis, pathology, immunohistochemistry, CyclinD1*

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Introduction

Mantle cell lymphoma is a distinct type of non-Hodgkin lymphoma with very aggressive clinical behavior (1). Although several variants with more indolent clinical course have been recognized (2, 3), mantle cell lymphoma (MCL) remains incurable and deadly disease. MCL accounts for 5-10% of all non-Hodgkin lymphomas.

MCL belongs to a group of mature B-cell neoplasms, and it is usually composed of monomorphic population of small to medium-sized cells with irregular nuclei. MCL cells are quite homogenous, uniformly appearing, usually there is no large, transformed cells within neoplastic population. MCL cells may have angulated nuclear contours or slightly polygonal shaped or cleaved nuclei, and scarce pale cytoplasm. Generally, MCL cells are just slightly larger, but very similar to normal lymphocyte. On a small biopsy, it is not difficult at all to miss the bland morphology of MCL. MCL can easily go unnoticed, especially on biopsies from organs that are normally rich in lymphoid tissue content, like mucosa of digestive system or Waldeyer's ring (1, 4).

Diagnostic hallmark of MCL is aberrant expression of CyclinD1, which reflects pivotal molecular event in pathogenesis of this lymphoma, the translocation t(11;14)(q13;q32). This chromosomal aberration results in juxtaposition of the bcl-1 locus on 11q13 to the immunoglobulin heavy chain gene region, which leads to deregulation of gene encoding the cell cycle protein CyclinD1. CyclinD1 overexpression further deranges cell cycle control by breaching the tumor suppressor function of RB1 and p27.

Additional secondary genomic alterations are required for complete malignant transformation. Predominantly these necessary alterations affect genes involved in cell cycle regulation and repair mechanisms in DNA damage cell response. Recently, a rare CyclinD1-negative subtype of MCL has been identified. This rare variant shares clinical, morphological, and phenotypic characteristics with classic MCL, and usually carries translocations involving *CCND2*/CyclinD2 gene (4, 5).

Clinical manifestations of MCL may be quite non-specific and diverse. Usually, they are associated with the anatomic region affected, and may represent as regional lymphadenopathy, asymmetric enlargement of palatine tonsil accompanied by odynophagia or hoarseness, abdominal discomfort or pain, gastrointestinal bleeding or bowel obstruction. Systemic symptoms like weight loss, general malaise, fever and night sweats are only seldom encountered. Occasionally, only abnormalities in peripheral blood analysis (leukocytosis with lymphocytosis, anaemia, pancitopenia) may lead to bone marrow biopsy and establishment of MCL diagnosis.

The data about the incidence, diagnosis, therapy, and outcome of MCL in our country is scarce (6-10). This study aimed to comprehensively analyze pathological features of MCL in patients from Southeastern Serbia and to determine the frequency of this devastating disease in our population.

Materials and methods

A total of 455 lymphomas that were diagnosed in the Center for Pathology, University Clinical Center Niš between January 2016 and December 2020 were analyzed, among which MCL cases were selected. The patients' data were retrospectively collected from archived medical records. Baseline clinical characteristics were evaluated, including patients' age, gender, and localization of lymphoma presentation.

Formalin-fixed paraffin-embedded tissue sections stained with hematoxylin and eosin were used for diagnosis and assessment of pathologic parameters. Pathologic diagnosis of MCL was based on the current 2017 World Health Organization classification (1).

Immunohistochemical (IHC) analysis in all cases where suspicion of MCL was made during the review of HE slides comprised the following IHC panel: CD20, PAX5, CD3, CD5, CD10, Bcl-2, Bcl-6, MUM1, CyclinD1 and Ki67. Only cases from year 2020 were stained to SOX11, because this antibody was not available in our Center during the previous years.

Results

During the five-year period the diagnosis of MCL was established in 47 cases, which constitutes 10.3% of all newly diagnosed lymphomas in our Center for Pathology, University Clinical Center Niš. The majority of the patients were men, 72.3%, and the average patients' age at the time of diagnosis was 66.1 years (Table 1).

The majority of MCL had extranodal clinical presentation (29 cases, 61.7%). Every fourth case of MCL was diagnosed on bone marrow biopsy. Oral cavity and gastrointestinal tract were equally represented as extranodal diagnostic location, with 17% respectively (Table 2).

Micromorphology and immunophenotypic characteristics of MCL are shown in Figures 1-3.

Table 1. Clinical-pathological characteristics of newly diagnosed cases of Mantle cell lymphoma

Year	Total lymphoma	**Mantle cell lymphoma**		Average patients' age (yrs)	Male patients (N)	%
		(N)	**(%)**			
2016	82	12	14.6	64.8	10	83.3
2017	84	9	10.7	73.0	6	66.6
2018	88	7	8.0	67.1	6	85.7
2019	69	8	11.6	59.4	6	75.0
2020	126	11	8.7	66.2	6	54.5
Total	455	47	10.3	66.1	34	72.3

Table 2. Structure of diagnosed cases of Mantle cell lymphoma by initial primary nodal and extranodal locations

MCL diagnostic site	N	%
Total	47	100
Nodal localization	18	38.3
Cervical lymph nodes	13	27.7
Inguinal lymph nodes	5	10.6
Extranodal site	29	61.7
Bone marrow	13	27.7
Oral cavity	8	17.0
Tonsil	4	8.6
Epipharynx	2	4.2
Non specified	2	4.2
Gastrointestinal tract	8	17.0
Stomach	4	8.5
Colon	3	6.4
Intestine	1	2.1

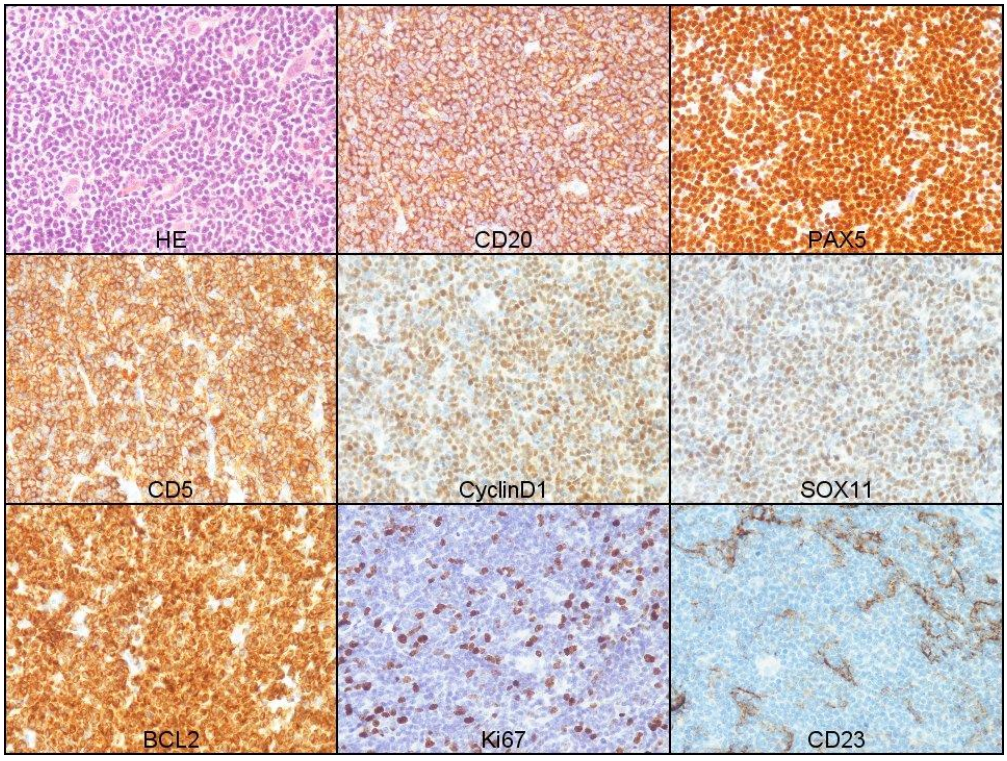

Figure 1. Morphology and immunophenotype of nodal Mantle cell lymphoma:
monomorphic medium-sized lymphoid cells population with scattered epithelioid histiocytes and hyalinized vessels.
Neoplastic cells express B-cell markers CD20 and PAX5 in addition to diagnostic CD5, CyclinD1 and SOX11 expression.
Bcl-2 is also positive, while Ki67 proliferative index is 15%.
CD23 highlights residues of follicular dendritic meshwork.
Original magnification x400.

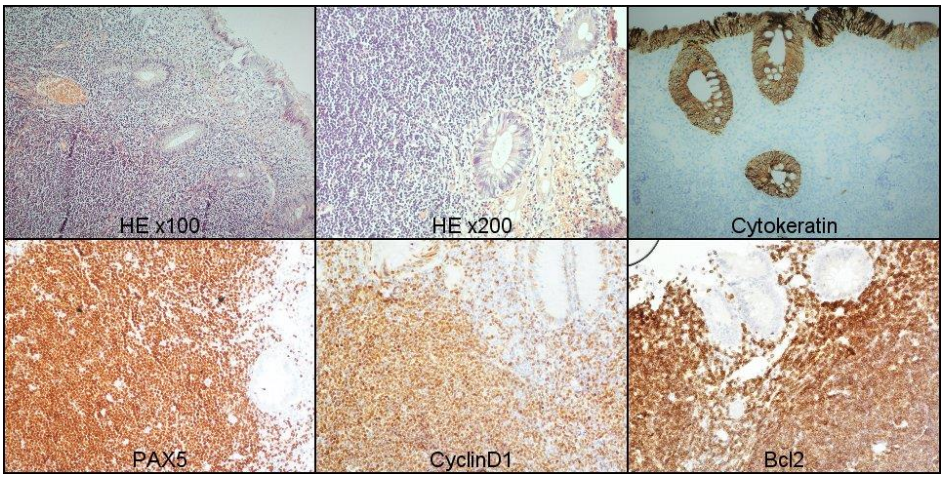

Figure 2. Lymphomatous polyposis of the colon – Mantle cell lymphoma of gastrointestinal tract.
Diffuse infiltration of colonic wall by small to medium sized neoplastic lymphoid cells.
Cytokeratin stains preserved superficial and cryptal epithelium.
Tumor cells strongly and diffusely express PAX5, CyclinD1 and Bcl-2.
Original magnification x100 and x200.

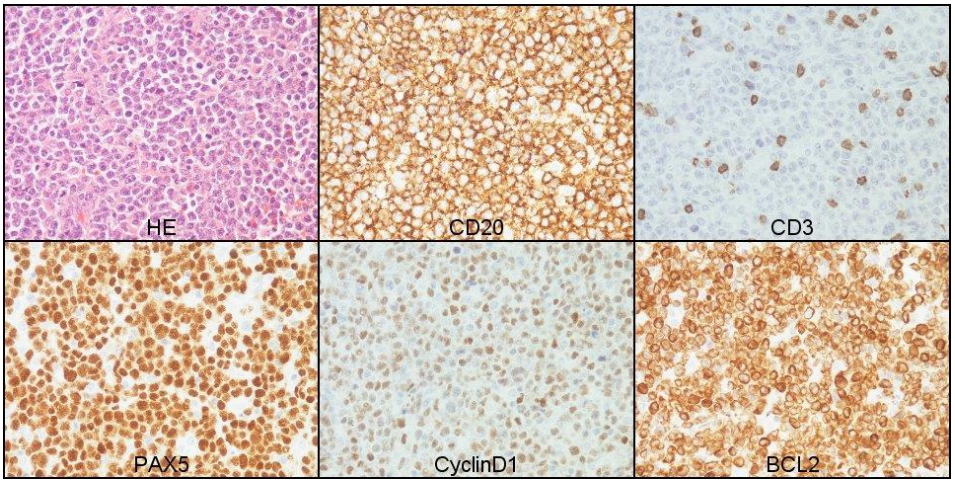

Figure 3. Pleomorphic mantle cell lymphoma, aggressive variant of MCL that closely resembles diffuse large B-cell lymphoma. Large lymphoid cells have irregular nuclei with prominent nucleoli,
and abundant cytoplasm, and are immunohistochemically positive to B-cell markers,
CyclinD1 and Bcl-2, and negative to T-cell marker CD3.
Original magnification x400.

Discussion

MCL most frequently occurs in older men, compared to other types of non-Hodgkin lymphoma, and the average patients' age is 65. The results of our study are in accordance with these findings. The majority of MCL in our Center was diagnosed in male patients, who comprised 72.3% of cases, with the average patients' age 66.1 years. In 2020, the percentage of female patients with MCL increased, which has no explanation other than pure stochastic probability, considering the fact that MCL is not significantly associated with autoimmune diseases, smoking, alcohol consumption, exposure to ultra-violet radiation or body mass index, unlike some other lymphoma types (11). The only significant risk factor, associated with a twofold increased risk of MCL, is family history positive for hematological malignancy among first-degree relatives.

Unfortunately, the data about family history of our patients was not available.

During the recent years, the definition of MCL has evolved from aggressive B-cell lymphoma with constitutive overexpression of CyclinD1 into a pathological spectrum of several clinical and biological subtypes. Major breakthrough was made with identification of CyclinD1 negative MCL and discovery of MCL with CyclinD2 translocations (5, 12, 13). Two distinct clinical subtypes are recognized: more aggressive, conventional MCL, and more indolent form, leukemic, non-nodal MCL. Conventional MCL originates from naïve B-cell of mantle zone, expresses transcription factor SOX11, develops numerous genetic aberrations because of high chromosomal instability, and usually presents with generalized lymphadenopathy. Leukemic non-nodal MCL originates from a B-cell that has experienced germinal center microenvironment, retains memory B-cell phenotype with more stable karyotype, and is negative for SOX11. Leukemic MCL shows minimal involvement of lymph nodes, it presents with leukemia and splenomegaly and can remain clinically indolent for years, but progression to aggressive disease may occur (3, 5, 14, 15). We initially diagnosed MCL from bone marrow biopsy in 27.7% of the cases, which is not surprising, since bone marrow infiltration and peripheral blood involvement with notable lymphocytosis are seen in the majority of MCL, up to 90%. These patients had hematologic disturbances, some had enlarged spleen, and the treating clinicians chose bone marrow biopsy as a diagnostic method of choice.

Mantle cell lymphoma *in situ* is a recently introduced entity in WHO classification of mature B-cell neoplasms (1). This lesion is usually an incidental finding of CyclinD1-positive B-cells in mantle zones of reactive lymphoid follicles, who carry translocation t(11;14) (2). Clinical significance of this lesion is not well understood. Its course may be associated with overt MCL, other small B-cell lymphoma or may remain silent long after diagnosis, even without any treatment (1, 2).

Differential diagnosis of MCL is not straightforward based on its histology, because MCL encompasses large spectrum of architectural patterns and cytological variants. Involvement of lymph nodes may represent in a form of mantle zone, nodular and diffuse pattern, and requires immunohistochemical analysis to CyclinD1 and SOX11 for correct diagnosis and distinction from other lymphoid neoplasms and reactive and hyperplastic conditions. Variant morphology may be easily confused with potentially curable or indolent lymphomas. Small cell MCL variant can be easily misdiagnosed as small lymphocytic lymphoma/chronic lymphocytic leukemia, while blastoid and pleomorphic variants (Figure 3) of MCL can mimic DLBCL or acute myeloid leukemia (16, 17). Opposite to DLBC which is curable in most of the cases, these aggressive variants of MCL are quite rare but deadly. Among our cases of MCL, only one had alarming morphology of pleomorphic variant, while two cases were small cell MCL.

We diagnosed MCL in gastrointestinal system in 17% of the cases, which is in accordance with the notion that MCL involves GIT in 10-25%. MCL can manifest in a form of multifocal lymphomatous polyposis, multiple polyps most frequently involving ileocecal region of the bowl (Figure 2). This gross finding may resemble much less perilous diagnostic possibilities, thus diagnostic biopsy is always indicated. Nevertheless, multifocal lymphomatous polyposis of small or large bowel is not specific for MCL only, and can be morphologic expression of other lymphomas (18, 19). Diagnosis of MCL in digestive tube is quite challenging, especially because of the presence of MALT, mucosa-associated lymphoid tissue, which may show hyperplastic and reactive changes in numerous conditions. Bland morphology of MCL can mimic benign entities. Therefore, MCL must be always considered in biopsies of polyps with large lymphoid aggregates (20, 21).

Clinical prognosis of MCL is poor, excluding the rare indolent forms. MCL usually follows the course of a very aggressive refractory disease. Patients are stratified according to the clinical prognostic factors clustered under MCL international prognostic index (MIPI) into low, intermediate, and high risk groups, and treated accordingly with various immunochemotherapy combinations, preferably with rituximab maintenance. In refractory cases or in early relapses newer targeted approaches are strongly recommended. Genomic landscape information, such as mutational status of *TP53* and *CCND1*, are gaining increased recognition as factors that increase prognostic value of MIPI index. Further understanding of molecular events in MCL which influence its clinical behavior could allow personalized therapeutic approach (22, 23).

Currently, MCL is an incurable disease, a killer with a child's face. However, accurate and precise diagnosis may improve patients' outcome through timely application of new and promising treatment strategies. The pathologist's role in a proper recognition and the rapid diagnosis of MCL and its subtype, especially in biopsies from extranodal locations, including endoscopic biopsies, and distinction from other less aggressive non-Hodgkin lymphomas may contribute significantly to longer survival and better clinical outcome of the disease.

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MANTLE ĆELIJSKI LIMFOM – UBICA DEČIJEG LICA

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Mantle ćelijski limfom (MCL) je poseban tip non-Hodgkinovog limfoma sa veoma agresivnim kliničkim ponašanjem. Uprkos blagoj, uniformnoj morfologiji, MCL je neizlečiva i smrtonosna bolest, iako je prepoznato nekoliko varijanti sa indolentnijim kliničkim tokom. Cilj ove studije je sveobuhvatna analiza patomorfoloških karakteristika MCL-a kod bolesnika iz jugoistočne Srbije i određivanje učestalosti ovog limfoma u našoj populaciji. Tokom petogodišnjeg perioda, dijagnoza MCL-a postavljena je u 47 slučajeva, što čini 10,3% svih novodijagnostikovanih limfoma u Centru za patologiju Univerzitetskog kliničkog centra Niš. Većina obolelih bili su muškarci (72,3%), a prosečna starost bolesnika, u trenutku postavljanja dijagnoze, bila je 66,1 godina. Ekstranodalna prezentacija bila je prisutna kod 61,7% obolelih. Svaki četvrti slučaj MCL-a dijagnostikovan je biopsijom koštane srži. Usna duplja i gastrointestinalni trakt podjednako su zastupljeni kao ekstranodalna dijagnostička lokacija, sa po 17%. MCL obuhvata širok spektar histoarhitektonskih obrazaca i citoloških varijanti, te dijagnostika zahteva imunohistohemijsku analizu ekspresije CyclinD1 i SOX11 gena za tačnu dijagnozu i diferencijaciju od drugih limfoidnih neoplazmi i reaktivnih i hiperplastičnih stanja. Morfološke varijante MCL-a mogu se lako pomešati sa potencijalno izlečivim ili indolentnim limfomima. Tačna i precizna dijagnoza MCL-a može poboljšati ishod bolesti blagovremenom primenom novih i obećavajućih strategija lečenja. Uloga patologa u pravilnom prepoznavanju i brzoj dijagnozi MCL-a i određivanju njegovog podtipa, posebno u biopsijama sa ekstranodalnim lokacijama, uključujući endoskopske biopsije, može značajno doprineti dužem preživljavanju obolelih i boljem kliničkom ishodu bolesti.

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Ključne reči: *mantle ćelijski limfom, dijagnoza, patologija, imunohistohemija, CiclinD1*

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DETERMINATION OF LYCOPENE CONTENT IN CULTIVARS OF *SOLANUM LICOPERSICUM* GROWN IN GREENHOUSE CONDITIONS

Vojkan Miljković[1], Violeta Rakić[2], Jelena Zvezdanović[1], Ljubiša Nikolić[1]

Lycopene is carotenoid, the pigment responsible for the color of ripe tomato.

The aim of this study was to determinate and compare lycopene content in fresh tomato grown under greenhouse conditions in Serbia (cultivar Hector-F_1), North Macedonia (cultivar Hamzali-F_1), Greece (cultivar Optima-F_1) and Turkey (cultivar Benetar-F_1). For this purpose, spectrophotometric method was used. The highest lycopene content is found to be in tomato grown in Serbia, followed by Turkey, Greece and North Macedonia with values given mg/kg of fresh fruit 81.53, 76.33, 27.92 and 13.49 respectively. The results confirmed that fresh tomato is good source of lycopene.

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Key words: *lycopene, tomato, spectrophotometric method*

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Introduction

Lycopene is a substance classified as phytochemical, bright red colored, carotenoid pigment, which can be found in tomatoes and other red fruits such as watermelon, pink grapefruit, pink guava, red bell pepper, papaya. In an organism that has the ability of photosynthesis, such as plants, algae and others, it plays an important role as a mediator in biochemical reactions of many carotenoids. Thus, beta carotene is responsible for yellow, orange or red pigmentation. Because of its strong color and non-toxicity, lycopene has found usage as a food colorant (1).

Lycopene is a polyene hydrocarbon, actually an acyclic open-chain unsaturated carotenoid having 13 double bonds, with 11 conjugated double bonds that are arranged linearly. It has molecular formula C_{40} H_{56}. Two central methyl groups are placed in the 1, 6 position, while the remaining methyl groups are in the 1, 5 position by each other. A series of conjugated double bonds in structure forms a chromatophore of variable length. The color and antioxidant activities of lycopene are a consequence of its unique structure, an extended system of conjugated double bonds (2) (Figure 1).

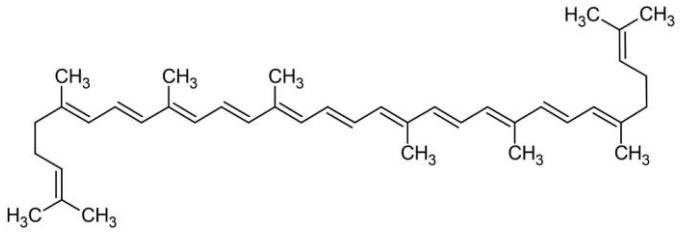

Figure 1. Structural formula of lycopene

Primary dietary source of lycopene in everyday nutrition is tomato and tomato based products (more than 80%) (3). Because of their composition, tomatoes and tomato based foods are considered as healthy food. They are low in fat and calories, cholesterol free and present a good source of fiber and protein. Also, tomatoes are rich in vitamins A, C, β-carotene, potassium, and lycopene. Well known red color of ripe tomato fruits and tomato based food products, which is considered as a measure of total quality, is due to lycopene (4). Some of the agricultural products that have high content of lycopene are: tomatoes, pink grapefruit, papaya and rosehip. The *Solanaceae* family consists of many species and its representative is *Lycopersicum esculentum* – tomato (5).

Lycopene is soluble in oils, so they help its absorption. Neither World Health Organization nor European Food Safety Authority gives recommendations for daily lycopene intake. Agarwal et al. in their work give recommendation for optimal daily intake of lycopene 3.35-4.82 mg (6). According to the work published by Uylaşer, daily intake of lycopene in amounts 5-7 mg is recommended for healthy humans in order to negate oxidative stress and for prevention of chronic diseases (7). Unlike some amino acids, lycopene is not an essential nutrient for humans. The intake of lycopene is through common diet, mostly from dishes with tomato sauce (8). Reduced risk of cancer is associated with daily intake of lycopene from food (9). Experimental researches showed that lycopene had high antioxidant activity and singlet oxygen quenching ability (10). It is obvious that it has properties which can improve human health and therefore deserves attention (7).

The aim

The aim of this work was to determinate and compare the lycopene content of fresh tomato of different species grown in Serbia, North Macedonia, Greece and Turkey (Figure 2).

Figure 2. Tomato cultivars grown in:
Serbia – Hector F_1; North Macedonia – Hamzali F_1, Greece – Optima F_1, Turkey – Benetar F_1

Materials and methods

Fruit material

For this experimental research tomato bought in April 2021 on the open market in Niš, Serbia was used. All species were cultivated in greenhouse, and their growth was observed. The tomatoes were washed, sliced and after that homogenized in Brown® blender.

Chemicals

Acetone was obtained from Fisher Scientific (Loughborough, United Kingdom), hexane, butylated hydroxytoluene and ethanol were purchased from Sigma-Aldrich (Steinheim, Germany).

Lycopene content determination

Spectrophotometrical method was used for total lycopene content determination as described (11). A 1 g of sample (fruit material) was added to a mixture consisting of 25 ml of hexane, 12.5 ml of acetone, 12.5 ml of ethanol and 0.05% (w/v) butylated hydroxytoluene. Afterwards, the mixture was stoppered and placed on an orbital shaker to mix at 180 rpm for 15 minutes (temperature of mixing was 5 ^{O}C). Process was followed with shaking, 7.5 ml of cold deionized water was added and the mixture was agitated for another 5 min. The suspension was left at room temperature for 10 minutes for separation of polar and non-polar layers to happen.

The absorbance of upper (non-polar) layer was mea-sured in a 1 cm path length glass cuvette at 503 nm versus a blank of hexane solvent on Jenway 6105 UV/Vis spectrophotometer (Jenway,

United Kingdom). Final calculation of the lycopene content was done using following equation:

$$A = \varepsilon\, b \cdot c$$

- (**ε**) the molar extinction coefficient of 17.2 x 10^4 M/cm is that reported (12),
- b is a 1 cm path length glass cuvette and c is concentration of lycopene.

All measurements were conducted in triplicate and data were expressed as mean values.

Results and Discussion

The lycopene content of the tomato fresh fruit grown in greenhouse conditions in the territory of Serbia, North Macedonia, Greece and Turkey was determined as follows: 81.53 mg/kg, 13.49 mg/kg, 27.92 mg/kg and 76.33 mg/kg in each examined cultivar respectively (Figures 3, 4).

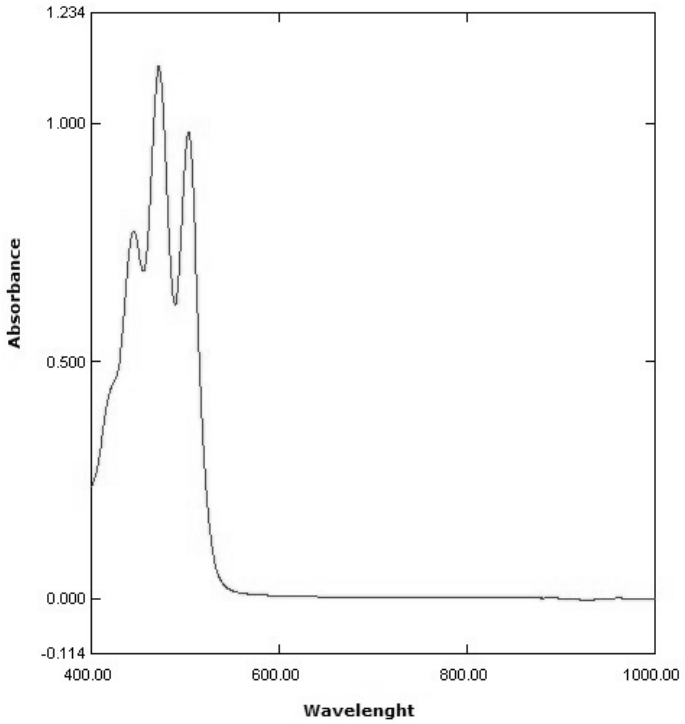

Figure 3. UV/VIS spectra of lycopene

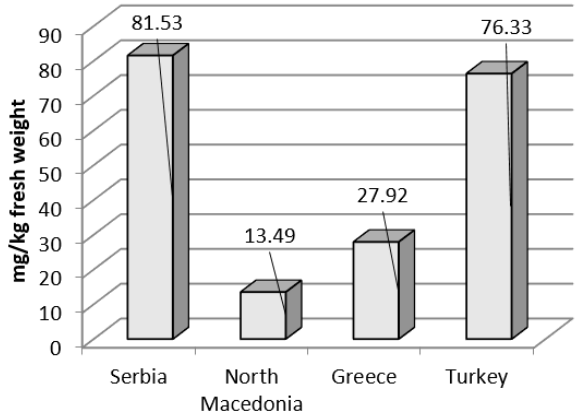

Figure 4. Lycopene content of tomato cultivated in greenhouse conditions in Serbia, North Macedonia, Greece and Turkey

It can be observed that there is a difference in lycopene content between cultivars. Hart and Scott reported that the amount of lycopene depended on variety, maturity and environmental conditions. They found that lycopene content in fresh raw tomato was 29.37 mg/kg and 37.03 mg/kg. Samples were kept frozen until the start of analysis and different composition of solvents was used (50 ml of tetrahydrofuran and methanol in ratio 1:1). These results were obtained by using HPLC with UV-VIS detector. For a reminder, UV-VIS method was used in our experimental research. However, cultivar conditions are not mentioned. Difference in results may be due to different cultivars which were analyzed (13). Gould researched how growing affected lycopene content of tomato. He showed that fruits grown in greenhouse during no matter what season (summer or winter) had lower lycopene content than fruits produced outdoors during summer time. Also, fruits picked up earlier and ripened during storage period are lower in lycopene content than ones that are fully ripened (14).

Results of our research are in agreement and very similar with the results for fresh tomato from Serbia. Veljović et al. calculated lycopene content in fresh tomato and found that fresh tomato had 79.66 mg/kg of fresh fruit (15). Jovanovski et al. Determined lycopene content in tomato growing in North Macedonia and it was 11.26 mg/dm^3 (16). Kapoulas et al. made a research about tomato cultivars Robin-F_1, Amati-F_1 and Elpida-F_1. The lycopene content was in range 24-37.5 mg/kg of fresh weight with the highest value for Elpida-F_1 cultivar (17). In the study by Karakaya and Yilmaz, the lycopene content of fresh tomatoes (cultivar Rio Grande) grown in Turkey was found to be 17.4 mg/kg (18).

Conclusion

We have reported results on lycopene content for tomatoes cultivated under greenhouse conditions in Serbia, North Macedonia, Greece and Turkey, determined using UV/Vis spectrophotometric method. Our results have shown lycopene content variation in different tomato cultivars grown in four diverse geographic locations. In our research, the highest lycopene content (81.53 mg/kg fresh fruit) was determined in tomato sample grown in Serbia. Results of our study are recommending fresh tomato as a good source of lycopene, where with everyday consumption the recommended daily intake can be easily achieved.

Acknowledgement

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ODREĐIVANJE SADRŽAJA LIKOPENA U SORTAMA *SOLANUM LICOPERSICUM* UZGAJANIM U PLASTENICIMA

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Likopen je karotenoid, pigment odgovoran za boju zrelog paradajza. Cilj ovog istraživanja je određivanje i upoređivanje sadržaja likopena u svežem paradajzu uzgajanom u uslovima plastenika u Srbiji (sorta Hector-F_1), Severnoj Makedoniji (Hamzali-F_1), Grčkoj (Optima-F_1) i Turskoj (Benetar-F_1). U tu svrhu, primenjena je spektrofotometrijska metoda. Najveći sadržaj likopena određen je u paradajzu uzgojenom u Srbiji, zatim u Turskoj, Grčkoj i Severnoj Makedoniji, sa vrednostima datim u jedinicama mg/kg svežeg paradajza 81,53, 76, 33, 27,92 i 13,49 respektivno. Rezultati su pokazali to da je svež paradajz dobar izvor likopena.

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Ključne reči: *likopen, paradajz, spektrofotometrijska metoda*

Original article

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EFFICACY OF FLUORIDE VARNISHES IN PREVENTING ENAMEL DEMINERALIZATION

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Demineralization is a process in which the inorganic content of the enamel structure is lost leading to occurrence of white spot lesions. The purpose of this study was to examine fluoride varnish effect on enamel. The study involved 20 premolars extracted for orthodontic reasons. Before the extractions, brackets were bonded with one type of adhesive according to manufacturers 'bonding instructions. After bracket bonding, ten left premolars (the test group) were kept dried by careful tooth isolation and the enamel received a topical application of fluoride varnish (Duraphat®, Germany). Ten right premolars (the control group) did not receive any varnish application and brackets were fixed using identical procedures. After two months, the premolars were extracted and prepared for SEM analysis. Samples treated with fluoride varnish showed a nearly smooth surface, with complete obtrusion of interrod spaces in some fields. The rods appeared as they were fused together with some globules deposited on the surface, relatively no evidence of porosities or irregularities. Within control group demineralization started on enamel surface, but still with adequate and genuine prisms together within interprismatic space. Micro-morphological surface observation of the enamel surfaces showed demineralized surface as rough and uneven tooth enamel (shrinking of prisms, due to the widening of the prismatic spaces). Fluoride varnish application on enamel surface prevents demineralization processes. Fluoride application could act as a 'barrier' against the demineralization processes on enamel.

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Key words: *fluoride varnish, demineralization*

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Introduction

Caries lesions result from the demineralization process of the tooth enamel, which primarily causes white spots on enamel surface and it is caused by bacterial products such as acids in cariogenic environment. White spots can clinically be found on the tooth surface when it comes to mineral dissolution of the enamel. Their appearance changes the color of the tooth on that particular spot from translucent to opaque. Because of that it can be considered as an esthetic problem knowing that they can't spontaneously disappear (1, 2).

Demineralization and remineralization are dynamical but also balanced processes that normally happen in oral cavity. Many factors can cause disbalance of these two processes such as diet variations, oral hygiene or microbial activity and result in the predominance of demineralization. The remineralization process represents the buffering capacity of the saliva with calcium and phosphate ions that form minerals for enriching tooth enamel.

Fluoride has been recognized as the main propriety for the decline in caries due to its cariostatic potential. Even though its major efficiency is in preventing caries, there are certain limitations when it comes to its effects. It means that fluoride cannot eliminate caries completely. On the contrary, it can even cause harmful effects on the tooth if applied in high concentrations (3, 4).

At a neutral pH of 7, low ion concentrations are sufficient to keep dental hard tissues in equilibrium. When pH drops below 7 as the result of acidogenic bacterial production and the presence of plaque, higher ion concentrations are needed to prevent appearance of incipient caries lesions. Enamel

starts to dissolve when pH drops to 5.5 or less. When that happens undersaturation begins, which means that calcium and phosphate ion concentrations in saliva and plaque fluid are not sufficient to provide minerals for enamel and keep it in balance. On the contrary, fluorhydroxyapatite (FHAP) and fluorapatite (FAP) dissolution begins at much lower pHs of about 4.7. Supersaturation begins when pH starts to increase. First, it begins within the FHAP followed by the FAP, all that it takes is some fluoride present in the oral cavity, which represents and explains the process of remineralization. Consequently, during remineralization after acid attack, a redistribution of mineral phases occurs, in which the proportion of stable, carbonate-poor FHAP in the enamel increases at the expense of carbonate-rich HAP. After this redistribution of minerals in processes of demineralization and remineralization, tooth enamel becomes more reluctant and acid resistant in comparison to undamaged enamel. During remineralization, the contribution of saliva with Ca^{2+}, PO_4^{3-} and OH^- ions in addition to the presence of dissolved F–is important.

Nowadays there are products that can be used for remineralization such as toothpastes and tooth mousses. From the clinical point of view the use of fluoride varnish could be more effective. The explanation lies in the fact that fluoride varnish will remain adhered to the tooth surface longer than toothpaste or dental cream prefunding its contact with the enamel (5, 6).

In the process of remineralization comes to ionic release which shows us that these products can act like a physical barrier and also show protective effect on the enamel when it comes to acid attack. Despite the fact that the use of varnishes can prevent white spots during orthodontic treatment, their effects regarding interproximal reduction procedures cannot be neglected (7, 8). It has been studied by Peng et al. (9) by measuring microhardness, density and mineral loss after applying fluoride varnish and resin infiltration on demineralized enamel surfaces. Fluoride catalyzes the diffusion of Ca and phosphate over the dental surface, which causes remineralization of the enamel crystalline structure to create fluorapatite crystals, which is the most resistant crystalline phase (10).

The purpose of this study was to examine the preventive effect of fluoride varnish on the enamel.

Materials and methods

The study involved 20 healthy premolars extracted for orthodontic reasons. Before extractions brackets were bonded with one type of adhesive according to manufacturers bonding instructions. The adhesive used in this study for bonding brackets was Con Tec LC (Dentaurum, Germany). After bracket bonding, ten left premolars (the test group) were kept dried by careful tooth isolation and the enamel received a topical application of fluoride varnish (Duraphat®, Germany) with the aid of a brush applicator. Ten right premolars (the control group) did not receive any varnish application and brackets were fixed using identical procedures.

After two months, the premolars were extracted and the teeth were disinfected by keeping them in 10% formalin for 48 hours and then stored at room temperature in distilled water till the time of SEM analysis. For the SEM analysis, the samples were coated with 40 nm to 60 nm of gold using a sputter coater and then observed in the microscope (VEGA3 LMU; TESCAN, a.s., Brno, Czech Republic) with the magnification ranging from x2000 to x3000.

By SEM analysis, micromorphologic changes in the enamel structure were monitored in the places where the brackets had been previously bonded.

Results

The initial demineralization of enamel prisms was observed in the control group (Figure 1).

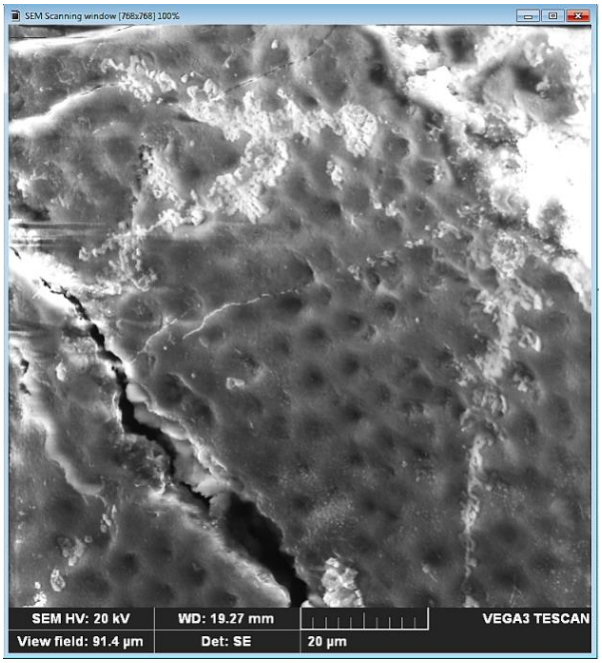

Figure 1. Initial demineralization of enamel prisms

The demineralized enamel showed a rough surface with a honeycomb appearance. Shallow depressions and fine porosities within these depressions were observed.

SEM examination of enamel surfaces adjacent to orthodontic brackets revealed calcium fluoride-like material (CaF_2) deposition as a product of topical fluoride varnish application. An adhered thin layer of fluoride varnish was also seen in some teeth of the test group, which was in close contact with the enamel around the orthodontic brackets (Figure 2 a, b).

Samples treated with fluoride varnish showed a nearly smooth surface, with complete obtrusion of inter-rod spaces in some fields. The rods appeared as they were fused together with some globules deposited on the surface, there were relatively no evidence of porosities or irregularities.

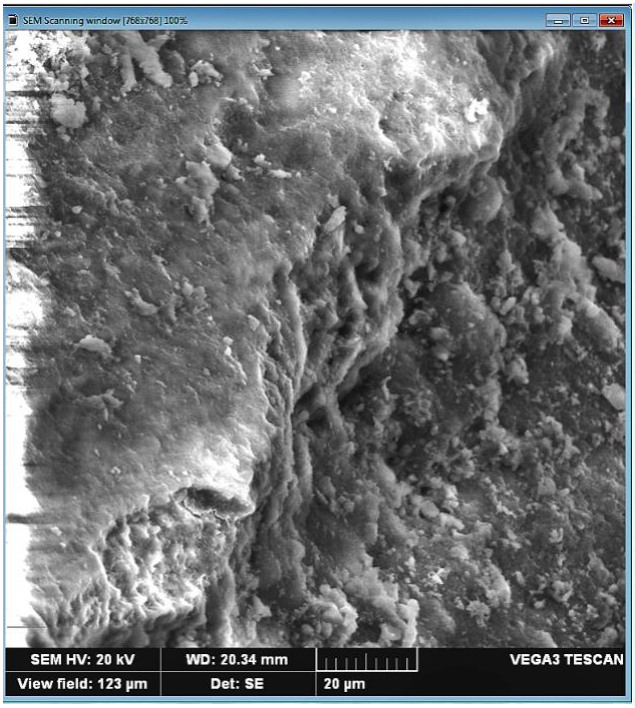

Figure 2 a. Calcium fluoride-like material (CaF_2) deposition

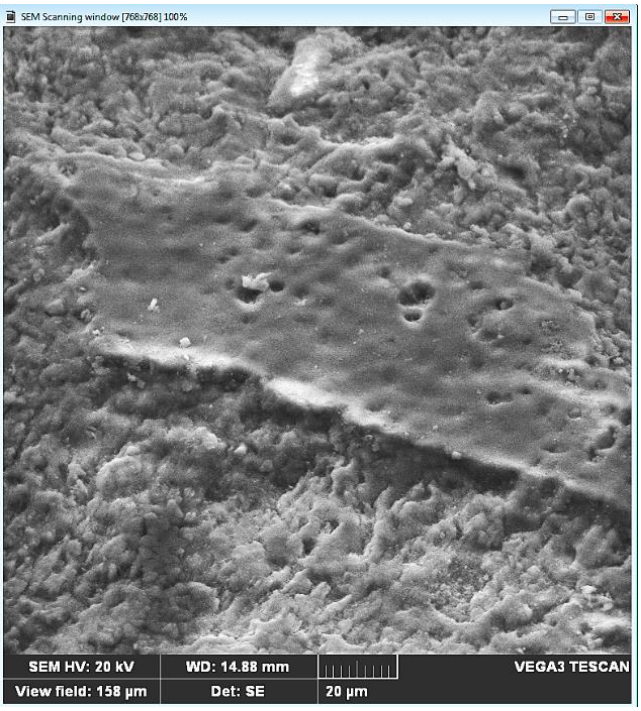

Figure 2 b. Smooth surface, with complete obtrusion of interrod spaces in some fields

Figure 3. Shrinking of prisms, due to the widening of the prismatic spaces

SEM examination showed multilayer surface dissolution with a minor honeycomb pattern of demineralization. The demineralization started on an enamel surface, but still with adequate and genuine prisms together within interprismatic space. We can see the teeth enamel in Figure 3, as demineralized surface showed through the uneven and rough surface of teeth enamel (shrinking of prisms, due to the widening of the prismatic spaces).

Discussion

Demineralization is a process which damages tooth surface, primarily enamel by obtaining its minerals and causing the loss of strength and hardness of the structure. White spots appear where demineralization takes place. Critical point for starting demineralization process is dropping pH at 5.5. In such conditions of oral environment, hydroxyapatite becomes more soluble because of more acidic environment. In the process of remineralization comes to replacement of the lost minerals by regaining fluoride ions and forming structure of fluorapatite crystals that are more resistant to acidic dissolution and subsequently larger than the original crystals. If pH continues to drop because of the acid attacks and gets to the point of 4.5, the efficiency of

'F' may be disputable yet ineffective in controlling caries progression (11, 12).

Early carries lesions such as white spots can be effectively cured with fluoride varnishes which can push the remineralization process to the formation of fluorapatite. However, if there is no certain amount of available ions of calcium and phosphate when applying fluoride topically then the process of remineralization can meet some limitations. If present in small amounts in solution around the tooth, fluoride has greater inhibition power regarding demineralization than incorporated fluoride. It means that small amounts of fluoride in solution have better impact on tooth demineralization and higher caries protective potential than large amounts of FAP in enamel (13).

The hypothesis above was confirmed. In those circumstances fluoride ions are adsorbed onto the crystalline surface and are in dynamic equilibrium with the fluoride ions that remain in solution in the immediate vicinity. In fluid that surrounds crystals this can lead to either equilibrium or supersaturation of fluorhydroxyapatite and due to that to reprecipitation of minerals. This adsorption of fluoride to the crystals shows the direct protection from demineralization. As for fluoride unprotected areas, enamel structure can easily be disrupted when acid attack occurs. Low fluoride concentrations could also be attained when consuming foods and beverages that contain fluoride salts. Only 30 minutes after the intake, the amount of fluoride concentration in saliva notably increases (14).

When observing CaF_2 in SEM its morphology appears as spherical globules which can come in different size and amount. Using an acidic amine fluoride solution first CaF_2 globules are formed within 20 s, but using acidic sodium fluoride or sodium monofluorophosphate (MFP) no CaF_2 globules can be formed *in vitro* at all (15). Since fluoride in MFP is covalently bound, it is very important for it to be released before its reaction with calcium in the oral cavity. After applying low dosage of amine fluoride dentifrice (250 ppm), significant amounts of soluble fluoride were found on the enamel which was not the case after applying a toothpaste with MFP. This facilitation of CaF_2 formation by low pH was confirmed in an in-situ study comparing a neutral-pH toothpaste containing sodium fluoride with an amine fluoride-containing toothpaste of pH 5.5.

After topical application of fluoride varnishes on dental tissues one of the most important products that comes out as the reaction product is calcium fluoride and as some may say the only product (16). Calcium fluoride that covers tooth enamel shows equal protective effects which is explained by releasing fluoride ions from it depending on pH and has major role in caries prophylaxis.

Pure CaF_2 does not form in vivo because of the substances and minerals that are deposited on it, which also makes it more resistant to acids. This stability comes from adsorption of hydrogen phosphate ions HPO_4^{2-} on the surface of CaF_2 crystals by creating protective film which inhibits solubility. When acid attack occurs, CaF_2 depot releases fluoride ions because of the reduced phosphate ions concentration which normally happens in acidic environment. Consequently, CaF_2 performs as fluoride depot dependent of pH level, which releases F^- at low pH but remains stable at neutral pH. By knowing these mechanisms, CaF_2 is considered to be the main source of free F^- ions during acid attack. These free F^- ions take part in both processes of demineralization and remineralization. In addition to that, they are far more important than high fluoride content of the enamel crystalline structure especially when it comes to caries attack (17).

Since most fluoride in Duraphat® varnish is insoluble and the CaF^{2-} like fluoride reservoirs chemically formed on enamel from soluble fluoride reactivity are considered responsible for the anticaries mechanism of action of professional fluoride application, in principle, it is challenging to explain how this product is effective to control caries (18).

This study explains the reaction of fluoride fractions from the varnish soluble and insoluble with the enamel surface and in that way extends scientific discoveries of Retief et al. (19) and Bruun and Givskov (20) who pointed out how important retaining of the fluoride varnish applied to dental surfaces for a longer time could be (21).

Duraphat® is often used fluoride varnish product and has been tested in many experiments by different authors (22). There has been *in vitro* study carried out by Shen et al. (23) who examined fluoride ion release of Duraphat® into artificial saliva. That study has shown that Duraphat® released roughly 30% of its total fluoride only 7 days after exposure. Fluoride ion release can occur in a much easier way from the varnish than from saliva because of the undersaturation of distilled deionized water. Having acknowledged this fact, it is important to understand what the addition of calcium and phosphate actually does. They do not reduce the availability of fluoride ions since fluoride from the varnish has provided great caries preventive efficacy in clinical trials.

Conclusion

Tooth surfaces that were treated with fluoride varnish could offer protection against demineralization of enamel.

Fluoride varnish applied to the tooth surfaces could act as a barrier against demineralization and also be recommended for caries prophylaxis to high caries risk patients.

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EFIKASNOST LAKA SA FLUOROM U PREVENCIJI DEMINERALIZACIJE GLEĐI

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Demineralizacija gleđi predstavlja proces gubitka mineralnih materija, što dovodi do nastanka belih mrlja. Cilj rada bio je da se ispita dejstvo lakova sa fluoridima na gleđ, koja je demineralizovana.

Ispitivanjem je obuhvaćeno 20 premolara, ekstrahovanih iz ortodontskih razloga. Pre ekstrakcije, na premolare su fiksirane bravice adhezivnim materijalom, po uputstvu proizvođača. Nakon fiksiranja bravica, gleđ 10 levih premolara (ispitivana grupa) tretirana je lakom sa fluoridima (Duraphat®, Nemačka). Deset desnih premolara (kontrolna grupa) nije bilo tretirano lakom sa fluoridima. Nakon dva meseca, premolari su ekstrahovani i pripremljeni za SEM analizu.

Gleđ premolara u ispitivanoj grupi imala je na izgled skoro glatku površinu sa potpunom opstrukcijom interprizmatičnih prostora. Prizme su bile povezane depozitima globula na površini, bez znakova poroznosti i iregularnosti. U premolarima iz kontrolne grupe zapažena je demineralizacija na površini gleđi, ali su prizme i interprizmatični prostori još uvek bili očuvani. Mikromorfološkom analizom površine gleđi, uočena je demineralizacija (hrapava i neravna površina gleđi).

Lak sa fluoridima, koji je aplikovan na površinu zuba, predstavlja barijeru i na taj način sprečava procese demineralizacije gleđi.

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Ključne reči: *lak sa fluoridima, demineralizacija*

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OBESITY AND SLEEP DURATION – INVESTIGATION OF DISORDERS OF GLYCOREGULATION AND SYSTEMIC INFLAMMATION

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Sleep quality and sleep duration are significantly associated with obesity onset and its progression. A prospective clinical study was conducted by analyzing 129 patients referred to polysomnography, out of whom 76 were obese. According to modified sleep quality survey ("National Health and Nutrition Examination Survey" - NHANES), patients were divided into two groups based on similar demographic and morphometric characteristics of sleep duration, so the group I comprised subjects with poor sleep quality, sleeping 4 hours or less on average, and group II enrolled subjects with moderate and good sleep quality, with 6 hours or more of sleep duration on average. It has been reported that all the subjects had elevated levels of C-reactive protein (CRP), and the subjects from group I with shorter sleep duration and poor sleep quality had statistically significant rise of CRP in comparison to the subjects from group II. It has also been proved that the subjects from both groups had elevated levels of glycated hemoglobin (HbA1c) as a parameter of poor glycoregulation. In obese persons, sleep duration and quality play a significant role in increasing inflammatory processes in the body. Obesity is a risk factor of impaired glycoregulation.

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Key words: *respiratory polygraphy, obesity, sleep duration, glycoregulation, systemic inflammation*

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Introduction

The World Health Organization (WHO) define overweight and obesity as abnormal or excessive fat accumulation that is a risk to health. The prevalence of overweight has significantly increased worldwide. In 2016 more than 1.9 billion adults over the age of 18 were overweight and out of them over 650 million were obese. Globally, 39% of adults (39% of men and 40% of women) were overweight, about 13% of adults (11% of men and 15% of women) were obese. Worldwide obesity has almost tripled between 1975 and 2016. Body mass index (BMI) is a simple index of body weight that is usually used for overweight and obesity classification in adults, and it is the same for both sexes and all ages of adults. It is defined as a person's weight in kilograms divided by the square of their height in meters (kg/m^2). As for adults, WHO defines overweight and obesity in the following way:

- overweight is a BMI 25 or greater;
- obesity is a BMI 30 or greater (1).

Short sleep duration has been increasing in many countries. The results from an analysis of 250,000 sleep questionnaires worldwide indicate that sleep duration on weekdays has dropped by about 37 minutes in the last decade (2). In the last 40 years sleep duration in the USA has declined for 1.5 to 2 hours. National Sleep Foundation of America survey in 2009 showed that American adults sleep 6h, 40 min on workdays on average, and 7h, 7 min at weekends, while in 1960 average sleep duration was 8h 30 min. The percentage of young people who sleep less than 7h a day has increased from 15.6% to 43% in the period from 1960 to 2009. In Brazil, sleep duration has declined for about 20 min in the period between 1987 and 2007 (3).

Experimental and clinical studies have recently demonstrated that sleep disorder and/or

sleep duration might affect glycoregulation and obesity onset and progression at one hand, and elevate levels of CRP, a marker of inflammation, on the other hand (4-6). Many of these studies are cohort, epidemiological, or cross-sectional ones enrolling large pools of participants, but very often these studies are inconsistent, conducted in inhomogeneous groups of participants, so this above mentioned association has not been clearly defined yet (7, 8). Sleep disorders and/or sleep duration are not the only factors responsible for obesity pandemic, but sleep affects energy balance and its effects should be taken seriously. Good sleep could be part of global approach in dealing with ongoing obesity pandemic (9). It has been proved in young, healthy volunteers that total sleep deprivation results in statistically significant increase in CRP levels at every 12-hour of total sleep deprivation, three-fold, or even four-fold in comparison to normal, average values in persons who sleep 7 hours a night (8, 10). It has also been undoubtedly confirmed that insomnia, obstructive sleep apnea/hypopnea syndrome (OSAHS), or 'restless leg syndrome' defined by reported symptoms and polysomnography are linked with disorders of metabolic and endocrine function, such as impaired glucose tolerance, leptin concentration levels decrease or increase, along with the development of leptin resistance, elevation of evening concentrations of cortisol, alterations in autonomic sympathetic activities, leading to increased appetite, body weight and its progression (11, 12).

The aim

The aim of this prospective clinical study was to establish HbA1c values as parameters of effective glycoregulation, and CRP values as markers of inflammation in obese individuals who sleep 4 hours or less in comparison to obese persons who sleep 6 to 7 hours per night.

Materials and methods

At the Clinic of Lung Diseases, University Clinical Center Niš, 129 patients underwent respiratory polygraphy from June 2016 to December 2017, aiming at confirming OSAHS. The procedures performed were as follows:

I. height and weight measurement, BMI calculation; individuals with BMI ≥ 30 were considered obese according to the WHO definition

II. modified NHANES survey on sleep quality in the last 12 months had 8 questions:

1. How often did you have trouble falling asleep?
2. How often did you wake up during night and did you find it difficult to fall asleep again?
3. How often did you wake up too early and were not able to fall asleep again?
4. How often did you feel tired during the day regardless the duration of sleep?
5. How often did you feel overly sleepy during the day?
6. How often did you not have enough sleep?
7. How often did you have leg twitching that woke you up?
8. How much sleep did you usually have at night?

The subjects who responded to any of 8 questions with 'almost always' (16 to 30 times a month) had poor sleep quality, in those who mostly responded 'often' (5-15 times a month) sleep quality was classified as moderate, and all the others had good sleep quality.

III. HbA1c values and CRP levels from blood samples were measured in all individuals.

Results

Out of 129 consecutive individuals, this study enrolled 76 obese ones with BMI ≥ 30, who were not diagnosed as diabetic patients before respiratory polygraphy, and who were divided into two groups according to average sleep duration and sleep quality based on given questionnaire. The first group comprised 40 individuals who slept 4 hours or less on average and assessed their sleep as poor quality, and the second group included 36 individuals with average sleep duration of 6 hours or more and who considered their sleep as moderately good and good sleep quality. Demographic and morphometric characteristics of all the subjects are given in Table 1.

Table 1. Demographic and morphometric characteristics of the subjects

Characteristics	Total - 76	I group – 40	II group – 36	p
Age	50.5 ± 7.1	51.7 ± 6.8	49.1 ± 9.4	p - n.s.
Men	49	20	19	p - n.s.
BMI	39.7 ± 10.6	40.1 ± 5.8	41.2 ± 4.9	p - n.s.
Smokers	38	17	21	p - n.s.
Alcohol consumption	40	18	22	p - n.s.
Marital	54	28	26	p - n.s.
Higher education	14	8	6	p - n.s.
Live in towns	48	25	23	p - n.s.

There was no statistically significant difference between the groups, both in morphometric and demographic characteristics. The groups were well homogenized according to obesity risk factors distribution and obesity itself in comparison to average sleep duration.

Statistical processing of systemic inflammation parameters, CRP levels and glycoregulation – HbA1c values in comparison to sleep duration in the groups of subjects are shown in Table 2.

Table 2. Values of systemic inflammation and glycoregulation parameters in both groups according to average sleep duration

	I group	II group	p
CRP (mg/l)	11.7 ± 9.4	7.92 ± 4.91	**p < 0.05**
HbA1c (%)	6.1 ± 4.8	5.9 ± 3.7	**n.s**

This study demonstrated statistically significant difference in the values of early systemic inflammation, CRP parameters, in obese patients with poor sleep quality and sleep duration of 4 hours or less in comparison to obese patients who slept 6 hours or more on average. In subjects of both groups statistically significant increase in CRP levels was registered in comparison to upper limit of the reference range for CRP of 5mg/l.

The results of this study have not confirmed statistically significant difference in HbA1c values between the groups of our obese patients in comparison to average sleep duration, but it has been proved that the subjects from both groups had elevated HbA1c values and poor glycoregulation, so further diagnostics and potential diabetes treatment is required.

Discussion

Sleep structure, duration and quality have been investigated for a long time, using different instruments, questionnaires, polygraphic recordings, polysomnography, aiming at better and more objective understanding of sleep influence in metabolic and endocrine body functions (11). Considering the fact that there are different sleep disorders, from obstructive and/or central sleep apnea, insomnia, narcolepsy, 'restless leg syndrome', 'jet lag', shift work disorder, 'Cheyne-Stokes' respirations, and alike, there are varieties of results and differences in examined populations that are still confusing (13). Black racial group, males over 65, obese, smokers, and heavy alcohol consumers are believed to be the most common risk factors for different sleep disorders, both in developed and developing countries (13, 14). The burden of modern times can be seen in disturbed sleep architectonics and sleep quality, consequently resulting in many cardiac, respiratory, endocrine and mental diseases (14). Knutson et al. found that sleep duration and quality were significant predictors of HbA1c, a key marker of glycemic control. In combination with existing evidence that link sleep loss with increased risk of diabetes onset, the authors suggest that optimizing sleep duration and sleep quality should be tested as a possible intervention for improving glucose metabolism control in patients with type 2 diabetes (15). Cappuccio et al. in their meta-analysis showed that a reduction of one hour sleep per day was associated with 0.35 kg/m^2 increase in BMI (16). Spiegel et al. found that six consecutive nights of sleep restrictions (4 hours sleep per night) resulted in a 30% reduction in acute insulin response to glucose (11). Irwin et al. Analyzed 72 studies (n-50000) by assessing CRP, interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF). They concluded that sleep disturbance was associated with higher levels of CRP. Shorter sleep duration, but not extremely short sleep duration, was associated with higher levels of CRP, but not IL-6. Extremely long sleep duration was associated with higher levels of CRP and IL-6. Sleep disorders and sleep duration were not associated with TNF-α alterations. Experimental sleep deprivation and restriction were not associated with CRP, IL-6, or TNF-α alterations (17). Patel et al. demonstrated in their study that an increase in usual sleep duration was associated with elevations in CRP levels and IL-6, while polysomnography revealed reduced sleep duration associated with elevated TNF-α levels. Activation of pro-inflammatory pathway may be a mechanism by which extreme sleep habits may affect our health (18). Dowd et al. examined association between inflammation and sleep characteristics in 1020 subjects from the Social Environment and Biomarkers of Aging Study from 2000 to 2006 in Taiwanese population aged 53 and over. They concluded that long sleep duration may be a cause of inflammatory diseases in older population (29).

Richardson et al. analyzed the sample of 5033 male and 4917 female individuals aged 20 and over in whom sleep duration was classified as short (6 or less hours a day), adequate (7-8 hours a day), or long (9 or more hours a day), the samples were homogeneous regarding age, race, smoking status, physical activity, and waist circumference. They concluded that short sleep duration was significantly associated with elevated serum CRP concentrations, regardless of the waist circumference and moderate physical activity in males, but not in females (20). Guilleminault et al. reviewed CRP levels in new

patients with OSAHS, upper airway resistance syndrome-UARS, and absence of important comorbidities, as well as in normal, healthy controls, in the period over 2 months and they concluded that obesity was a risk factor for high serum CRP levels in patients with sleep-disordered breathing and in general population as well, and that BMI was significantly associated with CRP levels in both genders (21). Having in mind the fact that sleep quality is one of the most important indicators of health and well-being, Nag and Pradhan concluded in their study that CRP level might be a marker of sleep disorder and excessive daytime sleepiness (22). Experimental studies have shown that there are distinct, not fully understood mechanisms of neuroendocrine system that determine metabolic effects of sleep deprivation (23), which can be seen as neurobehavioral outcomes, such as excessive appetite, constant sensation of hunger and need for additional energy intake, resulting in the onset and progression of obesity (17, 24). Our prospective study was conducted in adequately homogenous groups of respondents who were referred to polysomnography by doctors of different specializations, ENT doctors, endocrinologists, and chosen primary care physician. Obtained values of CRP levels were elevated in respondents from both groups, which is consistent with the results of many studies dealing with obesity (24). It actually confirms the hypothesis that adipose tissue stimulates and increases inflammatory processes in the body. Statistically significant difference was confirmed in early systemic inflammation parameters CRP between obese patients with poor sleep quality and sleep that usually lasted 4 hours or less and obese patients that slept 6 hours or more on average, suggesting the importance and impact of sleep duration and quality on the development of inflammatory processes in the body, as has been shown by other studies which were discussing this association as well. In all our respondents and within the groups as well, increased HbA1c values were reported in relation to sleep duration, suggesting that our obese respondents had long been suffering from metabolic disorder and poor glycoregulation, so type 2 diabetes mellitus diagnosis was warranted, which is consistent to the results of similar studies (17).

Conclusion

This prospective clinical study has confirmed that obese patients have elevated and statistically significant differences in values of CRP levels as a marker of inflammation, and elevated values of HbA1c as an indicator of prolonged poor glycoregulation, without statistically significant differences in relation to sleep duration and quality.

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GOJAZNOST I TRAJANJE SNA – ISTRAŽIVANJE POREMEĆAJA GLIKOREGULACIJE I SISTEMSKE INFLAMACIJE

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Kvalitet i dužina sna bitno utiču na nastanak i progresiju gojaznosti. Prospektivno kliničko istraživanje sprovedeno je na 129 bolesnika upućenih na polisomnografsko ispitivanje, od kojih je 76 bilo gojazno. Na osnovu modifikovanog upitnika o kvalitetu sna ("National Health and Nutrition Examination Survey" – NHANES upitnik) bolesnici su podeljeni u dve gupe sličnih demografskih i morfometrijskih karakteristika prema dužini sna, tako da I grupu čine oni sa lošim kvalitetom sna, koji prosečno spavaju 4 sata i kraće, a II grupu oni sa umereno dobrim i dobrim kvalitetom sna, koji spavaju prosečno 6 sati i duže. Nađeno je to da svi ispitanici imaju povećane vrednosti C-reaktivnog proteina (CRP), a ispitanici I grupe, sa kraćim snom lošeg kvaliteta, imaju statistički značajno veći CRP u odnosu na CRP ispitanika II grupe. Utvrđeno je to da ispitanici obe grupe imaju povećane vrednosti glikolizirajućeg hemoglobina (HbA1c), kao parametra loše glikoregulacije.

Kod gojaznih osoba dužina i kvalitet sna imaju značajnu ulogu u povećanju inflamatornih procesa u organizmu. Gojanost je faktor rizika za poremećaj glikoregulacije.

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Ključne reči: *respiratorna poligrafija, gojaznost, trajanje sna, glikortegulacija, sistemska inflamacija*

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THE IMPACT OF COVID-19 PANDEMIC ON TYPE 1 DIABETES MELLITUS INCIDENCE AND ITS CLINICAL PRESENTATION IN CHILDREN AND ADOLESCENTS: ONE CENTER'S EXPERIENCE

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The outbreak of the COVID-19 pandemic has a drastic impact on health systems worldwide. The aim of this research was to present the incidence and clinical picture of newly diagnosed diabetes in children in our institution during the first year COVID-19 pandemic and to compare obtained data with previous years' data. A total of 41 children were newly diagnosed with T1DM1. There were two non-significant join point periods with an annual percentage increase (2007-2012: APC 8.94%), followed by a decrease (2012-2015: APC -6.88%) and a statistically significant increase (2015-2020: APC 14.20%). During the first COVID year, there was a larger number of newly diagnosed children, but without statistical significance. The percentage of children presenting with DKA in the time of T1DM diagnosis was significantly higher compared to pre COVID-19 period (84.8% vs. 34.6%; $p < 0.001$). Our results suggest a potential diabetogenic effect of the COVID-19 infection. Larger trials with long term follow ups are needed. Drastic increase in DKA at the onset of T1DM during the first COVID year urge need for better prevention measures.

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Key words: *COVID-19, child, DKA*

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Introduction

The outbreak of the Covid-19 pandemic has a drastic impact on health systems around the world. The introduced measures of isolation and social distancing aim to reduce the infection rate and huge influx of patients with Covid-19, hinder the normal functioning of the health care systems and increase the risk of inadequate treatment of patients with "non Covid" health problems (1).

Since the outbreak of the Covid-19 pandemic, there has been an increased influx of patients with newly diagnosed diabetes type 1 (T1DM) in a state of severe acute complication of diabetes, diabetic ketoacidosis (DKA), observed in the everyday practice at the Pediatric Clinic in Niš. Diabetic ketoacidosis is a potentially severe, life-threatening complication of diabetes (2, 3).

The papers published thus far on this topic have provided different results regarding the incidence of type 1 diabetes in children and adolescents during the Covid-19 pandemic (4, 5). However, an increased incidence of diabetic ketoacidosis as a severe acute complication of diabetes has been reported by all cited authors (4-6).

The aim

The aim of this research was to present the incidence and clinical picture of newly diagnosed diabetes in children and adolescents in our institution during the Covid-19 pandemic between April 1st, 2020 and April 1st, 2021 and to compare the obtained data with the data of the period between 2007 and 2020.

Materials and methods

In our research, we collected and analyzed the data off patients younger than 19 years with newly diagnosed T1DM. All newly diagnosed children

with T1DM from the Southeast Serbia were referred to the Pediatric Clinic in Niš. The diagnosis of T1DM and DKA was made according to the International Society for Pediatric and Adolescent Diabetes (ISPAD) guidelines. Data regarding subject's gender, date of birth, date of diagnosis, duration of symptoms, DKA at clinical presentation (yes/no), venous pH, glucose, and glycated hemoglobin (HbA1c) levels at diagnosis were collected retrospectively from the medical records. The methodology of our data collection was explained in detail, in an already published paper (7). Informed consents were obtained from the parents or guardians of all participants for admission to hospitals and the procedures performed during hospitalization. The study was approved by the Hospital Ethics Committee and data were retrospectively collected in accordance with the 1964 Declaration of Helsinki.

Statistical analysis

Results are expressed as percentages or means ± standard deviation (SD). The differences in the means of variables between groups were tested using the independent T test in case of normally distributed continuous variables and for non-normally distributed continuous variables, Mann-Whitney U test. The chi squared test was used to compare occurrence of DKA and DKA severity by age categories. A statistical analysis was performed in EPI INFO v7.2.2.6 (CDC, Atlanta, USA). The trend of new T1DM cases was estimated by the join point regression analysis. Join point analyses were performed using an available software: "Join point Regression Program", Version 4.8.0.1 (Surveillance Research program, National cancer Institute, USA) (8). A p-value of $p < 0.05$ was selected as statistically significant.

Results

There were 349 children newly diagnosed with T1DM1 in the period from 2007 to 2020 (Table 1). During this period there were two non-significant join point periods with an annual percentage increase (2007-2012: APC 8.94%), followed by a decrease (2012-2015: APC -6.88%) and a statistically significant increase (2015-2020: APC 14.20%) (Figure 1).

During the first Covid-19 year there were a larger number of newly diagnosed children, but without statistical significance. Most newly diagnosed cases were during the fall and winter months (Figure 2).

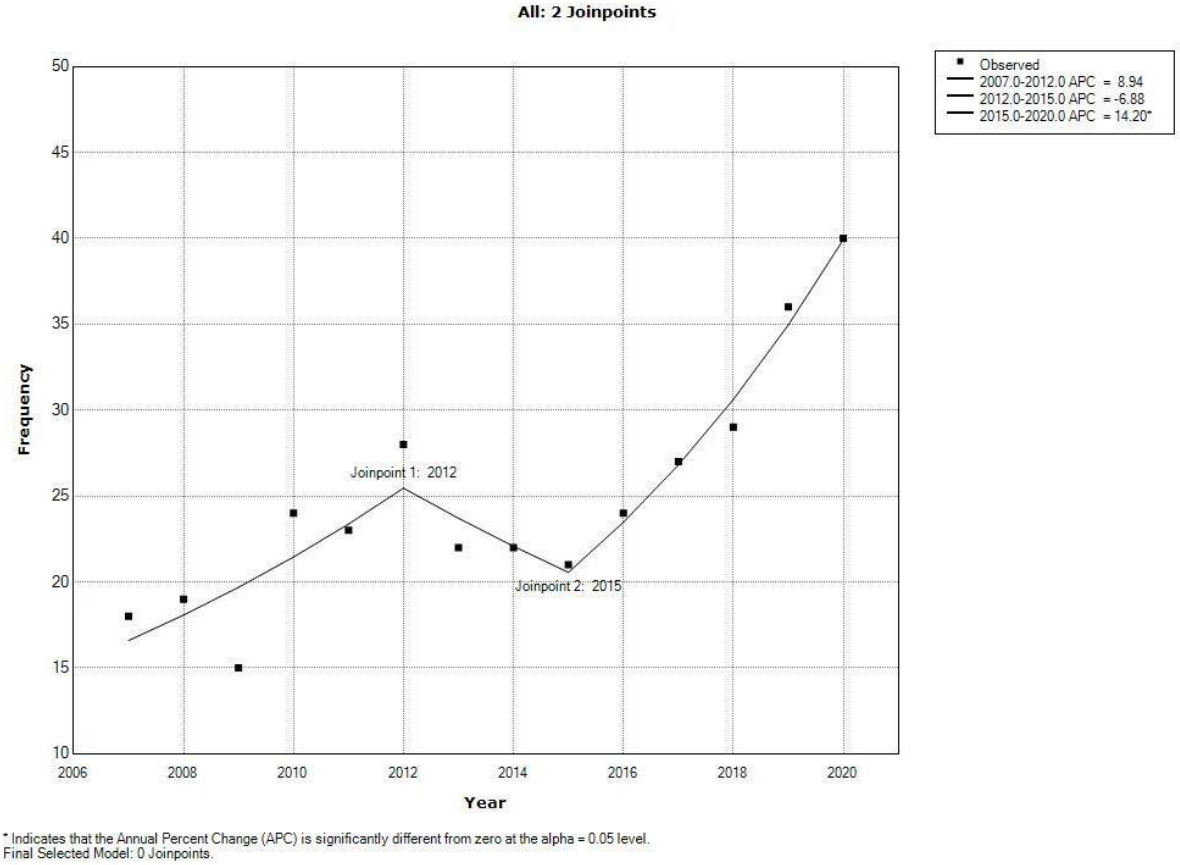

Figure 1. Join-point regression analysis of new cases in the period 2007-2020

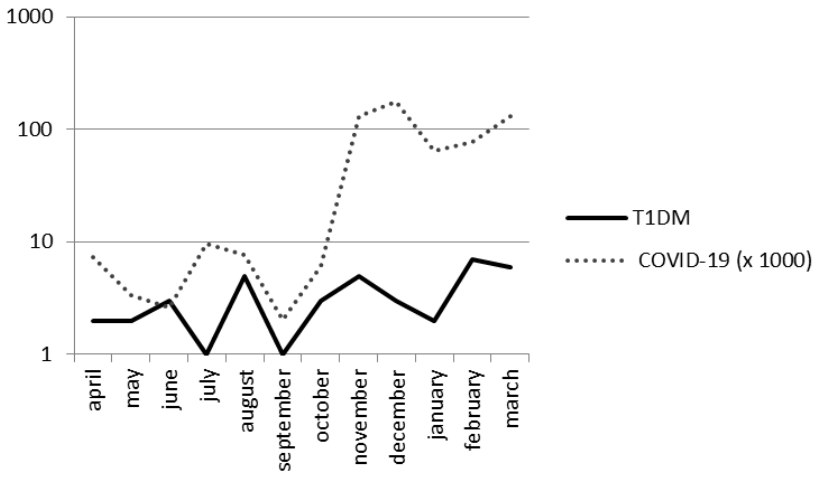

Figure 2. Number of T1DM and COVID-19 patients 2020/21

Table 1. Total number of newly T1DM diagnosed patients

Year	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	Covid 19	TOTAL
Count	18	19	15	24	23	28	22	22	21	24	27	29	36	41	349
%	5.0	5.3	4.2	6.7	6.4	7.8	6.1	6.1	5.9	6.7	7.5	8.1	10.3	11.7	100.0

Table 2. Demographic and clinical characteristics

	Before 2020		Covid year		p[1]
Gender					
Boys	173	54.6	23	56.1	0.986
Girls	144	45.4	18	43.9	
Age					
Mean ± SD	9.61 ± 4.21		9.20 ± 4.64		0.785[2]
Median (IQ)	9.63 (6.72)		10.39 (8.66)		
Min-Max	0,34-18,40		0.52-15.79		
Age sroups					
< 6	71	23.6	12	29,3	0.254
6-11	112	37.2	10	24.4	
> 12	118	39.2	19	46.3	
Clinical status					
Compensate state	187	63.4	5	15.2	< 0.01
DKA	99	34.6	30	84.8	
DKA severity					
Mild DKA	128	44.8	9	27.3	
Moderate DKA	25	8.7	7	21.2	
Severe DKA	34	11.9	12	36.4	< 0.001
Blood glucose	22.04 ± 8.93		24.28 ± 8.92		0.135[2]
Hba1c	12.25 ± 8.02		12.06 ± 2.12		0.252[2]
Duration of symptoms	19.37 ± 18.87		19.32 ± 15.01		0.732[2]

[1] Chi-square test, [2] Mann-Whitney test

The baseline demographics and clinical charac-teristics at the time of diagnosis are presented in Table 1. The mean age at presentation, gender distribution, and duration of self-reported symptoms were similar between the two periods. Blood glucose and HbA1 levels were comparable.

During the Covid-19 pandemic, the number of children presenting with DKA in the time of T1DM diagnosis was significantly higher compared to the pre Covid-19 period (84.8% vs. 34.6%; $p < 0.001$), representing an absolute increase of 50.5% (Table 1).

The prevalence of severe DKA at DM1 onset was also significantly higher during the pandemic period, compared to the control (36.4% vs. 11.9%; $p < 0.001$), representing an absolute increase of 24.5% (Table 2).

Discussion

In the Republic of Serbia, there is a significant increase in the incidence of diabetes in children and adolescents aged 0 to 19 in the period between 2007 (8.09/100,000) and 2016 (16.31/100,000) (7, 9, 10). In our research, there is a significant increase of newly diagnosed patients between 2015 and 2020 year. This increase is also evident during the first Covid-19 year, but with no statistical difference. Such a rapid increase in the incidence cannot be attributed to changes in the genetic structure of the population. The main reasons for the increase in the incidence can be attributed to the change in exposure to environmental factors (11, 12). There are numerous risk factors for the development of T1DM, and viral infections certainly play one of the major roles in its pathogenesis. Since the COVID-19 pandemic was announced by the World Health Organization on March 11th, 2020, a large number of patients have been diagnosed with this infection in our region. On the other hand, due to the closure of collective spaces, there are fewer cases of other virus infections. Stress exposure is increased, children are isolated and physical activity is reduced, which are additional risk factors (13-15). During the Covid-19 pandemic, most newly diagnosed cases were during the fall and winter months (14). Figure 2 displays the parallel number of Covid-19 patients and newly diagnosed T1DM children. Our results suggest a potential diabetogenic effect of the COVID-19 infection. The expression of ACE2 on pancreatic β cells may potentially lead to β cell damage by the virus and result in insulin deficiency (16, 17). The sparse literature data however cannot exclude the delay between immunologic factors activation due to Covid-19 infections and the onset of T1DM (18).

Recent papers investigating the incidence of T1DM during the Covid-19 pandemic have given different results. German study demonstrated an increasing incidence of T1DM in this country since 2011 (5). However, this study failed to demonstrate any pandemic related short-term changes in T1DM. Unsworth et al. found an increase in new T1DM cases between March, 2020 and June, 2020 comparing with T1DM cases in the previous five years (4).

Previously published studies have shown the prevalence of DKA in about 35% of newly diagnosed patients with T1DM in our hospital (10, 19). A delay in diagnosis of children with new onset DM1 during the COVID-19 pandemic could be due to possible fears regarding the viral transmission during healthcare appointments. Additionally, the under-recognition of secondary symptoms could be due to reduced face-to-face interactions with medical providers. Clinicians may have been more prone to assess infectious and respiratory symptoms for a possible Covid-19 diagnosis, rather than considering the new onset of DM1. With laboratory resources being targeted towards Covid-19 diagnosis, clinicians may have been reluctant to pursue lab testing for blood glucose or urinalysis.

Conclusion

Our results suggest a potential diabetogenic effect of the Covid-19 infection. The sparse literature data however cannot exclude the delay between immunologic factors activation due to Covid-19 infections and the onset of T1DM. Larger trials with long term follow ups are needed. Drastic increase in DKA at the onset of T1DM during Covid-19 year urge the need for better prevention trough patients and medical stuff re-education.

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UTICAJ PANDEMIJE VIRUSA COVID-19 NA UČESTALOST I KLINIČKU PREZENTACIJU DIJABETESA TIPA 1 KOD DECE I ADOLESCENATA: ISKUSTVO JEDNOG CENTRA

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Pandemija virusa COVID-19 drastično je promenila funkcionisanje zdravstvenog sistema. Cilj našeg istraživanja je da prikaže incidenciju i kliničku prezentaciju dece i adolescenata sa novootkrivenim tipom 1 šećerne bolesti (DM tip 1) tokom prve godine pandemije virusa COVID-19 i da dobijene podatke uporedi sa podacima iz prethodnih godina. Tokom ispitivanog perioda, dijagnostikovan je 41 bolesnik. Joint point analizom utvrđen je godišnji porast u periodu od 2007. do 2012. godine (APC 8,94%), praćen smanjenjem broja u periodu od 2012. od 2015. godine (APC 6,88%) i statistički značajnim uvećanjem od 2015. do 2020. godine (APC 14,20%). Broj novoobolele dece tokom prve godine COVID-19 pandemije bio je veći, ali bez statističke značajnosti. Procenat dece sa dijabetesnom ketoacidozom (DKA) u vreme postavljanja dijagnoze DM tip 1, u ispitivanom periodu, bio je značajno veći nego u prethodnim godinama (84,8% prema 34,6%; $p < 0,001$). Naši rezultati ukazuju na potencijalno dijabetogeni efekat COVID-19 virusa. Potrebne su opsežnije i dugotrajnije studije da bi se utvrdile prave dimenzije ovog uticaja. Drastičan porast DKA u vreme postavljanja dijagnoze DM tip 1, tokom pandemije COVID-19 virusa zahteva poboljšanje preventivnih mera.

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Ključne reči: *COVID-19, deca, DKA*

Review article

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SPECIFICITY OF AIRWAY MANAGEMENT IN THORACIC ANESTHESIA

Aleksandar Nikolić[1], Vladan Cvetanović[1], Milena Stojanović[1], Tijana Maričić[1], Marija Stošić[1], Milica Marković[1]

Before the advancement of anesthesia techniques in the mid-1930s, chest operations were short and difficult. Anesthesia during thoracic surgery is in itself very demanding and complicated to work with and represents a real challenge for the anesthesiologist. In order to perform the operation smoothly in patients whose respiratory reserve has already been reduced, it is necessary to exclude the lung that is being operated on and to isolate the lung that is ventilated during the surgical intervention. Double lumen tubes and endobronchial blockers are used to secure the airway and to achieve collapse and unilateral ventilation. Fiberoptic bronchoscopy is the gold standard in the world of modern thoracic anesthesia for checking the position of a double lumen tube and endobronchial blocker.

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Key words: *airway, thoracic anesthesia, double lumen tubes, endobronchial blockers*

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Introduction

The first clinical surgery on the chest was performed by Dr. Huber Block in 1882 when he performed a lung resection on his cousin with bilateral pulmonary tuberculosis, who unfortunately died soon after the operation, which caused a lot of legal problems (1). The era of modern and safe surgery followed, and the advancement of general and regional anesthesia techniques, the introduction of aseptic conditions during surgery, the introduction of new instruments and surgical techniques, which contributed to greater survival, but this was not the case with patients who were subjected to thoracic surgery. Before the advancement of anesthesia techniques in the mid-1930s, chest surgeries were short and difficult. The development of anesthesia techniques and procedures has led to thoracic surgery experiencing expansion (2). In 1928, Guedel and Waters patented an endotracheal tube with cuff, which was used for positive pressure ventilation (Figure 1) (3).

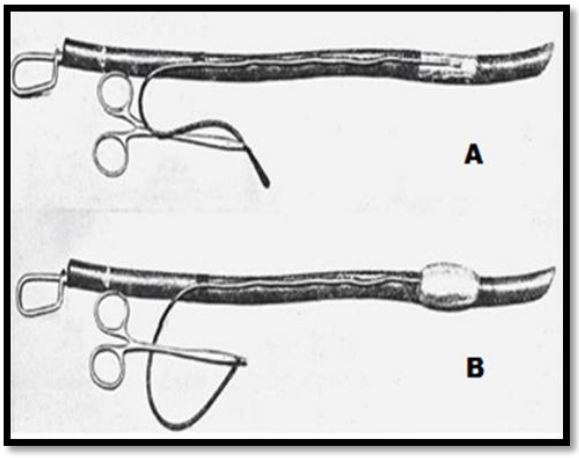

Figure 1. Guedel and Waters - Endotracheal tube with cuff, patented in 1928

A. Tracheal balloon released.
B. Inflated tracheal balloon.
Tube length 35.56cm, made of rubber.

Three years later, in 1931, Joseph W. Gale and Ralph M. Waters patented an endotracheal tube with cuff that provided unilateral lung ventilation, where the tube advanced into one bronchus and the inflated cuff closed the other bronchus isolating it, essentially serving as a bronchial blocker (4). The first successful pneumonectomy for lung cancer was performed in 1933 by Dr. Evarts Graham (5).

Isolation and one-sided pulmonary ventilation

Thoracic surgery in itself is very demanding and complicated to work with, and for the smooth performance of the operation, it is necessary to exclude the lung that is being operated on and to isolate the lung that is ventilated during the surgical intervention. This method of isolating the ventilated lung and collapsing the lung being operated on is called unilateral lung ventilation and can be reported with a double lumen endobronchial tube (left, right) or with a bronchial blocker when a double lumen tube is contraindicated (2).

Double-lumen tubes (DLT)

The design of the double lumen tube changed over the years, the first double lumen cuff tube was patented by Carlens in 1949, and it was a left double lumen tube with endotracheal and endobronchial cuff, which also had a hook that positioned itself on the carina (6). Since the hook that was placed on the carina caused damage to the carina and its rupture, 10 years later, in 1959, Brian Smith modified the left double lumen Carlens tube and removed the hook (7). A year later, in 1960, White modified the Carlens tubes and thus patented the right double lumen tube without a hook (8). In 1962, Robetshaw patented the right and left double lumen tubes with large ventilation lumens, without a hook (9).

Double lumen tubes can be left or right, depending on the bronchus they are designed to intubate (Figure 2). The difference is that the right-sided double lumen tubes have a slit called the so-called Murphy's eye, which should be placed so as to ventilate the upper right lung lobe. The installation of the right double lumen tube is technically more demanding, so that the left double lumen tubes are used more often except when there is no surgical contraindication for their installation (2).

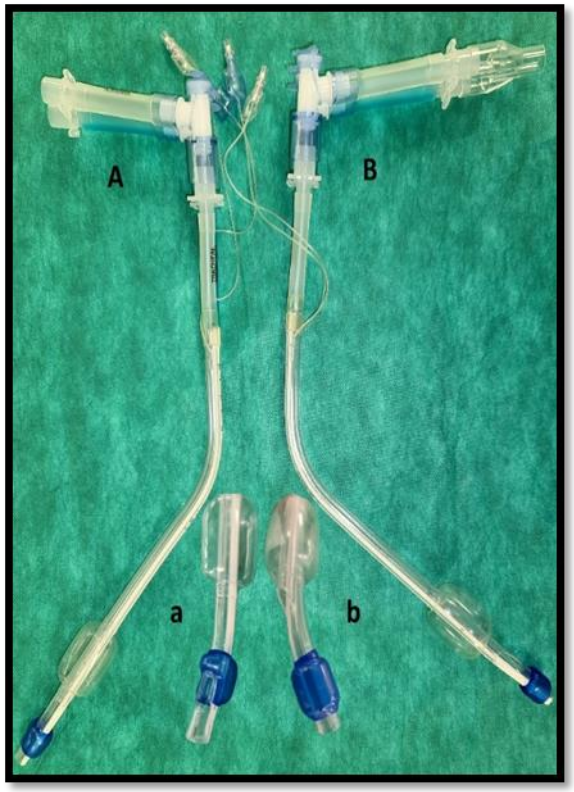

Figure 2. Double lumen tube (Rusch)

A - Rusch right double lumen tube
a - endotracheal and endobronchial (with Murphy's eye) cuff of the right double lumen tube;
B - Rusch left double lumen tube
b - endobrotracheal and endobronchial cuff of the left double lumen tube

Many anesthesiologists prefer the left double lumen tube for both right and left lung surgery to reduce the possibility of bronchial obstruction of the upper lobe of the right lung (10). The right bicuspid tube must be used if there is intrinsic (tumor, stenosis) or extrinsic (tumor, aortic aneurysm) obstruction of the left bronchus. The right double lumen tube is also used for left bronchial resections and during left lung transplantation (11).

Selection of DLT size

When choosing the size, you should choose the largest double lumen tube with a bronchial lumen that corresponds to the desired bronchus. Many problems can arise when choosing the wrong DLT size. For example, a selected smaller DLT can often be placed too deep in the bronchus, making it more likely to clog the bronchus of the upper lobe. Then, during unilateral lung ventilation, there is greater resistance to air flow through the small lumen of the tube, and this is manifested as a high level of auto peep pressure in patients with COPD (12).

There are various measurements for choosing the ideal DLT size, and some of them are:

1. Computed tomography of the chest, where the bronchus can be accurately measured, but this requires additional involvement of a radiologist (13, 14).

2. X-ray of the lungs, measuring the width of the trachea that can be used to determine the size of the left bronchus (15) (Figure 3). The width of the left bronchus is directly proportional to the size of the trachea (16). Because the trachea is visible and can be easily measured on a chest radiograph, the width of the trachea can be used to predict the size of the left bronchus. This allows the selection of the appropriate size of the left double lumen tube (17) (Table 1).

The use of DLT (39fr, 41fr) was initiated in most men, and the use of DLT (37, 39fr) was indicated for most women (18).

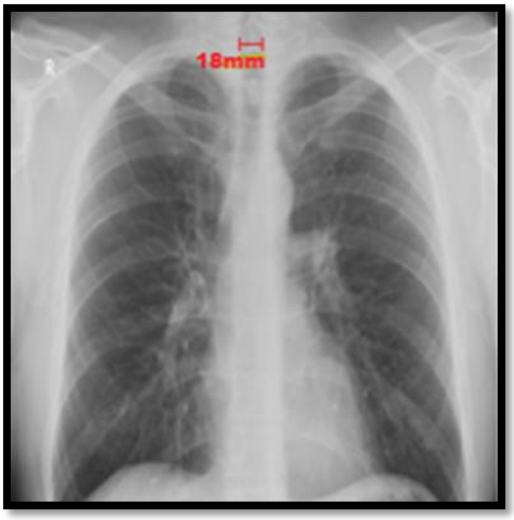

Figure 3. X-ray of the lungs - measurement of tracheal diameter

Table 1. Guidelines for selecting the left DLT

MEASURED WIDTH OF THE TRACHEA (mm)	PREDICTED LEFT BRONCH WIDTH (mm)	RECOMMENDED DLT SIZE	LEFT DLT OUTER DIAMETER (mm)	DLT LUMEN INNER DIAMETER (mm)
≥ 18	≥ 12.2	41 Fr	14 – 15	10.6
≥ 16	≥ 10.9	39 Fr	13 – 14	10.1
≥ 15	≥ 10.2	37 Fr	13 – 14	10.1
≥ 14	≥ 9.5	35 Fr	12 – 13	9.5
≥ 12.5	≥ 8.5	32 Fr	10 – 11	8.3
≥ 11	≥ 7.5	28 Fr	9.4	7.4

* Tracheal width measured on an X-ray of the lungs

* Predicted left bronchial width tracheal width (mm) x 0.68

Placing DLT

Endotracheal intubation and placement of DLT before adequate preoxygenation and induction under general anesthesia and after achieving adequate relaxation can be reported using two methods:

1. *By blind technique*, so that the DLT, after passing the distal part of the tube (endobronchial cuff) through the vocal cords, rotates the whole DLT 90 degrees to the left if it is the left DLT, or rotates to the right if it is the right DLT. The recommended end point for placing DLT through the trachea is when we encounter resistance. In both men and

women, the depth of placement of the DLT is directly proportional to the height. For a man or woman 170 cm tall, the DLT should be placed in the airways up to 29 cm, and for each ± 10cm height change the DLT should be placed deeper or pulled by ± 1cm (19, 20).

2. *Fiber optic technique*, after passing the DLT through the vocal cords, the pediatric fiber-optic bronchoscope is placed through the DLT, then advances further through the trachea and enters the main left bronchus using a bronchoscope, and then through the bronchoscope that serves as a guide, the DLT is placed in the left bronchus if we place the left DLT (21, 22).

After placement of the DLT (Figure 4), the tracheal cuff is inflated first (5-10 cc) and then the bronchial (3 cc max). The position of the tube is checked by physical examination of the chest, including auscultation and observation of chest lift (23).

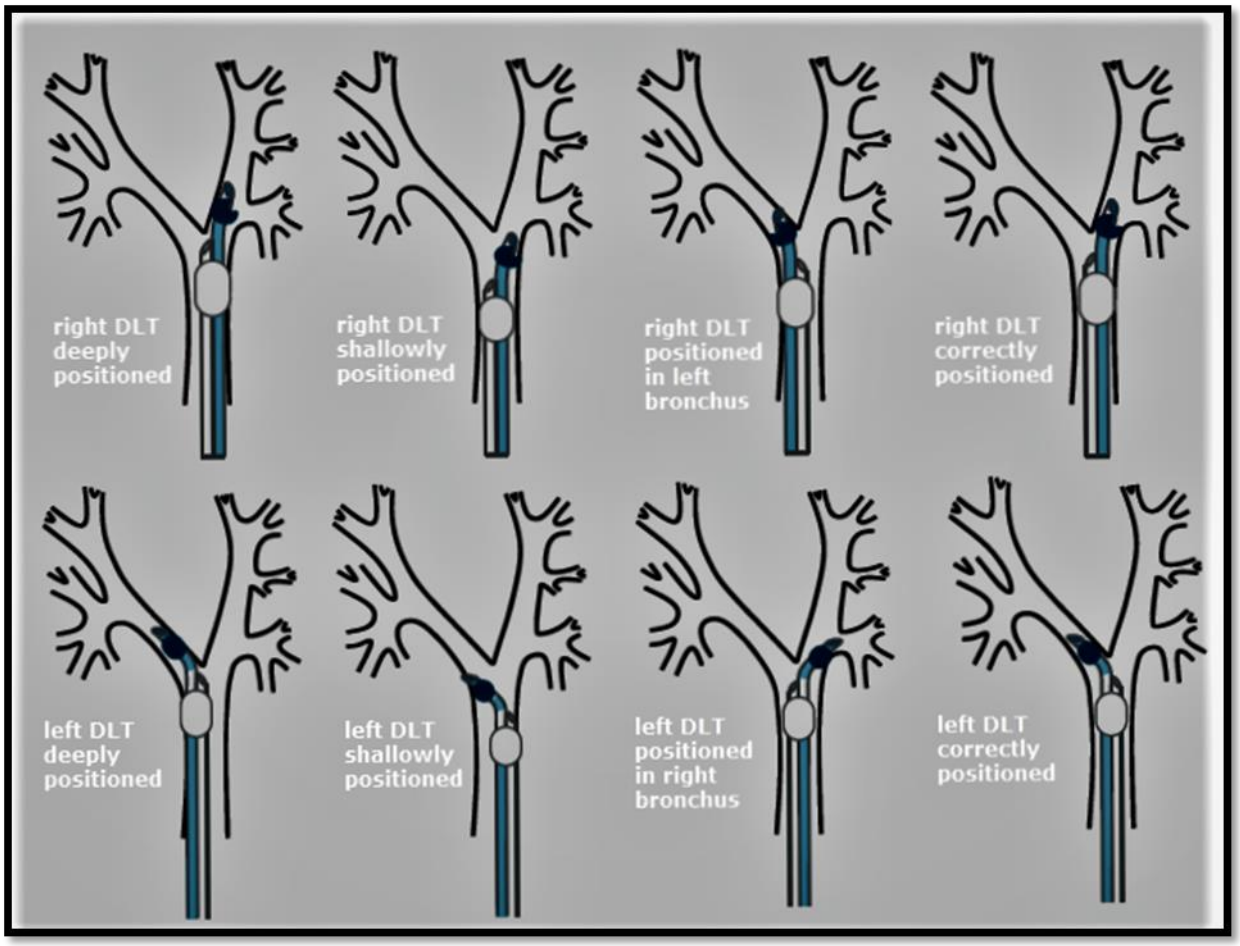

Figure 4. Position of the left and right DLT in relation to the carina

In the world of modern thoracic anesthesia, bronchoscopy is the gold standard for checking the position of the DLT, both in the supine and lateral position, and at the same time the safest method of checking the position of the DLT (24) (Figure 5, 6 and 7).

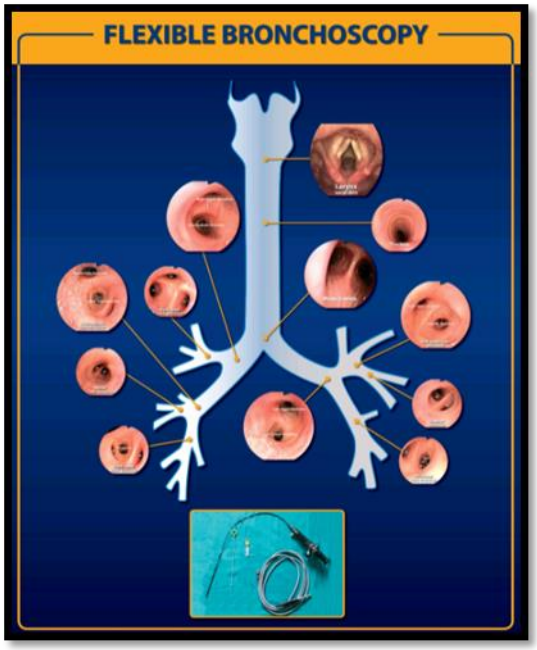

Figure 5. Visualization of anatomical structures using flexible bronchoscopy

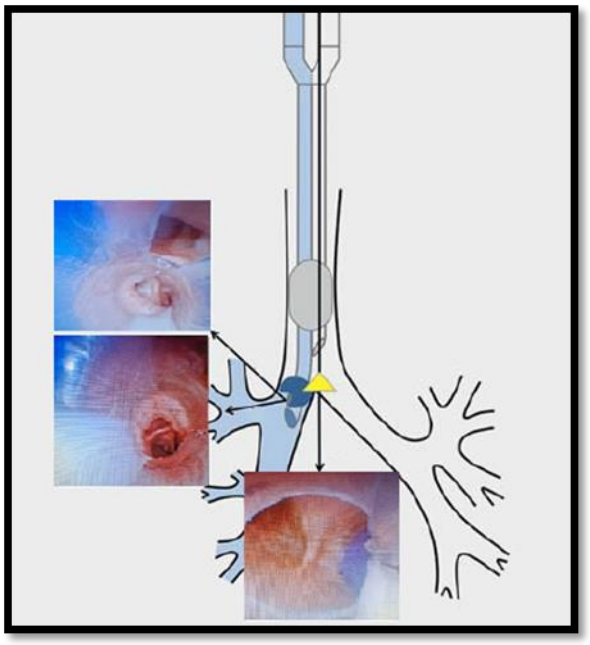

Figure 6. Right-sided intubation with right DLT - fiberoptic presentation of anatomical structures

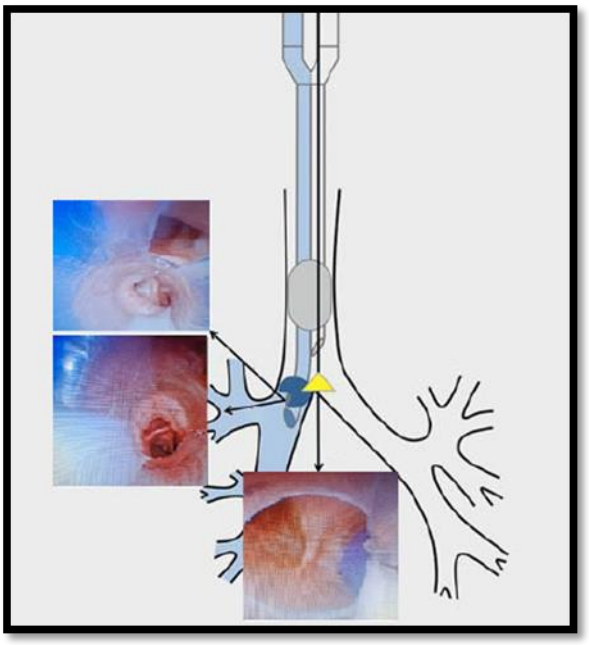

Figure 7. Left-sided intubation with left DLT - fiberoptic presentation of anatomical structures

Complications due to intubation with DLT

In general, DLTs are safe and easy to use, but complications can certainly occur (Table 2). The most common problems are related to the position of DLT. Poorly placed DLT can lead to airway damage, hypoxemia, or compromise surgery if adequate lung collapse does not occur (23).

Table 2. Complications due to intubation of DLT

INTUBATION	TRAUMA	PROBLEMS RELATED TO DLT POSITION
✓ carinal hook cannot pass through epiglottis (Carlens DLT) ✓ inability to place DLT in the bronchi due to excessive airway obstruction (intrinsic, extinct)	✓ tracheobronchial rupture ✓ rupture of thoracic aneurysm ✓ tooth trauma ✓ airway injury ✓ laryngeal mucosal injury ✓ vocal cord injury ✓ arytenoid dislocation	✓change of DLT position when positioning the patient in the lateral position ✓Flexion or extension of the head leads to a change in DLT position ✓shallowly placed DLT, bronchial cuff in front of the carina ✓deep placed DLT ✓change of DLT position during surgical manipulation

To avoid any complication related to DLT placement, the following recommendations should be followed (23):

1. Choose the largest plastic DLT that will fit in the airways,
2. Remove the intruder as soon as the DLT passes behind the vocal cords,
3. Caution in patients with pathologically altered tracheobronchial wall, leukemia, on corticosteroid therapy,
4. Slowly inflate tracheal and bronchial cuff, never over-inflate cuff,
5. If it is necessary to inflate the cuff further, check the position of the DLT and perform bronchoscopy and auscultation,
6. Do not use nitric oxide, and if you use it, inflate the cuff with saline,
7. Be sure to measure the pressure in the cuff (Figure 8),
8. When moving patients, be sure to drain both cuffs, dispense bronchial cuff when isolation or selective ventilation is no longer required,
9. After the operation, be sure to do a bronchoscopy to determine if the injury occurred, its location and extent of the injury.

Figure 8. Checking the pressure in cuffs (endobronchial, endotracheal) using a cuff pressure control device (Mallinckrodt)

Endobronchial blockers

Historically, bronchial blockade has been achieved with gauze tampons, urinary catheters, and kits used for embolectomy (25, 26).

Nowadays, endobronchial blockers (Figure 9) are used exclusively to block the bronchi, and there are several types:
EZ blocker, ARNDT blocker, COHEN blocker, FUJI blocker, COOPDEH blocker.

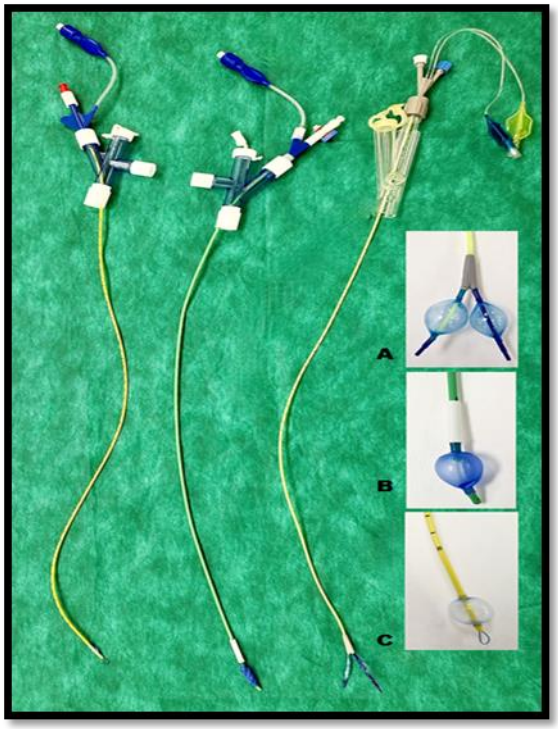

Figure 9. Endobronchial blockers

A. EC blocker,
B. Cohen blocker,
C. Arnd blocker

In thoracic anesthesia, double lumen tubes are mainly used for lung isolation and unilateral ventilation. Some endobronchial blockers should be used in certain groups of patients, such as patients with limited mouth opening, patients with previous laryngectomy, patients with permanent tracheostomy when the stoma is too small to place a double lumen tube (27, 28). In children and pediatric patients, bronchial blockers should be the first choice, because even the smallest DLT may be too large (29).

After endotracheal intubation, and placement of the one-lumen tube in the trachea, an endobronchial blocker can be placed in the appropriate bronchus to achieve lung collapse (23). The procedure of placing endobronchial blockers is performed under the control of bronchoscopy, except for urgent conditions when lung collapse is necessary, EZ blocker can be placed blindly (30).

Although they are more practical and easier to use than DLT, they also have their drawbacks: they can be easily moved when changing the patient's position or during surgical manipulation, the lung cannot collapse and expand again during surgery, and CPAP ventilation cannot be applied to the collapsed lung if the patient becomes hypoxemic during unilateral ventilation (23).

Conclusion

The use of a double lumen endobronchial tube is the standard of isolation and unilateral lung ventilation during thoracic surgery. The use of endobronchial blockers may be an alternative in special cases when the use of a double lumen tube is contraindicated. In order to avoid complications that occur during the airway management and after the placement of the double lumen tube, it is necessary to follow the recommendations and choose the appropriate size of the double lumen tube. Fiberoptic bronchoscopy is the gold standard of modern thoracic anesthesia and it is necessary for: insight into the anatomy of the bronchial tree, positioning of the endobronchial tube, lung isolation and unilateral ventilation as well as the toilet of the bronchial tree.

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SPECIFIČNOST OBEZBEĐIVANJA DISAJNOG PUTA U GRUDNOJ ANESTEZIJI

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Sve dok tehnike anestezije nisu napredovale sredinom tridesetih godina dvadesetog veka operacije na grudnom košu bile su kratke i teške. Anestezija tokom torakohirurških intervencija, sama po sebi jako je zahtevna i komplikovana za izvođenje i predstavlja pravi izazov za anesteziologa. Za nesmetano izvođenje operacije kod bolesnika kod kojih je respiratorna rezerva već snižena, potrebno je isključiti plućno krilo koje se operiše i izolovati plućno krilo koje se ventilira tokom hirurške intervnecije. Za obezbeđivanje disajnog puta i za postizanje kolapsa i jednostrane ventilacije, koriste se dvolumenski tubusi i endobronhijalni blokeri. Fiberoptička bronhoskopija je u svetu moderne grudne anestezije zlatni standard za proveru pozicije dvolumenskog tubusa i endobronhijalnog blokera.

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Ključne reči: *disajni put, grudna anestezija, dvolumenski tubusi, endobronhijalni blokeri*

Review article

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MORPHOLOGY AND FUNCTIONAL ABILITIES OF THE TEMPOROMANDIBULAR JOINT

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The mandible and the cranium are integrated by a unique joint juncture which is a special structure in the human body for most of its characteristics. From the other joints, it differs in morphology, the potential of the variability of all of the structures related to the joint, special functions, vast freedom of movement, and the close relationship between the joint and vital organs. Articular surfaces are covered by a special type of fibrous tissue which consists of four different zones: the articular zone, the zone of the fibrous cartilage, and the zone of the calcified cartilage. The fibrous capsule of the joint is covered by the synovial membrane. The inside of the joint cavity is filled by the synovial fluid that serves as the metabolic medium and ensures lubrication. The articular disc inserted between the articular surfaces has a complex structure and very important roles ensuring the vast mobility of the joint and the physiological pressure transfer. It divides the joint into two different portions that serve as a uniform functional unit. Morphologically it has a biconcave shape and can be divided into three portions. The intermedial zone is the thinnest, while the posterior, bilaminar, zone has the most complex structure containing the well vascularised retrodiscal tissue between its laminae. Disc constantly changes its shape according to the new configuration inside of the joint which is ensured by a specific alignment of the collagen fibers but also the presence of elastic and oxytalan fibers.

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Key words: *temporomandibular joint, morphology, articular disk, histology*

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Introduction

Starting with the histology, going over the anatomical landmarks and the physiology of the oral cavity, we will explain the function of the temporomandibular joint which participates in our ability to chew, swallow, and speak (1).

The mandible and the cranium are integrated by a unique joint juncture which is a special structure in the human body for most of its characteristics. The fact that the two temporomandibular joints (TMJ) are connected by the same bone complicates the function of the whole masticatory apparatus since the action in one joint results in a reaction in the other (2).

From the other joints, it differs in morphology, the potential of the variability of all of the structures related to the joint, special functions, vast freedom of movement, and the close relationship between the joint and vital organs. The binding tissue of the joint is not the hyaline cartilage but instead a dense fibrous tissue while the secondary condylar cartilage is persisting on the head of the condyle until adolescence (3).

Due to the position of the articular disc, the TMJ is divided into two parts which in the sense of functionality operate as a single functional unit:

- The lower joint space is defined by the condylar processes of the mandible and the articular disc. The disc is firmly bonded to the condyle by the lateral and medial discal ligament which only allows for the rotational movement of the disc over the surface of the condyle;
- The upper joint space is defined by the articular disc as the lower border and the mandibular fossa as the upper. In this space, the only possible movement is gliding since the disc is not that firmly bonded to the mandibular fossa (2, 4).

Histology of the articular surfaces of the TMJ

The tissue that covers the bone surfaces of the joint consists of four different zones:

- the articular zone,
- the zone of the fibrous cartilage, and
- the zone of the calcified cartilage.

The first zone that opposes the articular space is named the articular zone and it represents the outer functional surface. It is built of dense fibrous connective tissue. Most of the collagen fibers in this zone are tightly packed in binds and aligned almost parallel to the surface. Due to this specific structure, the articular surface of the TMJ has many advantages over the hyaline cartilage, aging, and degenerative disorders progress much more slowly and the potential for regeneration is much larger. All of these structures serve the purpose in a joint that is highly mobile and constantly participating in some functionality, whether it comes to chewing, speaking or some parafunction such as grinding of the teeth during the night (5).

The second zone in order is the zone of proliferation and is built of non-differentiated mesenchymal tissue which produces cartilage cells based on the functional requirements or the stress on the joint.

The third zone is the zone of fibrous cartilage. In this zone, the binds of collagen are mostly crossed while some are radially aligned which creates a special kind of tri-dimensional net that counteracts compressive and lateral forces (6-8).

The deep zone is the zone of calcified cartilage. This zone contains chondrocytes and an extracellular matrix. Chondrocytes produce collagen, proteoglycans, glycoproteins, and enzymes. Proteoglycans are complex molecules that consist of the protein core and glycosaminoglycan chains. Proteoglycans are bonded to the hyaluronic acid chains which create matrix protein aggregates. These aggregates are intercrossed throughout the whole collagen net and due to their hydrophilic traits, the matrix is expanding while the pressure inside of the fibers protects from the expansion pressure of the proteoglycan aggregates. Hydrophilic traits are of high importance when it comes to nutrient delivery and maintaining the healthy articular cartilage (2, 9).

Synovial membrane

The synovial membrane covers the internal surface of the fibrous capsule and doesn't cover the articular surfaces since it would quickly tear. It is already presented that the TMJ consists of two articular spaces, the upper and the lower. Internal surfaces of these spaces are covered by the specialized endothelial cells which lie over the vascular layer. The cells that build the superficial layer are macrophages and fibroblasts. The upper and the lower articular space are filled by the synovial fluid which is produced by the synovial membrane and the synovial fold on the anterior end of the retrodiscal tissue. The lower articular space is filled with roughly 1 ml of the synovial fluid, while in the upper there is slightly more fluid since it is more voluminous (10).

The main functions of the synovial fluid are as follows:

- The metabolic medium which is needed since the articular surfaces do not have blood vessels,
- Lubrication during the function of the joint (11).

The synovial membrane establishes lubrication in two possible mechanisms. Boundary lubrication is the primary mechanism and relieves the friction inside of a moving joint as the synovial fluid moves throughout the joint cavity covering the articular surfaces (12). Weeping lubrication ensures the exchange of the metabolites. The joint in function creates tensions between the articular surfaces which press on the synovial membrane to excrete a small amount of the fluid inside of the joint cavity ensuring the exchange of metabolites. Important constituents of the synovial fluid that provide the lubrication function are proteoglycans, together with hyaluron which binds to fibronectin and helps keep the surfaces smooth. Surface-active phospholipids together with the glycoprotein lubricin provide friction relief in the joint (13). The synovial membrane also contains pro-inflammatory cytokines such as interleukins and tumor necrosis factor which elevate in concentration in TMJ disorders that are present in all age groups with the prevalence in females (14). The hydrostatic pressure of the synovial fluid is lower than the atmospheric pressure but raises in levels in patients with bruxism since the articular surfaces are under higher pressure during these para functions.

Morphology of the TMJ articular disc

TMJ articular disc transfers the pressure and enables complex mobility of the joint. The biconcave shape gives it the ability to adapt to the morphology of the articular surface on the condyle on one side and the mandibular fossa and the articular tubercle on the other. While in the function the shape of the disc changes and in rest it turns back to normal unless the joint was impacted by non-physiological forces, such as bruxism and trauma (3, 15). Observed in the sagittal plane the articular disc can be divided into three sections based on its thickness. The thinnest is the central section and it is named the intermedial zone. The anterior section is slightly thicker while the posterior zone is the thickest. Based on the transverse cross-section it can be concluded that the intermedial zone is the thinnest and the medial zone is the thickest (3). If the joint is in its normal anatomical configuration the condyle is set up against the intermedial zone.

The posterior portion of the disc can be divided into two laminae:

1) Superior retrodiscal lamina is built out of the fibrous and elastic fibers which are connected with the tympanic plate;

2) Inferior retrodiscal lamina which is built out of collagen with the downwards junction direction to ensure binding to the condyle.

This region is named the bilaminar zone. The retrodiscal tissue between the two laminae contains a large number of nerves and blood vessels. A significant blood flow can be seen in the venous plexus of the disc as the veins enlarge multiple times when the condyle moves to the front when the jaw is open.

The anterior part of the disc also contains two laminae which are connected to the capsular

ligament, the superior being connected to the anterior end of the articular tubercle on the temporal bone and the inferior lamina that is connected to the front side of the condylar neck. Both of these laminae consist of collagen fibers. In-between the junctures to the capsular ligament the disc is connected to the lateral pterygoid muscle. The articular disc is not only connected to the capsule in the front and back rather all around the borders dividing that way the joint cavity into two. The synovial membrane covers the surface of these parts (2, 3).

Cells and the extracellular matrix of the articular disc

TMJ disc is rich in cells and vascularization before birth. The vascularization quickly becomes scarce and the only source of nutrients become the retrodiscal tissue and the synovial fluid. The articular disc embodies three types of cells, mostly fibroblasts, but also fibrochondrocytes and chondrocytes. The more rounded the body of the cell is, the more cytoplasm it contains. Fibroblasts contain a certain amount of endoplasmatic reticulum, mitochondria, Golgi apparatus and vesicles. Extracellular collagen fibers surround cellular membranes. Extensions of cellular membranes on the cells inside of the disc that are 100 μm and longer have been observed. The role of these extensions is to supply nutrients from the peripheral blood vessels and transport them to the central portion which is avascular (13, 16). This functionality is ensured by the small transmembrane protein molecules classified as connexins which provide the transport route for the small molecules to go from one end of the cell to the other.

The extracellular matrix consists mostly of water (roughly 80%), collagen and oxytalan fibers, glycosaminoglycans and proteoglycans. Collagen fibers serve the purpose of keeping a certain shape of the disc. In the intermedial zone, collagen fibers are stacked in the anteroposterior direction while in the anterior and the posterior zones, which are thicker, fibers are stacked in the superoinferior and transversal directions. Peripheral portions of the disc contain collagen fibers that are arranged circumferentially (17).

While the joint performs its movements, the articular disc constantly adapts to the shape of the mandibular condyle and the fossa. This adaptability stems from the position of the collagen fibers as well as their wavy configuration (18). Elastic fibers are present inside of the disc but their amount lowers with aging except in the superior retrodiscal lamina. Oxytalan fibers are binds of microfibrils and are specific for the intra-articular discs in humans. Primary glycosaminoglycans are dermatan sulfate and chondroitin sulfate as well as a certain amount of hyaluronic acid and heparan sulfate (19). Proteoglycans provide viscoelasticity to the disc as well as the ability to maintain the pressure of the interstitial fluid. Proteoglycans intercross collagen fibers throughout the whole collagen net. Larger molecules of proteoglycans can be found in the intermedial zone of the disc, but also in the anterior and posterior portions where the pressure on the disc is higher (20). In the lateral and medial zones rather small proteoglycan molecules can be found, such as decorin and biglycan (21).

Conclusion

The exceptional specificity of the TMJ stems from the complex structure of all of its constituents. This complexity provides a vast range of motion and the ability to participate in a large number of important functions. Even though the joint is biologically designed to sustain constant function and intense functional pressures, aging causes the lowering of adaptation capacities of the tissues inside of the joint even when physiological relationships among all of the related structures are optimal. Connection with various oral tissues alongside the fact that two joints are connected by the same bone additionally complicates the structure and functions. Severe damage of the joint requests surgical intervention and replacement with implants that in most cases don't satisfy the biological criteria, therefore not improving the quality of the patient's life. Possibilities for inventing new therapeutic procedures are hard due to the complexity of the joint. Many sources state that the hope for the future lays in tissue engineering which accentuates the importance of acquiring knowledge about temporomandibular joint structures for all biomedical researchers in this field.

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GRAĐA I FUNKCIJA TEMPOROMANDIBULARNOG ZGLOBA

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Mandibula i kranijum povezani su kompleksnom zglobnom vezom, koja je po mnogim osobinama jedinstvena u ljudskom organizmu. Od drugih zglobova razlikuje se građom, morfološkom varijabilnošću zglobnih komponenata, specijalizovanim funkcijama, izuzetnom slobodom pokreta i blizinom vitalnih organa. Zglobne površine prekrivene su gustim fibroznim tkivom, koje se sastoji iz četiri histološki različite zone: artikulaciona zona, proliferativna zona, zona fibrozne hrskavice i zona kalcifikovane hrskavice. Fibrozna kapsula zgloba prekrivena je sinovijalnom membranom. Zglobna šupljina ispunjena je sinovijalnom tečnošću, koja ima ulogu metaboličkog medijuma i omogućava lubrikaciju. Temporomandibularni disk ima kompleksnu strukturu i veoma važne uloge, koje omogućavaju širok spektar pokreta u zglobu i fiziološki prenos pritiska. Disk deli zglobnu šupljinu na dva zasebna sprata, koji predstavljaju jednu funkcionalnu jedinicu; morfološki ima bikonkavan oblik. Po debljini, može se podeliti na zone od kojih je intermedijalna najtanja. Najkompleksniju strukturu ima posteriorna, bilaminarna zona, između čijih lamina se nalazi dobrovaskularizovano retrodiskalno tkivo. Disk se u toku funkcije stalno prilagođava izmenjenom položaju zglobnih površina, zahvaljujući specifičnom rasporedu i valovitoj strukturi kolagenih vlakana, kao i prisustvu elastičnih i oksitalanskih vlakana.

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Ključne reči: *temporomandibularni zglob, morfologija, temporomandibularni disk, histologija*

Review article

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IMAGING STRATEGY IN ACUTE ISCHEMIC STROKE: DIAGNOSTIC CHALLENGE

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Stroke is the third leading cause of death and the most common cause of permanent disability among the world population. Imaging plays a central role in assessing this pathology and various techniques can be used to examine brain and its vessels in acute stroke.

Angio- and perfusion computer tomography (CT) are widely used due to their high availability worldwide. However, MRI diffusion-weighted imaging (DWI) allows earlier and more accurate detection of ischemia. It is used in conjunction with other sequences, especially with susceptibility-weighted imaging (SWI), 3D-time-of-flight MR angiography (MRA), and contrast-enhanced MRA of supra-aortic arteries (CE-MRA), which allows diagnosing occluded arteries or hemorrhage and localizing thrombosis. By means of non-contrast arterial spin labeling (ASL) perfusion, which is of special interest, it is possible to diagnose ischemic penumbra and suggest prognosis in a noninvasive way.

MRI is the most accurate and the most reliable technique for diagnosing ischemic stroke and, in combination with the ASL sequence, for differentiating it from other diseases such as epilepsy and migraine aura. However, CT may be a valuable alternative in case MR is non-available or contraindicated.

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Key words: *acute ischemic stroke, magnetic resonance imaging, arterial spin labeling*

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Introduction

During the last ten years, the incidence of stroke in Europe has ranged from 95 to 290/ 100,000 per year, 80% of cases having the ischemic origin (1). Recently, the overall incidence of stroke has declined and the overall patient outcome improved (2). However, an increased stroke incidence has been observed in patients aged 30-65 years, mostly due to cardiovascular risk factors such as hypercholesterolemia, diabetes, smoking, and consuming alcohol (3). Moreover, the population aging leads to a progressive increase of the stroke rate. Consequently, stroke is among the most common causes of death, being in the 3rd place after heart diseases and cancer, and the most common cause of permanent disability in the world. Since the therapeutic window of opportunity in early stroke treatment is as narrow as the first six hours, it is necessary to develop strategies for early diagnosis, the crucial factor for successful treatment (1, 4-7).

Imaging is essential for the assessment of patients suspected of acute stroke, particularly before the initiation of therapy (8). First, it is necessary to distinguish between hemorrhagic and ischemic stroke, second, to locate the potential intravascular clot and assess its extent, and, lastly, to evaluate the extension of the ischemic core and the penumbra (7, 9).

Currently, non-contrast computer tomography (NCCT) remains the most widespread and low-cost imaging technique of those being widely available. It is highly sensitive to acute intracerebral hemorrhage (ICH), but is not sensitive to acute ischemia in the first hours of onset. CT angiography is combined with NCCT in order to detect arterial occlusion. However, MRI is increasingly used in acute stroke, improving the imaging diagnostic accuracy.

The conventional MRI sequences include difusion-weighted imaging (DWI), fluid-attenuated inversion-recovery imaging (FLAIR), T2-weighted

(T2-w) gradient-echo imaging (GRE), or susceptibility-weighted imaging (SWI), as well as MR angiography (MRA) of the intracranial arteries using 3D time-of-flight (TOF) sequence. In some cases, TOF or gadolinium-enhanced neck-vessel MRA is performed, especially prior to thrombectomy. If dissection is suspected, the neck is examined using fat-suppressed T1- or T2-w sequences (10), the method being more sensitive 3-5 days after the event, when the intra-mural thrombus can be evidenced. Additionally, the advanced MRI techniques, such as post-contrast perfusion-weighted imaging (PWI) or contrast-free arterial spin labeling (ASL) perfusion, are helpful in assessing ischemic penumbra (i.e., the hypo-perfused territory that is not yet infarcted and may be preserved with early recanalization) and also in differential diagnosis.

Diverse imaging techniques, such as positron emission tomography (PET), single photon emission computed tomography (SPECT), xenon-enhanced computed tomography (XeCT), dynamic perfusion CT, MRI-based dynamic susceptibility contrast (DSC) perfusion, ASL, and Doppler ultrasound, can be applied to assess brain hemodynamics. Among the above-mentioned perfusion techniques, perfusion CT and DSC allow, after contrast material administration, evaluating cerebral blood flow (CBF), cerebral blood volume (CBV), and mean transit time (MTT) parameters (11), while ASL allows measuring CBF without contrast injection. It also should be mentioned that, out of all the above methods, only MRI and CT can be used for fast perfusion evaluation in an emergency context.

As for acute-stroke patients, there are numerous imaging techniques suitable for the evaluation of vascular lesions and brain tissue status. However, in order to provide the most appropriate emergent management to a stroke patient, physicians should be aware of and well familiar with advantages and challenges of each technique and have the most appropriate standard imaging protocol for stoke management in each center.

Computed tomography

Among the advantages of CT are its fast performance, low cost, and wide availability. It can be safely used in patients in severe condition, agitated, being on support or with implanted monitoring devices, such as cardiac pacemakers, that make MRI contraindicate. Multimodal CT, in which NCCT, CT angiography (CTA), and perfusion CT are combined, is also commonly used. Performing NCCT and CTA takes less than 10 min, which is enough to rule out ICH and detect potential arterial occlusion. In addition, perfusion CT makes it possible to reveal acute infarction, localize the infarct core and salvageable brain tissues and estimate the degree of collateral circulation (12-14).

Early ischemia signs on NCCT have been described, which allows assessing the Alberta Stroke Program Early CT (ASPECT) score (14, 15). Although the early signs of stroke may not be always observed, NCCT can exclude ICH, which is most important. This technique can also be helpful for detecting such lesions as tumors that may mimic an acute ischemic stroke. The diagnosis of acute posterior fossa and brainstem infarctions remains problematic due to beam-hardening artifacts (Figure 1 a-d) (16).

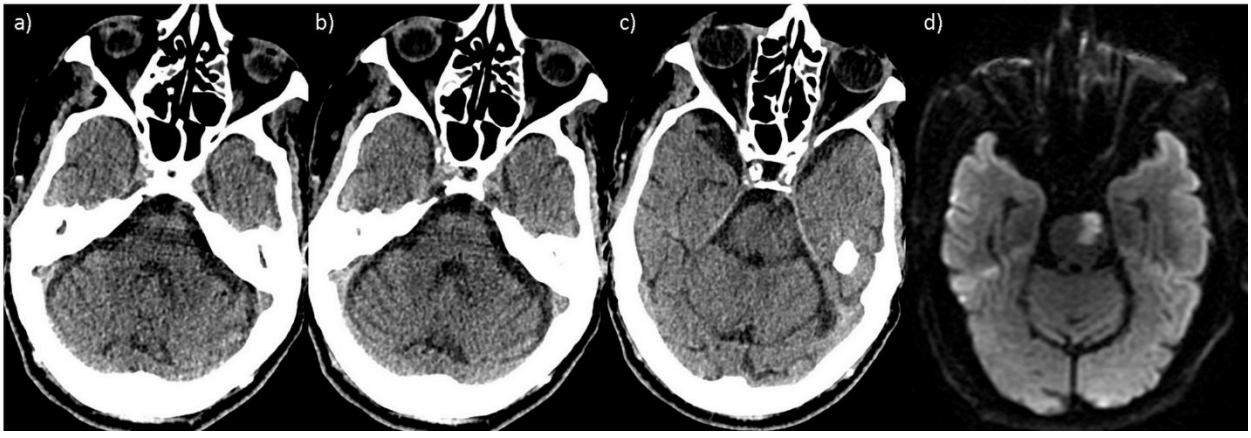

Figure 1. Axial brain CT tomograms (**a, b**) show posterior fossa beam-hardening artifacts;
(**c**) suspected brainsteam acute ischemic lesion;
axial MRI 3D DWI image (**d**) confirms the diagnosis of acute ischemic stroke, showing a restricted diffusion

Additional information related to vessel patency and the hemodynamic consequences of vessel occlusion can be obtained by CTA and perfusion CT (17).

Perfusion CT increases the sensitivity of ischemic lesion detection by evidencing systematic pathological hypo-perfusion. The previous perfusion CT acquisition gave only limited brain coverage,

which could lead to false negative results. This drawback has been greatly reduced by using the latest CT-scanners, which allow evaluating a much larger or the complete brain volume.

Perfusion CT acquisition enables the physician to distinguish between penumbra and infarcted tissue. Increased MTT with decreased CBF and normal or mildly increased CBV, resulting from autoregulatory mechanisms, suggest penumbra in the early stage of ischemia, whereas increased MTT with markedly decreased CBV and CBF suggest irreversibly infarcted tissue (18). The color-coded perfusion maps are helpful for visual assessment of perfusion CT (Figure 2 a-e).

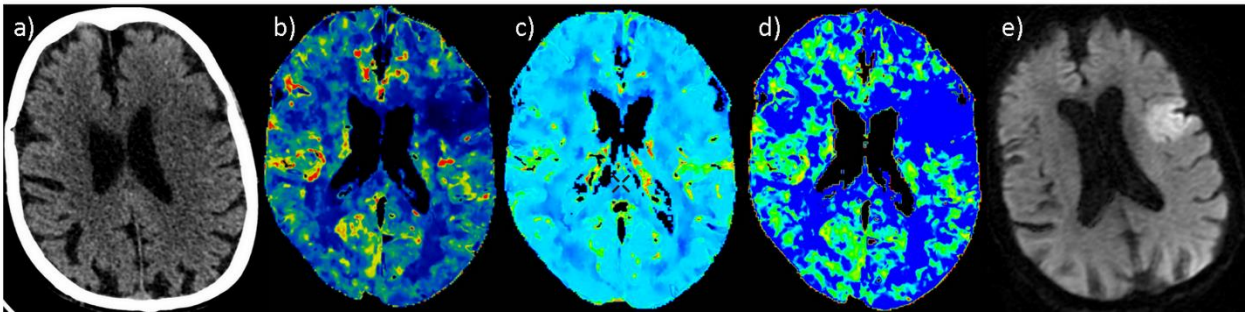

Figure 2. Axial brain CT tomogram (**a**) shows normal brain parenchyma at supratentorial levels (ASPECTS 10); PCT (**b, c, d**) show decreased CBF, normal CBV, and increased MTT, suggest early stage of ischemia; axial MRI 3D DWI image (**e**) confirms the diagnosis of acute ischemic stroke, showing a restricted diffusion (ASPECTS 9)

However, during the first hours after the onset of symptoms, perfusion CT is of limited value because CBV either stays within normal limits or may increase due to vasodilatation in response to CBF decrease (19). Moreover, the perfusion coverage of posterior fossa remains limited. Finally, the detection of small lesions remains challenging and, in contrast to MRI, CT is an irradiating examination.

CTA allows to study cervical and intracranial arteries at the same time, thus assessing the arterial occlusion site, dissection, potential collateral arteries, and atherosclerotic disease. The evaluation of arterial status is highly important before interatrial treatment (17). A thrombus may be identified as a filling defect by CTA.

However, distal thromboses are often missed, especially in older patients with vascular encephalopathy when it is challenging to distinguish the newly created lesion from the old one. Furthermore, while the prognosis seems to be better for patients with rich collateral leptomeningeal arterial network, its evaluation is limited if only CT imaging is used (17).

Finally, on CT images, there may be a number of potential stroke mimics, which would give false positive results (20, 21).

Magnetic resonance imaging

When available, brain MRI is the examination modality of choice for acute stroke management. In emergency context, it is sufficient to acquire four sequences (DWI, FLAIR, 3D TOF MRA and T2-w GRE), which takes less than 10 min.

These sequences allow to confirm the ischemic stroke diagnosis, locate it precisely, approximately evaluate the stroke date, detect occluded arteries, locate the thrombus, and rule out hemorrhage or other lesions.

DWI is the most sensitive sequence for identifying acute ischemia shortly after the stroke onset (22). Ischemic lesions that appear in high DWI signal intensity, resulting from diffusion restriction with decreased apparent diffusion coefficient (ADC) are generally considered to be irreversible (23). However, the precise threshold of the ADC values for declaring a lesion as irreversible has not been established. Although there is no exact threshold of the DWI lesion volume for deciding on further treatment, still it is important to quantify DWI lesions either by visual determination of the DWI ASPECT score or by automatical software measurements, such as rapid processing of perfusion and diffusion (RAPID) or perfusion mismatch analyzer (PMA) software (24-27).

FLAIR sequence plays a significant role in the selection of patients for further treatment. It helps to approximately date the stroke event and determine whether or not the patient is within the therapeutic window for arterial recanalization by means of thrombolysis or thrombectomy. Indeed, the FLAIR hyper-intensity usually appears within 4-6 hours after the stroke onset (28).

A stroke in the early phase can be confirmed on the basis of FLAIR-diffusion mismatch, defined as a negative FLAIR signal in a zone of restricted diffusion (29). In addition, FLAIR sequence may reveal hemodynamic stenosis or an occluded artery by showing downstream hyper-intense arteries also known as slow flows. This hyperintense vessel sign is suggestive of brain tissue at risk of ischemia and has a prognosis value (Figure 3 a-c) (30).

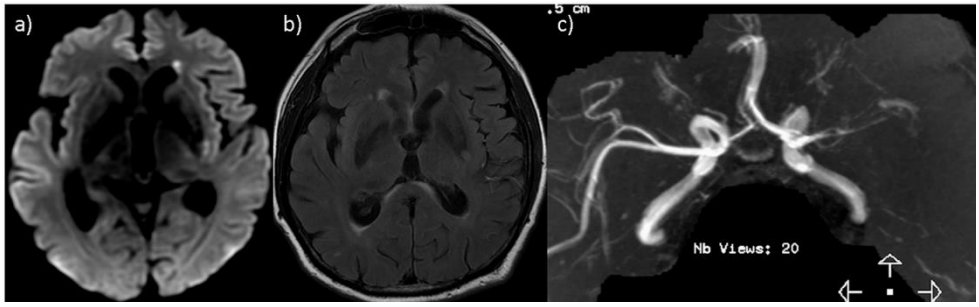

Figure 3. Patient with suspected acute ischemic stroke.
Axial 3D DWI image (**a**) shows a lake of zone of restricted diffusion;
with negative FLAIR (**b**) hyper-signal, showing downstream hyper-intense arteries, indicating slow flow;
3D TOF MRA (**c**) confirms occlusion of the left M1 segment

Both MRI and CT may provide information about irreversible brain ischemia, but MRI provides more accurate data and is recommended as the exam of choice by the American Academy of Neurology (AAN). However, if the access to MRI is limited, it should be performed in the first place for those patients who are within twelve hours after the stroke onset (31).

DWI is more accurate and sensitive as compared to CT in detecting acute infarction. Using just NCCT, it is neither possible to rule out ischemic stroke nor to differentiate between and epilepsy or migraine. Moreover, there is strong correlation of the lesion volume on DWI acquired in the acute phase of stroke with the final infarct volume, which is not the case with CT (32). For posterior fossa stroke, NCCT is usually normal, while DWI allows making an early stroke diagnosis (19).

On the T2-w GRE sequence, early hemorrhage appears as a rim of hypo-intense signal surrounding the isointense core (33), the sensitivity of this technique and of CT being the same. More sensitive sequences, such as SWI, which have recently been developed, allow identifying an acute thrombus owing to its blooming effect, the sensitivity being higher than that of NCCT (34).

3D-TOF MRA, in contrast to CTA, is non-invasive since a contrast agent injection is not needed in the case of the former. This technique allows detecting occlusion or stenosis as well as its extension and localization. It is also helpful for evaluating collateral circulation, an important predictor of the outcome (35).

Extracranial vascular imaging plays an important role in the case of acute stroke and transient ischemic attack since it allows detecting atherosclerosis, which causes 40% of all ischemic strokes, but is most widely used in acute context for thrombectomy planning (36).

In view of the above, some medical centers have recently introduced contrast- enhanced MRA of supraaortic arteries (CE-MRA) as part of standard protocol in patients older than 70, with acute ischemia, in order to provide diagnostic information on steno-occlusive diseases within 2 min of acquisition (37). The signal-to-noise ratio of CE-MRA being higher than that of TOF MRA, the former technique is more advantageous than the latter, allowing better visualization of small intracranial vessels. Finally, CE-MRA can provide a morphological image of vessels by giving insight into plaque composition (38).

Adding CE-MRA to the imaging protocol has also some disadvantages, such as the need of using a larger coil, which can cause inconvenience for the acute patients, the more so that the need of perfusion and contrast injection extends the duration of the examination. Finally, CE-MRA alone does not provide dynamic information about the vascular supply of the infarcted territory. In order to overcome the above-mentioned shortcomings, a novel time-resolved broad-use linear acquisition speed-up (kt BLAST) CE MRA technique has been proposed. It allows using a non-dedicated body coil, has a shorter acquisition time and provides high-temporal-resolution dynamic data, which can help to differentiate occlusion from high-grade stenosis (39).

DSC perfusion weighted imaging

PWI provides information about brain hemodynamics and allows early detection of penumbra and infarcted areas. In contrast to perfusion CT, MR PWI gives the whole-brain coverage (40).

DSC-MRI, the most widely used perfusion technique in clinical practice, is based on measuring the T2* decrease during the first pass of an exogenous endovascular tracer through the capillary bed. The other perfusion method, dynamic contrast-enhanced (DCE) MRI T1-weighted (T1-w) sequence, is not commonly used in stroke examination, due to a lower signal-to-noise ratio (41, 42).

The mismatch between DWI and PWI is used to identify penumbra in acute stroke. The DWI abnormality represents the infarcted zone, whereas the PWI abnormalities, which are not yet seen at this stage on DWI, correspond mostly to penumbra (43).

Parametric DSC-PWI maps provide the time-to-peak (TTP), MTT, CBV, and CBF parameters. TTP is considered a PWI marker of the highest accuracy for detecting penumbra in hyper-acute stroke (44), whereas CBV and CBF values have the best correlation with the final infarct volume and core. Since the DSC PWI sequence is not fully quantitative, the CBF and CBV are relative values, calculated as the

ratio of the values in the region of interest (ROI) located in the pathological area and in the contralateral ROI, which is used as a normal reference.

This method is not always used in stroke protocols since it requires perfusion and contrast enhancement and is time-consuming. Moreover, the results may depend on the post-processing tools. Errors may also be introduced by the variability in the choice of arterial input function, partial volume averaging caused by mixing the tissue and vessel signal intensities, as well as by the orientation of the vessel with respect to the direction of the magnetic field. Another drawback is that, the parameter values being relative, it is not possible to determine the zone of benign olighemia, which does not represent a part of the tissues at risk of ischemia (44, 45). The relative perfusion values may also vary due to proximal carotid occlusion.

Arterial spin labeling imaging

ASL perfusion is a particularly interesting non-invasive, contrast-free perfusion method, which allows identifying cerebrovascular abnormalities and hypo-perfused regions. ASL is currently gaining an increasing interest in the management of cerebrovascular diseases and ischemic events.

In contrast to perfusion CT and DSC MRI perfusion, ASL is non-irradiating and allows the evaluation of the whole brain, without contrast injection required, which is particularly advantageous in pediatric population (46). Moreover, ASL allows the follow-up of the CBF changes within ischemic regions over time, while DSC PWI perfusion requires repeated contrast administration for that (47).

ASL can be performed using continuous ASL (CASL), pulsed ASL (PASL), or pseudo-continuous (pCASL) techniques, each of them being based on different excitation methods. When constant radiofrequency (RF) pulses under constant gradient are used, CASL tends to continuously invert the blood water spin as it passes a certain plane. In PASL, a single pulse is employed in order to determine the volume containing arterial blood for labeling. In PASL, unlike the case of CASL, there is an inversion of a large slab along the feeding arteries, occurring over the very brief labeling phase. While CASL, similar to PASL, produces a greater signal-to-noise ratio (SNR), the labeling efficiency is higher with PASL. In order to reduce the technical restrictions of CASL and PASL, a novel approach, called pCASL, has been introduced. It employs a discrete RF pulse train in conjunction with a synchronous gradient field, which results in both greater SNR and labeling efficiency. Thus pCASL is more advantageous than both CASL and PASL (48, 49).

In pCASL, the time required for the labeled protons to enter the area of interest (called post-labeling delay (PLD)) is an important parameter for achieving good acquisition. If PLD is too short, the signal may remain in the vessels, producing arterial transit artifacts, while excessive PLD length leads to the signal loss. Consequently, PLD should be adapted to the patient's age since the circulation is faster in young as compared to elderly patients. The appearance of hypo-perfused regions may be actually connected with supra-aortic stenosis (48, 50). Finally, one should be aware that anatomical variations, such as fetal origin of cerebral posterior artery, may lead to perfusion map asymmetry (51). Obviously, all the above factors may lead to misdiagnosis.

Although the 3T scanner is recommended in clinical practice for increasing the SNR, ASL may also be performed on 1.5 T, providing images of satisfactory quality (48). The use of ASL in clinical practice is increasing, especially thanks to the availability of 3T MR scanners as well as to the development of improved pulse sequences and multichannel receiver array coils (50).

In the ASL, labelled blood water molecules are used as free diffusible tracers and their movement is followed from the arterial compartment to the tissue capillaries. Labelling is performed by applying radiofrequency waves in the neck area. The perfusion images are generated by subtracting control images from the labeled ones. By means of ASL, it is possible to measure absolute values of cerebral blood flow. ASL sequences are acquired by fast techniques, such as echo-planar, gradient- and spin-echo, or three-dimensional fast spin-echo imaging (50, 52). The turbo spin-echo 3D ASL acquisition is preferable to the echo-planar one since the former reduces artifacts.

The labeling plane must be located in the region where the relevant feeding arteries are perpendicular to the labeling plane and not at the level where dental artifacts may be produced. In order to appropriately position the labeling plane, a low-resolution MRA should be performed before ASL, so that tortuous artery segments that may cause protons defection can be avoided (50).

Since acquiring an ASL sequence takes rather long time (around 4 min), it is difficult to obtain good-quality ASL images in highly agitated or confused patients. In order to decrease motion artifacts, background suppression should be used (50).

In the case of stroke related to brain haemodynamics disruption, ASL allows non-invasive contrast-free evaluation of brain damage and potential assessment of differential diagnoses (53). Using ASL, it is also possible to evaluate cerebral parenchymal perfusion by detecting both hypo- and hyper-perfused regions and delayed transit effects, which may predict clinical outcome in acute ischemic stroke (54, 55). Delayed transit effects are caused by the longer arterial transit time (the time it takes the labeled blood to reach the region of interest) in patients with cerebrovascular disease. The arterial transit time increases due to the decrease in perfusion pressure and the consequent development of collateral circulation. Spontaneous recanalization, therapeutic recanalization, and improved collateral flow without recanalization may be mistakenly interpreted as hyper-perfusion in the cases of acute ischemic stroke. Hyper-perfusion indicates greater proneness of the regions to hemorrhagic transformation (48, 54, 55).

Luxury perfusion refers to the CBF increase which occurs later on within the ischemic tissue and is considered to be a good prognostic factor, related

to the collateral flow, though with increased risk of bleeding (56).

An arterial bright signal (ABS) at the level of vascular occlusion in the ASL sequence can be helpful in identifying and locating a thrombus (Figure 4 a, b). The ABS corresponds to the labelled blood that is trapped in the vessel due to the vascular occlusion. In acute ischemic stroke, the thrombus and the vessel occlusion site can be detected as ABS localized proximal to the hypo-perfused area, related to the trapped labelled spins (57, 58). ABS may also appear due to a stagnant flow upstream from the stenosis since the bright intravascular signal may be attributed to slow flowing blood (59). If TOF and SWI are not conclusive, ABS may be helpful for the detection of vascular occlusion or narrow stenosis, especially in the small arteries (Figure 5 a-f) (59, 60).

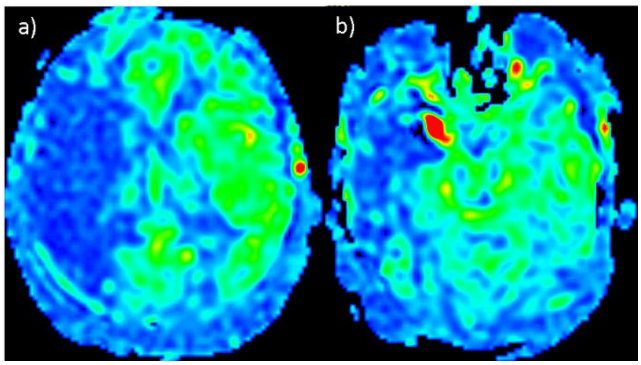

Figure 4. 3D ASL perfusion (**a**) shows whole right hemisphere hypo-perfusion;
with ABS (**b**) indicating the thrombus and the vessel occlusion site in right internal carotid artery

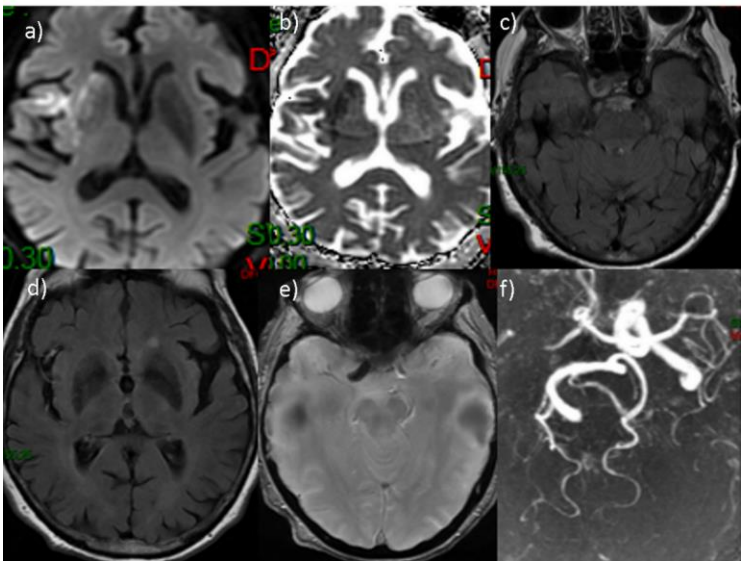

Figure 5. Axial 3D DWI image shows restricted diffusion, with low ADC values (**a, b**), representing ischemic lesion;
3D FLAIR axial reconstructions (**c, d**) show the hyper-intense vessel sign and downstream hyper-intense arteries, indicating occlusion, and slow flows;
T2* axial image (**e**) shows the blooming low signal within the right internal carotid artery, indicating a clot;
3D TOF MRA (**f**) confirms occlusion of the internal carotid artery

In patients with acute ischemic stroke, due to delayed arterial transit time the late-arriving flow within or around the hypo-perfused territory is presented as a bright intravascular signal. This signal is usually referred to as arterial transit artifact (ATA). It can be an indication of stenosis or a stagnant flow, but also can represent the collateral flow. Thus ATA may signify both a better outcome after an acute stroke and the lack of its progression to infarct (Figure 6 a-d) (58).

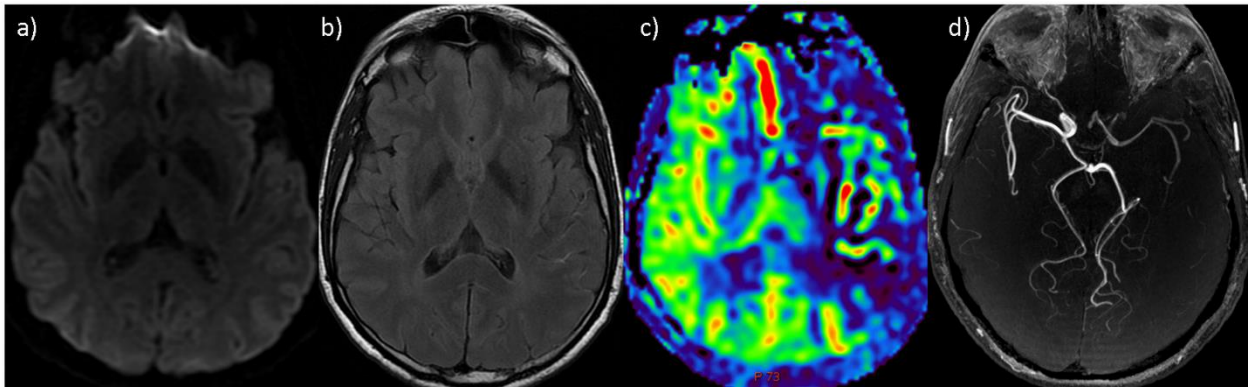

Figure 6. The patient with negative DWI and FLAIR (**a, b**);
3D ASL perfusion (**c**) shows left hemisphere hypo-perfusion, with ATA indicating slow flow, or collateral circulation;
3D TOF MRA (**d**) confirms occlusion of the internal carotid artery

Still another sign that may be seen on ASL images is the so-called border zone sign, presented as cortical areas of high signal intensity and resulting from ATA surrounding the normal low ASL signal in the watershed border zones. This signal is often found in elderly people with cardiovascular diseases and should not be confused with pathological hypo-perfusion. Otherwise, the lesion may be overestimated when the hypo-perfused area is adjacent to the border zone. Therefore, correlation with the contralateral hemisphere may help to avoid erroneous diagnosis (61).

To sum up, these artifacts are of significant interest in stroke patients. However, in cases requiring optimal CBF quantification, it is possible to avoid ATA by applying the vascular suppression method (62). Precise quantification of CBF can also be achieved by applying the multi-PLD method, which provides greater accuracy of CBF quantification as well as improved visualization of collateral flow through series of dynamic images. Moreover, multi-delay pCASL makes it possible to differentiate between temporal profiles of hyper-intensities on ASL images due to different conditions such as hyperemic responses or delayed transit effects (63).

ASL can be used to evaluate penumbra volume in acute stroke, which is observed as zones of hypo-perfusion. In recent studies, positive correlation between DSC, PCT, and ASL hypo-perfusion volumes was found (64). Moreover, it was shown that the ASL-CBF decrease of more than 40% had about the same diagnostic performance as DSC thresholds of PWI Tmax > 6 sec and CTP Tmax > 5.5 sec, which are considered to be reliable marker of penumbra (64).

ASL can also be helpful for eliminating other differential diagnoses such as epilepsy or migraine. During the ictal phases in epileptic patients, focal hypo-perfusion in the epileptogenic grey matter zones, which is not systematized to a vascular territory, can be seen on ASL imaging, at the stage following the early post-ictal hyper-perfusion. Furthermore, in patients suspected of a recurrent stroke in the previous-infarction zone, ASL identifies epileptogenic foci in the ischemic scar tissue by showing high flow rate zones located on the borders of parenchymatous sequelae (48).

In addition, ASL makes it possible to detect decreased CBF areas with the brush sign in acute migraine aura, which follows the CBF increase during the headache phase, with no vascular systematization (in contrast to ischemia), and is predominantly observed in posterior regions (65).

Conclusion

There is a growing number of imaging techniques for assessing the parenchymal and vascular status in acute stroke. The chosen method should provide a fast and precise diagnosis, improve the outcome, and provide timely intravenous thrombolysis and/or mechanical thrombectomy. In different medical centers, the choice of a particular method may depend on several factors such as MRI availability, cost and local preferences. MRI is more accurate and more reliable than CT in diagnosing acute ischemic stroke. Advanced imagings, such as the ASL sequence, allow assessment of penumbra and differential diagnoses (e.g., epilepsy or migraine aura).

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IMIDŽING STRATEGIJA U AKUTNOM ISHEMIJSKOM MOŽDANOM UDARU: DIJAGNOSTIČKI IZAZOV

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Akutni ishemijski moždani udar je treći vodeći uzrok smrti i najčešći uzrok trajne invalidnosti u svetu. Različiti modaliteti imidžinga imaju važnu ulogu u evaluaciji parenhima mozga i intrakranijalnih krvnih sudova u akutnom ishemijskom moždanom udaru.

Angiografija i perfuziona kompjuterska tomografija (CT) imaju široku primenu, zbog dostupnosti širom sveta. Međutim, MRI difuzioni imidžing (DWI) omogućava ranije i preciznije otkrivanje ishemije i koristi se zajedno sa drugim sekvencama protokola, kao što su imidžing susceptibilnosti (SWI), 3D MR angiografija 3D (MRA) i kontrastna MRA supraortalnih arterija (CE-MRA), što omogućava dijagnostikovanje okludiranih arterija ili otkrivanje hemoragije, kao i lokalizaciju tromboze. Pomoću nekontrastne perfuzije, sekvence arterijskog obeležavanja spinova (ASL), koja je od posebnog interesa, moguće je dijagnostikovati ishemijsku penumbru i predvideti prognozu ishoda na neinvazivan način.

MRI je najtačnija i najpouzdanija tehnika za dijagnostikovanje ishemijskog moždanog udara i u kombinaciji sa ASL sekvencom, značajna je za diferencijaciju ovog stanja od drugih bolesti, poput epilepsije i migrene. Međutim, CT može biti značajna alternativa u slučaju da MR nije dostupna ili je kontraindikovna.

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Ključne reči: *akutni ishemijski moždani udar, magnetno rezonantni imidžing, obeležavanje arterijskih spinova*

Case report

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EARLY VASOSPASM FOLLOWING SPONTANEOUS SUBARACHNOID HEMORRHAGE: A CASE REPORT

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Ultra early cerebral vasospasm (UEAV) occurs within 48 hours after aneurysm rupture, can be verified by angiographic imaging and usually has a poor outcome. The presented patient had clinical symptomatic UEAV, detected within a few hours of bleeding from anterior communicating artery aneurysm. The neurosurgical team decided on late operation, more precisely 18 days after the rupture of the aneurysm, because the patient was then giving clinical signs of cerebral vasospasm relief. Right pterygoid craniotomy was performed. The aneurysm was excluded from circulation by clipping without any incident. When treating patients with clinical signs of UEAV, the ruptured aneurysm site should be verified by angiographic imaging as soon as possible, while the appropriate treatment modality should be carefully and duly considered.

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Key words: *intracranial vasospasm, anterior communicating artery aneurysm, surgical clip*

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Introduction

Cerebral vasospasm (CVS) after spontaneous subarachnoid hemorrhage (SAH) occurs in 20 to 30% of patients and represents a life-threatening condition (1). It involves a temporary contraction of the arteries, which results in a focal or diffuse reduction in caliber of blood vessels, and usually occurs 3 to 14 days after SAH (2). Diagnosis is made by Transcranial Doppler ultrasound (TCD), Magnetic Resonance angiography (MRA) or Computed tomography angiography (CTA) (3). The clinical presentation of a patient with symptomatic CVS depends on the affected intradural or surface brain artery and causes cerebral ischemia, which can be marked as delayed ischemic deficit (4).

On the other hand, there is much less data in the literature on early CVS, which occurs within 48 hours after aneurysm rupture, can be verified by angiographic imaging and usually has a poor outcome (5, 6).

In this case report, we present a patient with clinical symptomatic ultra-early vasospasm (UEAV), detected within a few hours of bleeding from the anterior communicating artery (ACoA) aneurysm, with an emphasis on therapeutic dilemmas.

Case report

A 30-year-old female patient was admitted to the emergency department due to spontaneous SAH. The symptoms occurred in the evening, on the day of admission, followed by a neck stiffness and severe headache, which she described as the worst in her life, followed by the loss of consciousness and emesis. The measured arterial pressure was 180/120 mmHg, and we obtained heteroanamnestic data that she did not suffer from hypertension and did not take any oral medications by then. Initial examination at the emergency department was performed by a neurosurgeon, who indicated CTA scan of the brain. Clinical examination showed that the patient was somnolent but conscious (Glasgow Coma Scale Score of 14), oriented to time, place and person, with neck stiffness (Hunt & Hess Score of 1) and without any recorded gross neurological deficit. CTA recorded SAH sagittal frontally, which filled basal cisterns with blood and expanded to the prepontine cistern, as well as an anteriorly directed

aneurysm of the ACoA, measuring 4 mm in diameter.

Therefore, she was hospitalized at the Neurosurgical department, where she was treated with hyperdynamic therapy for CVS, a calcium channel blocker nimodipine (Nimotop® in the dose of 60 mg administered orally every 4 hours), antiedematous and prophylactic anticonvulsant therapy. Moreover, Digital subtraction angiography (DSA) was performed the next morning (Figure 1), which showed a saccular aneurysm with a maximal diameter of 3.8x5 mm on ACoA, with the neck 3.1 mm wide. The aneurysmal dome, directed anteriorly, had a smaller aneurysm with a diameter of 3x2.6 mm, and another aneurysm was originating from the neck of the aneurysm, measuring 2 mm in diameter. Furthermore, at the origin of the right pericallosal artery, there was another sacullar aneurysm with a wide neck, measuring 3.2x2 mm in diameter, directed posteriorly. Vasospasm of both anterior cerebral arteries (ACA) was verified. Initially, the patient responded well to the therapy. Control CT angiography was performed 11 days after DSA, given that the patient's mental state had deteriorated.

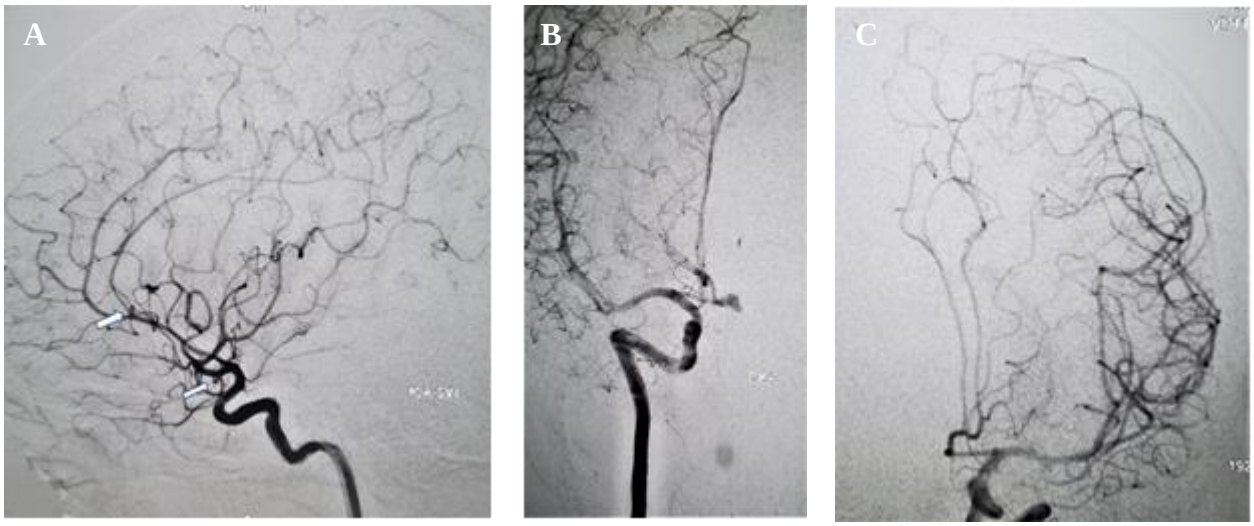

Figure 1. DSA finding (A, B, C) confirms previously seen aneurysms at ACoA and at the origin of pericallosal artery on the right. The CVS of both ACA is evident.

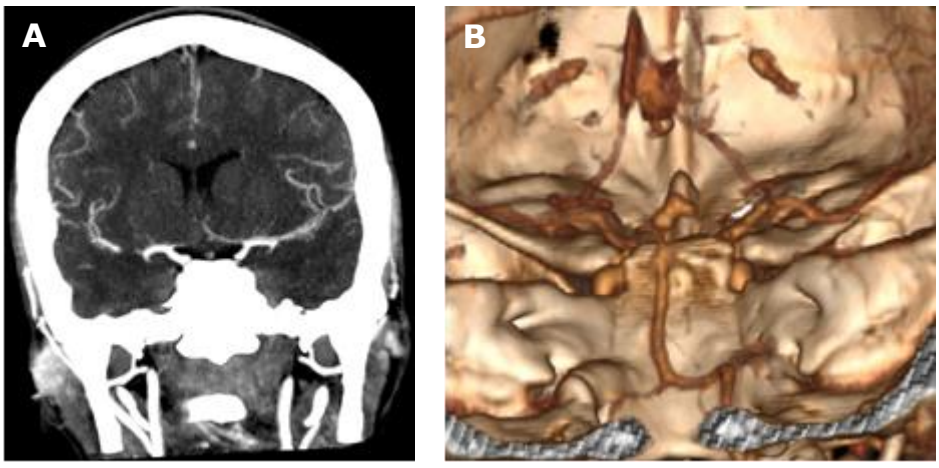

Figure 2. CTA (coronal plane- A; VR image- B) shows an ACoA aneurysm.
Bilaterally, the A1 segments of ACA are graceful, while the A2 segments are filiform in the proximal part, but distally without visible contrast, which indicates spasm of the aneurysm.

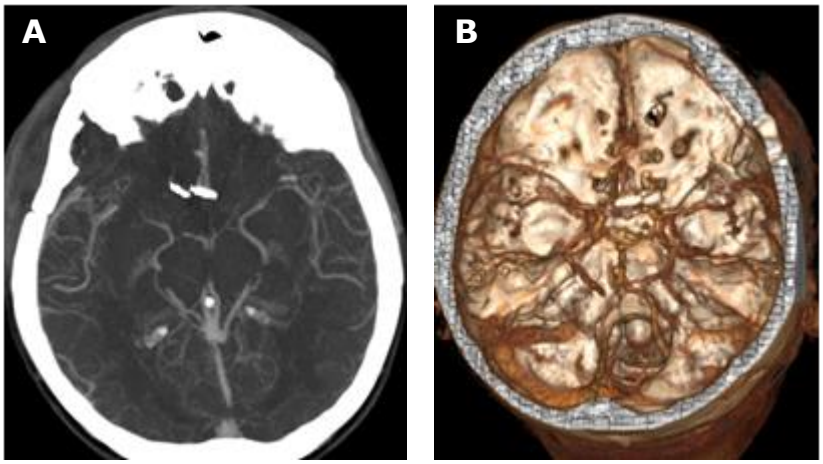

Figure 3. CT angiography (axial plane-A; VR imaging- B) shows surgical clips, which are placed correctly. Both ACA are transient and of a larger caliber in comparison with the previous CTA, but still in vasospasm, which indicates a relief of a CVS.

Compared with the initial CTA, regression of the amount of SAH was recorded and CVS of both ACA was still present. Since there was no re-hemorrhage and the CVS still persisted (Figure 2), the surgery was postponed. The patient was stabilized again after applied therapy.

After 7 days, due to the good clinical state of the patient, the neurosurgical council decided that the aneurysm should be treated surgically. Right pterygoid craniotomy was performed. The vasospasm of both ACA was verified. The aneurysm was excluded from the circulation by clipping without any incident. The bony lid was fixed back with surgical sutures and returned to the appropriate place and the soft tissues was sewn in layers. The postoperative recovery went smoothly. The patient was somnolent and without any gross neurological deficits. The further clinical course was complicated by the patient's mental state alteration and insipid diabetes. Due to the continued worsening of the patient's state to subcoma, as well as the respiratory insufficiency, the patients was transferred to the intensive care unit, when she was intubated and placed on mechanical ventilation in a BiPAP mode. The control CTA was performed (Figure 3), on which CVS of both ACA was still present. Six days after intubation, the patient became hypotensive, bradycardic, which resulted in cardiac arrest and death.

Discussion

Fawaz et al. reported UEAV in 4.6% of SAH patients, which were associated with younger age, poor neurological state during the first examination, as well as the presence of sentinel bleeding. Moreover, UEAV was linked with an increased chance of experiencing delayed cerebral ischemia (DCI), but was not related to poor neurological outcome after treatment (7). In contrast, Baldwin et al. found UEAV in 10% of SAH patients, which was not associated with symptomatic DCI (8). Furthermore, Qureshi et al. reported a significant relation between UEAV, symptomatic CVS and poor neurological outcome (9). Our patient, on the other hand, did not have a poor clinical grade on admission and during the first day of hospitalization, during which the vasospasm was verified by CTA and DSA, but she experienced cerebral ischemia and eventually a lethal outcome.

The etiology of UEAV has not been fully elucidated due to insufficient investigation. Predictive factors for the development of UEAV may be patient's poor clinical grade, hypertension, blood sodium level greater than 138 mM, high score (3 and 4) on the Fisher scale, larger dimensions of the ruptured aneurysm, and the previous history of SAH (7, 10). Some of the potential pathophysiological factors may be elevated intracranial pressure, inflammatory response to injury as well as the formation of subarachnoid blood clots. Moreover, key elements after subarachnoid clot formation are an increased concentration of nitric oxide (NO) scavengers, such as reactive oxygen species (ROS) and oxyhemoglobin, as well as increase in concentration of transmitters (serotonin, thromboxane A2, Endothelin-1 and thrombin), which are responsible for CVS (11). The patient we treated did not suffer from hypertension, did not have previous history of SAH, and had a lower score (2) on the Fisher CT scale.

Danura et al. consider that the incidence of CVS is more frequent after surgical clipping of a ruptured cerebral aneurysm. Furthermore, in a series of patients examined, they found that the CVS rate was 30% after clipping and 14% after endovascular treatment in patients with SAH, due to manipulation with arteries during surgery (12).

The open microsurgery has certain disadvantages, which could theoretically lead to CVS, such as the presence of new blood and its breakdown products during surgery in the subarachnoid space and the consequent formation of free radicals and lipid peroxides, as well as vasospasm after manipulation with arteries. The results of previously

conducted studies have shown a better outcome for patients and less often CVS after coiling compared to open microsurgery. These results could be explained by the fact that patients who were treated with coiling had a better neurological grade preoperatively (13-15). In our case, we opted for an open microsurgical approach in the patient, because of the possibility of irrigating the basal cisterns and removing blood from the subarachnoid space, guided by the experiences of some other authors (16, 17).

Ebeling et al. considered the right timing of surgery for the ruptured aneurysm, and pleaded that the patient should undergo a surgical treatment within 48-72 hours after rupture or within the 10-14 days following the rupture of an aneurysm (18). However, Ebeling et al. believe that the TCD examination in CVS could enable us to decide on the best and individual timing for surgery while daily examining the patient (18). Mahaney et al. Considered possible causes for unfavourable postsurgical outcomes in patients, which were operated on 3-6 days after rupture of an aneurysm. They explained it with secondary injuries due to impaired cerebral autoregulation. Furthermore, this pathophysiological mechanism has been described by some other authors as well (19). Patients with a high score of Fisher's scale (3 and 4) are considered to have better outcomes, if they undergo surgery during the first and second day after the aneurysm rupture, but the outcome is considered to be worse if they undergo surgery later. In contrast, patients with lower Fisher scale scores (1 and 2) experience more favorable outcomes if they undergo surgery early and later, while outcomes are less favorable with intermediate surgery. Consequently, Mahaney et al. Reported that during the follow-up examinations 90 days after surgery, patients who had undergone surgery during the first 2 days or after 6 days of aneurysm rupture had better outcomes (19). In accordance with these results, our neurosurgical team decided on late surgery, more precisely 18 days after the rupture of the aneurysm, because the patient was then giving clinical signs of CVS relief.

Conclusion

When treating a patient with clinical signs of UEAV, the ruptured aneurysm site should be verified by TCD, CTA or DSA as soon as possible, while the appropriate treatment modality should be carefully and duly considered.

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RANI VAZOSPAZAM NAKON SPONTANOG SUBARAHNOIDALNOG KRVARENJA: PRIKAZ SLUČAJA

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Rani cerebralni vazospazam javlja se u roku od 48 sati nakon rupture aneurizme, a može se potvrditi angiografskim snimanjem i obično ima loš ishod. Prikazani bolesnik imao je klinički simptomatski rani cerebralni vazospazam, otkriven u roku od nekoliko sati nakon krvarenja iz aneurizme prednje komunikantne arterije. Neurohirurški tim odlučio se za kasniju operaciju, tačnije 18 dana nakon rupture aneurizme, jer je bolesnik tada davao kliničke znake popuštanja cerebralnog vazospazma. Urađena je desna pterigoidna kraniotomija. Aneurizma je isključena iz cirkulacije pomoću klipa, bez ikakvih incidenata. Prilikom lečenja bolesnika koji imaju kliničke znake ranog cerebralnog vazospazma, mesto rupture aneurizme treba, što je pre moguće, verifikovati angiografskim snimanjem, dok treba pažljivo i propisno razmotriti odgovarajući modalitet lečenja.

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Ključne reči: *cerebralni vazospazam, aneurizma prednje komunikantne arterije, hirurški klip*

Case report **UDC: 616.15-073**
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STEPS IN DIAGNOSIS OF CHRONIC IDIOPATHIC NEUTROPENIA: IS IT THE TIME FOR SERBIAN PATIENT REGISTRY?

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Chronic neutropenia (CN) corresponds with absolute count of neutrophils below 1.8 × 10^9/L in last three months. Besides, neutropenia that persists more than three months and in absence of underlying diseases, chemical components, irradiation, use of particular drugs, inflammation, is defined as chronic idiopathic neutropenia (CIN). There are three types of CN - severe, moderate and mild, with or without extra-hematopoietic manifestations. The aim of this report was to define a stratified approach toward patient with hypothesis of CIN, including timely patient recognition with consequent follow-up and actions afterwards based on recommendation from EuNet INNOCHRON- European Network for Innovative Diagnosis and Treatment of Chronic Neutropenias. Case presented a 40-year-old female who went to the Hematology Department of the University Clinical Center Niš for regular health condition and blood count control. Since she was 21, she has been self-monitoring her neutropenia, which occurred immediately after first child birth. Laboratory parameters and peripheral blood smear values were within physiological range. Performed immune assays excluded the immune background of neutropenia. In the next step, we excluded viral infections as a cause of neutropenia with particular serological tests. Also, a bone marrow aspiration was performed with results in physiological range.

Chronic neutropenia can be associated with serious health complications. The Severe Chronic Neutropenia International Registry (SCNIR) is a global organization dedicated to finding the causes, consequences and best treatments for severe CN. Similar to other European countries, we suggest the introduction of defined approach to diagnosis, registration and monitoring of chronic neutropenia patients in Serbia through Register.

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Key words: *chronic neutropenia, chronic idiopathic neutropenia, diagnostic approach, register*

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Introduction

Chronic neutropenia (CN) is defined as reduced absolute neutrophil count below 1.8 × 10^9/L during three or more months. These latter categories of CN, include benign and uncomplicated forms of the disease but also pre-myeloid dysplastic syndrome cases, associated or not with clonal hematopoiesis (1). In addition, if low count of neutrophils persisted in absence of underlying diseases, chemical components, irradiation, use of drugs, infectious disease, inflammation, autoimmune diseases and malignancies, neutropenia can be defined as chronic idiopathic neutropenia (CIN) (1, 2).

In clinical practice there are patients with long lasting neutropenia, without any related health problems or serious life-threatening conditions. In addition, CIN can express extra-hematological symptoms, depending on the severity of neutropenia and characteristics of patients (2, 3).

Moderate to severe bacterial infections are common in patients with CN (3). However, infections are more common in patients with severe CIN when the absolute neutrophils count is less than 0.5 × 10^9/L. In that case, this hematological disease is often accompanied with extra-hematological disorders, fever, chronic inflammation of the oropharynx as well as severe infections (4, 5).

The treatment of patients with CNP depends on the type and severity of the underlying disease. There is currently no specific therapy for severe CN, but mostly patients are treated symptomatically. The approach towards patients may consist of a simple follow-up of uncomplicated cases, but also it may require the administration of granulocyte colony stimulating factor (G-CSF), antimicrobial and immunomodulatory agents, or haemopoietic stem cell (HSC) transplantation or even experimental gene-based interventions using CRISPR-Cas9 technology (5). More recently, G-CSF has been used in therapy to accelerate the growth and maturation of myeloid cells, especially neutrophils (5, 6).

An idea of registration of CIN patients and systematization of their data is realized in numerous European countries (Italy, Greece, France, etc). The registry is required form for adequate collection and analysis of clinical data, comparison in relation to geographical, ethnic, and social characteristics, diagnosis and treatment of all patients with CN. Therefore, an adequate approach to those patients can be achieved through introduction of worldwide registry of CN patients. The Severe Chronic Neutropenia International Registry (SCNIR) is a global organization dedicated to finding the causes, consequences and best treatments for severe CN. The SCNIR opened in 1994 after researchers discovered that the hematopoietic growth factor called granulocyte colony stimulating factor (G-CSF) is an effective treatment for these patients.

Regarding the needs of clinicians and patients, central collecting of data in National Registry has to be established in our country too. It is a great challenge to introduce and implement in clinical practice an adequate Registry suitable for the use by physicians. The establishment of the registry of CN patients in Serbia could help and facilitate the monitoring, diagnosis and treatment (1, 5).

Therefore, we presented a case which included steps in an approach towards diagnosis of CIN in one patient. Hence, the aim was to define a stratified approach towards CIN, including timely patient recognition with consequent follow-up and actions afterwards. Also, the case report represented the importance of introduction registry data regarding optimal monitoring and risk management in CN.

Case report

A 40-year-old female patient attended the Hematology Clinic, University Clinical Center Niš for regular monitoring of health condition and blood count control. Since she was 21, she has been following up neutropenia, which occurred immediately after giving birth for the first time. After a detailed anamnesis, we determined that the patient did not have a positive family history and comorbidities, except anemia, which persisted from first child birth, too.

The lastest results of the patient's blood analysis showed the presence of neutropenia which was similar to the earlier control. The neutrophils count was low during the previous years.

In the lastest visit, laboratory parameters showed:

- white blood count 2.2 x 10^9/L (3.9-10 x 10^9/L),
- neutrophils 1.2 x 10^9/L (1.6-7 x10^9/L),
- eosinophil 0.1 x 10^9/L (0.1 – 0.6 x 10^9/L),
- lymphocytes 0.8 x 10^9/L (0.8-5 x 10^9/L),
- monocytes 0.1 x 10^9/L (0.1-1 x 10^9/L),
- basophils 0 x 10^9/L (0-0.2 x 10^9/L),
- red blood count (5.13 10^{12}/L),
- hemoglobin (111 g/L),
- hematocrit (0.367 L/L),
- platelets (176 x 10^9/L)

were in physiological range.

As a second step, a peripheral blood smear was performed, whereas cells did not show any pathological changes. Further, we run immune analysis, serum immunoglobulin levels and ANA and Anti DNA screen aimed to exclude the immune background of neutrophils account disorder. Results were within the reference values:

- IgG 9.03 g/L (7.0 - 16 g/L),
- IgA 0.95 g/L (0.7 - 4 g/L),
- IgM 1.97 g/L (0.4 - 2.3g/L),
- anti dsDNK 6.2 (< 25),
- ANA 0.1 (< 1).

Direct and indirect COOMBS tests gave negative results too. Biochemical status of patient showed normal range of all biochemical parameters, although iron was low:

- serum glucose 5.7 mmol/L,
- urea 4.0 mmol/L,
- creatinine 73.7 mmol/L,
- total bilirubin 8.2 μmol/L,
- total bilirubin 1.5 μmol/L,
- total proteins 66.4 g/L,
- albumins 44 g/L,
- AST 15 U/L,
- ALT 15 U/L,
- folate 13.6 ng/mL,
- vitamin B12 522 pmol/L,
- hTSH 1.4188 mU/L.

In order to exclude viral infections as a cause of neutropenia, serological tests were performed, but the results were negative (HBs Ag, HCV Ag, CMV Ag, EBV Ag, Parvovirus, and HIV Ag).

A bone marrow aspiration was performed to remove the suspicion of a malignancy or other hematological disease presented with a decreased number of neutrophils. The results were as follows: myeloblast 0%, promyelocytes 6%, myelocytes 4%, neutrophil granulocytes 10%, metamyelocytes 9 %, no segments granulocytes 3%, segments granulocytes 25%, eosinophil granulocytes 2%, basophil granulocytes 2%, monocytes 6%, lymphocytes 14%, normoblasts 19 % .

The final step was the analysis of dietary habits. Nevertheless, malnutrition was not present (BMI was 25 kg/m^2) and hypoalbuminemia as well, but the analysis showed lack of proteins, particularly from red meat and sufficient carbohydrates.

Discussion

The diagnosis of CIN has to be made on the basis of the absolute number of neutrophils and the exclusion of other underlying diseases. Also, there is a need to collect as more information as you can, regarding family history, medical treatments, drugs, health status of patients. Nowadays the greatest challenge is to timely provide useful "point of care" information to clinicians and patients (5). The possibility of congenital neutropenia in an adult patient should be kept in mind whenever a patient presents with a life-long history of (severe) neutropenia even in the absence of infection, also if it has characteristic recurrent oral lesions and periodontal disease or features of organ malformation.

Frequently, the base of congenital neutropenia is disorders of neutrophil production associated with mutations in more than 20 recognized genes and more still unknown genetic aberrations. Since the results of these genetic changes are impaired neutrophil differentiation and/or survival, varying degree of propensity to malignity and frequent extra hematopoietic disorders (1, 6). In addition, ethnic variations in genetic polymorphisms may lead to lower number of neutrophils.

On the other hand, acquired CNP encompass diverse disease entities which are based on unknown pathogenic mechanisms or cellular immune processes of antibodies against neutrophils. With or without clonal hematopoiesis, every CN has to be timely recognized and requires close monitoring aimed at avoiding complications.

Severe CIN is a very rare disease that occurs without a known and clear cause, rather in adults than pediatric population, mostly in women. Very little is still known about its nature.

Studies showed that mutations on the ELANE gene showed changes on HAKS1, GFI1, WAS, CSF3R or G6PC3 genes especially related to severe neutropenia (7, 8). Most of *de novo* mutations and transmission can be autosomal dominant, recessive or X-linked. In previous studies, there is a strong evidence of the frequency of those mutations, and their dependence on the ethnicity of the patients (8, 9). Furthermore, a mutation in the JAGN1 gene has been identified in some patients, which disrupts further signaling at G-CSF receptors and explains the non-response to therapy with recombinant human G-CSF (10).

In adults, the use of particular drugs can cause the adverse effects and decreased count of neutrophils (antiinfectives, antipsychotics, anti-thyroids drugs) (11-13).

The variety of causes and symptoms of neutropenia makes diagnosis, monitoring and treatment of patients very difficult. For each follow-up, it is necessary to take a good anamnesis with detailed patient data, hematological, clinical data, medication list and other relevant medical and non-medical information. According to recommendation, detailed anamnesis was taken in the reported case. Our patient did not show any possibility of low neutrophil level explanation.

In addition, it is known that chronic or acute viral infections such as hepatitis, HIV, cytomegalovirus (CMV) or influenza may be associated with neutropenia. The performed tests in our case were negative, so we excluded virus-induced neutropenia.

Further, our patient had a healthy bone marrow aspiration finding. Since the bone marrow aspiration is currently without a pathological substrate, we plan to further monitor the patient in terms of periodic testing of blood smears.

The defined stepped approach includes investigation of autoimmune base on neutropenia. Regarding this step we performed immunological tests and excluded autoimmune disease. Testing of anti-neutrophil antibodies is performed more frequently in the pediatric population of patients, while in adults this type of testing is not very useful. In adults, an increased level of polyclonal gamma globulins supports the diagnosis of autoimmune neutropenia (14, 15).

As a final step we collected dietary habits data. Previous studies showed a high prevalence of anemia and neutropenia in a population of young American and Northern European patients with anorexia. Hematological abnormalities in patients with anorexia or diet were strictly related to the duration of the diseases, in particular CN (16). There are numerous studies on hematological disorders in anorexia or protein-deficient diet that prove an increased prevalence of anemia, leukopenia and thrombocytopenia. Lambert et al. explained the relationship between the index of total body fat mass and bone marrow consumption and the lower number of erythrocytes, leukocytes, neutrophils and platelets (17).

Caloric malnutrition also may lead to neutropenia, but it is usually mild. In addition, patients with folate or vitamin B12 deficiency can be exposed to low neutrophils number, too. For that reason, we control levels of vitamin B12 and folate in serum, and investigate dietary habits. Low calorie diet not only decreases the reserve of leukocytes in bone marrow but also leads to degeneration of leucocytes during their transition from BM to peripheral blood (18, 19).

In patients undergoing neutropenia treatment decisions should be patient-centered and based on the clinical presentation of the disease (20, 21). Results show an association between neutropenia and level of leptin and adiponectin. Leptin is a hormone that regulates nutrition and is produced from adipocytes (22). Leptin acts as a signaling molecule that transmits energy from food to the immune and neuroendocrine systems. Children with congenital leptin deficiency have reduced lymphocyte numbers and show an increased risk for infections and death caused by infection in childhood (23). We hypothesized a possible association between the type of diet and changes in adipokines, and will focus on intensive monitoring of diet-related behaviors and related biochemical monitoring.

Aimed to find link between leptin, adiponectin and number of neutrophyls, our next step will include quantification of serum leptin in patients with CIN. Also, we are planning genetic screening of patients which may help to illumination of neutropenia.

Creating the registry of patients with neutropenia is a big challenge for many reasons, particularly for better approach, risk control and achieving optimal health outcomes. There are already high-quality registries for neutropenia in Europe (24). The SCNIR is a voluntary organization, with over than 25 years of existence, supported primarily by government grants and private gifts. Participation benefits patients, their families and their physicians by providing up-to-date information about severe CN and its treatment options.

It is important to introduce this registry in Serbia similar to other countries. The registry data would help physicians in diagnosis, monitoring and treatment of patients with neutropenia.

The prognosis of patients with CNP is closely correlated to the underlying pathogenesis, the degree of neutropenia and the propensity for leukemic transformation. Accurate diagnosis is mandatory to avoid omissions and risk stratification as well as treatment choice. Hence, we can suggest to establish the register of chronic neutropenia patients in Serbia. (20, 24, 25).

Conclusion

Based on the results of performed examinations and analyses, we assumed chronic idiopathic neutropenia in our patients. The stepped approach gives to hematologists an opportunity to make right choice in monitoring and treatment. The registry of this and similar cases may provide their comparison and consequently better understanding, timely diagnosis and treatment of CNI.

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Prikaz bolesnika

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STEPENOVANI DIJAGNOSTIČKI POSTUPAK ZA HRONIČNU NEUTROPENIJU: DA LI JE VREME ZA REGISTAR U SRBIJI?

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Hronična neutropenija javlja se kada, minimum, tri meseca broj neutrofila bude ispod $1,8 \times 10^9$/L. Ukoliko neutropenija perzistira duže od tri meseca, bez prisutne druge bolesti, izlaganja hemijskim supstancama, zračenju, infekcijama, inflamacijama, različitim lekovima, koji mogu da uslove nastanak neutropenije, onda govorimo o hroničnoj idiopatskoj neutropeniji. Prema težini, razlikujemo ozbiljne, umereno ozbiljne i blage neutropenije, koje mogu biti sa ekstrahematološkim manifestacijama ili bez njih.

Cilj ovog rada je da prezentuje definisani stepenovani pristup kod bolesnika sa pretpostavljenom dijagnozom hronične neutropenije, uključujući pravovremeno prepoznavanje bolesti i kontinuirano praćenje bolesnika, u skladu sa preporukama Evropske mreže za inovativnu dijagnozu i tretman hronične neutropenije (European Network for Innovative Diagnosis and Treatment of Chronic Neutropenias). Slučaj prikazuje četrdesetogodišnju ženu, koja se javila Hematološkoj klinici Univerzitetskog kliničkog centra u Nišu radi redovne kontrole zdravlja i krvne slike. Naime, kod bolesnice su u 21. godini, nakon prvog porođaja, uočene neutropenija i anemija. Laboratorijski parametri, izuzev broja neutrofila, bili su u fiziološkim granicama. Periferni razmaz krvi takođe nije odstupao od fiziološkog izgleda. Rezultati imunoloških analiza bili su u okviru referentnih vrednosti. Zatim, sprovedeni su testovi, koji su pokazali da ne postoji virusna infekcija koja bi mogla da bude uzrok smanjenog broja neutrofila. Punkcija koštane srži pokazala je uredan nalaz. Hronična neutropenija može biti udružena sa nizom ozbiljnih zdravstvenih komplikacija. Stoga, sugerišemo uvođenje definisanog pristupa identifikaciji, registrovanju, praćenju bolesnika sa hroničnom neutropenijom kroz centralni registar, slično drugim evropskim zemljama.

Acta Medica Medianae 2022;61(1):85-90.

Ključne reči: *hronična idiopatska neutropenija, dijagnostički postupak, registar*

JEDINSTVENI KRITERIJUMI ZA OBJAVLJIVANJE NAUČNIH RADOVA U BIOMEDICINSKIM ČASOPISIMA

Ideja o postavljanju jedinstvenih kriterijuma za objavljivanje radova u časopisima za biomedicinske nauke iskristalisana je 1978. godine u Vankuveru. Ovi kriterijumi za rukopise, uključujući pravila za pisanje bibliografije, prvi put su objavljeni 1979. godine. Vankuverska grupa je vremenom prerasla u Međunarodni komitet urednika medicinskih časopisa — International Committee of Medical Journal Editors (ICMJE). Trenutno je na snazi peta revizija kriterijuma za objavljivanje radova u biomedicinskim časopisima, doneta 1997. godine.

Kriterijumi za citiranje i navođenje referenci

Reference se obeležavaju arapskim brojevima u zagradama, pri čemu se reference obele-žavaju brojevima onim redosledom kojim se pojavljuju u tekstu. Reference citirane jedino u tabelama ili legen-di moraju se obeležiti brojem u skladu sa redosledom pojavljivanja u tekstu.

Naslove medicinskih časopisa treba pisati u skraćenom obliku onako kako su navedeni u poglavlju **List of Journals Indexed in Index Medicus**. Lista skraćenih naziva medicinskih časopisa objavljuje se svake godine u januarskom broju ***Index Medicusa***. Ova lista se takođe može naći na adresi **www.nlm.nih.gov**

Izbegavati upotrebu apstrakata kao referenci, već koristiti samo izvorne tekstove (*in extenso* članci). Reference koje se odnose na radove koji su prihvaćeni, ali još nisu odštampani, treba označiti sa "u štampi", pri čemu autor mora imati pismeno odobrenje da citira takve radove i da priloži pismeni dokaz da je citirani rad prihvaćen za štampu. Informacije iz rukopisa koji nisu prihvaćeni za štampanje mogu se citirati u tekstu kao "neobjavljeni rezultati", ali sa pismenom dozvolom autora.

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Vega KJ, Pina I, Krevsky B. Heart transplantation is associated with an increased risk for pancreatobiliary disease. Ann Intern Med 1996; 124(11):980-3.

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The Cardiac Society of Australia and New Zealand. Clinical exercise stress testing. Safety and performance guidelines. Med J Aust 1996;164:282-4.

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Phillips SJ, Whisnant JP. Hypertension and stroke. In: Laragh JH, Brenner BM, editors. Hypertension: pathophysiology, diagnosis, and management. 2nd ed. New York: Raven Press; 1995. p. 465-78.

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Službeni izveštaji (Issued by funding / sponsoring agency):

Smith P, Golladay K. Payment for durable medical equipment billed during skilled nursing facility stays. Final report. Dallas (TX): Dept. of Health and Human Services (US), Office of Evaluation and Inspections; 1994 Oct. Report No.: HHSIGOEI69200860.

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Field MJ, Tranquada RE, Feasley JC, editors. Health services research: work force and educational issues. Washington: National Academy Press; 1995. Contract No.: AHCPR282942008. Sponsored by the Agency for Health Care Policy and Research.

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Kaplan SJ. Post-hospital home health care: the elderly's access and utilization [dissertation]. St. Louis (MO): Washington Univ.; 1995.

Druge vrste publikovanog materijala

Neobjavljeni materijal

19. U štampi (In press)

Leshner AI. Molecular mechanisms of cocaine addiction. N Engl J Med. In press 1996.

Elektronski zapisi

20. Internet članak u elektronskom formatu

Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis (serial online) 1995 Jan-Mar "cited 1996 Jun 5"; 1(1)(24 screens). Available from: URL: http://www.cdc.gov/ncidod/EID/eid.htm

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Hemodynamics III: the ups and downs of hemodynamics (computer program). Version 2.2. Orlando (FL): Computerized Educational Systems; 1993.

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