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DRUG-DRUG INTERACTIONS IN ACUTE CORONARY SYNDROME PATIENTS: SYSTEMATIC REVIEW

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ABREVIATIONS

ACE - Angiotensin-converting enzyme
ACS - Acute coronary syndrome
AHA - American Heart Association
CCB(s) - Calcium channel blocker(s)
CYP - Cytochrome P450
DAPT - Dual antiplatelet therapy
DDI(s) - Drug-drug interaction(s)
ESC - European Society of Cardiology
GP - Glycoprotein
NNH - Number needed to harm
NSAID(s) - Non-steroidal anti-inflammatory drug(s)
NSTEMI - Non-ST-segment elevation myocardial infarction
PCI - Percutaneous coronary intervention
PPI(s) - Proton pump inhibitor(s)
rTPA - Recombinant tissue-type plasminogen activator
SSRI(s) - Selective serotonin reuptake inhibitor(s)
STEMI - ST-segment elevation myocardial infarction

ABSTRACT

Drug-drug interaction (DDI) is defined as a clinically significant change in the exposure and/or response to a drug caused by co-administration of another drug which may result in a precipitation of an adverse event or alteration of its therapeutic effects. The aim of this systematic review was to provide an overview of DDIs that were actually observed or evaluated in acute coronary syndrome (ACS) patients with particular focus on DDIs with clinical relevance. Electronic searches of the literature were conducted in the following databases: MEDLINE, EBSCO, Scopus, Google Scholar and SCIndeks. A total of 117 articles were included in the review. This review showed that ACS patients can be exposed to a variety of DDIs with diverse outcomes which include decreased efficacy of antiplatelet drugs, thrombolytics or anticoagulants, increased risk of bleeding, rhabdomyolysis, hepatotoxicity, adverse effects on cardiovascular system (e.g. QT interval prolongation, arrhythmias, excessive bradycardia, severe hypotension), serotonin syndrome and drug-induced fever. Majority of the DDIs involved antiplatelet drugs (e.g. aspirin, clopidogrel and ticagrelor). Evidence of some of the reported DDIs is inconclusive as some of the studies have shown conflicting results. There is a need for additional post-marketing and population-based studies to evaluate the true effects of disease states and other factors on the clinical outcomes of DDIs. Clinicians should be attentive to the potential for DDIs and their associated harm in order to minimize or, if possible, avoid medication-related adverse events in ACS patients.

Keywords: *Drug-drug interactions, acute coronary syndrome, review.*

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INTRODUCTION

Acute coronary syndrome (ACS) is a high-risk manifestation of coronary artery disease associated with the rupture of an atherosclerotic plaque and partial or complete thrombosis of the coronary artery (1-3). Clinical manifestations of ACS include ST-segment elevation myocardial infarction (STEMI), non-ST-segment elevation myocardial infarction (NSTEMI) and unstable angina pectoris (4). Initial and long-term treatment of ACS involves multiple medications such as antiplatelet drugs, anticoagulants, analgesics, nitrates, beta-blockers, angiotensin-converting enzyme (ACE) inhibitors and lipid-lowering therapy (5-7). Additionally, ACS patients often have other comorbidities requiring long-term treatment which put them in high risk of drug-drug interactions (DDIs) (5-7).

DDI is defined as a clinically significant change in the exposure and/or response to a drug caused by co-administration of another drug which may result in a precipitation of an adverse event or alteration of its therapeutic effects (8, 9). Potential DDI is defined as a co-prescription or co-administration of two drugs known to interact (8). Studies have shown that about 99-100% of ACS patients are exposed to at least one potential DDI during different phases of treatment (10, 11). DDIs may have important implications in the setting of ACS, particularly because they may affect the balance of preventing thrombotic events versus the potential for increased bleeding risk (12). Although, there are many interaction checkers available, they have limited clinical utility (13), so it is important that prescribers are aware of the potential DDIs and associated harm in order to minimize or, if possible, avoid medication-related adverse events, decrease risk of hospitalization or longer hospital stay (14).

Previous systematic reviews have either focused only on the DDIs in the setting of ACS and dual antiplatelet therapy (DAPT) (5) or on the DDIs that have the potential to affect procedural outcomes of cardiovascular catheterizations and interventions with only a brief discussion of other DDIs that are highly likely in the coronary artery disease patients (12). Therefore, the aim of this systematic review was to provide an overview of DDIs that were actually observed or evaluated in ACS patients with particular focus on DDIs with clinical relevance.

METHODS

Electronic searches of the literature were conducted in the following online databases: MEDLINE available at https://www.ncbi.nlm.nih.gov/pubmed/, EBSCO available at https://ezproxy.nb.rs:2443/kobson.82.html, Scopus available at https://www.scopus.com, Google Scholar available at https://scholar.google.com/ and Serbian Citation Index (SCIndeks) available at https://scindeks.ceon.rs/.

The search and evaluation of the retrieved articles was performed from April 1st to April 17th, 2019. Details of the search strategies for each database are provided in Table 1. Inclusion criteria were: clinical trials or observational studies or case series or case reports which included patients with one of the manifestations of the ACS (unstable angina pectoris, myocardial infarction with or without ST-segment elevation) and which aimed to investigate or report a clinically relevant DDI. Exclusion criteria were: systematic reviews, meta-analysis, expert opinions, guidelines, “in vitro” studies, studies conducted on animals. There were no restrictions on publication date, format or language in the search strategy. The references of the retrieved articles were also searched for further similar studies.

A clinically relevant DDI for the purpose of this study was defined as a DDI that could be associated with any of the following: clinical or laboratory signs of toxicity causally associated with the concomitant administration of the combination of drugs (as estimated by the authors of the respective study); loss of efficacy of one or both drugs from the drug combination believed to be caused by an interaction between them (as estimated by the authors of the respective study); or change of the drug therapy because of the recognition of a DDI with adverse consequences.

Table 1. Search strategy for databases

Database	Search strategy
MEDLINE	(("drug interactions"[MeSH Terms] OR ("drug"[All Fields] AND "interactions"[All Fields]) OR "drug interactions"[All Fields] OR ("drug"[All Fields] AND "interaction"[All Fields]) OR "drug interaction"[All Fields]) OR ("drug interactions"[MeSH Terms] OR ("drug"[All Fields] AND "interactions"[All Fields]) OR "drug interactions"[All Fields]) OR ("drug interactions"[MeSH Terms] OR ("drug"[All Fields] AND "interactions"[All Fields]) OR "drug interactions"[All Fields] OR ("drug"[All Fields] AND "drug"[All Fields] AND "interaction"[All Fields]) OR "drug drug interaction"[All Fields]) OR (drug-drug[All Fields] AND interactions[All Fields]) OR (drug[All Fields] AND co-administration[All Fields])) AND (("acute coronary syndrome"[MeSH Terms] OR ("acute"[All Fields] AND "coronary"[All Fields] AND "syndrome"[All Fields]) OR "acute coronary syndrome"[All Fields]) OR ("st elevation myocardial infarction"[MeSH Terms] OR ("st"[All Fields] AND "elevation"[All Fields] AND "myocardial"[All Fields] AND "infarction"[All Fields]) OR "st elevation myocardial infarction"[All Fields] OR "stemi"[All Fields]) OR ("non-st elevated myocardial infarction"[MeSH Terms] OR ("non-st"[All Fields] AND "elevated"[All Fields] AND "myocardial"[All Fields] AND "infarction"[All Fields]) OR "non-st elevated myocardial infarction"[All Fields] OR "nstemi"[All Fields]) OR ("angina, unstable"[MeSH Terms] OR ("angina"[All Fields] AND "unstable"[All Fields]) OR "unstable angina"[All Fields] OR ("unstable"[All Fields] AND "angina"[All Fields] AND "pectoris"[All Fields]) OR "unstable angina pectoris"[All Fields]) OR "myocardial infarction"[ti]) NOT (("review"[Publication Type] OR "review literature as topic"[MeSH Terms] OR "review"[All Fields]) OR ("meta-analysis"[Publication Type] OR "meta-analysis as topic"[MeSH Terms] OR "meta-analysis"[All Fields]) OR "in vitro"[All Fields] OR ("animals"[MeSH Terms:noexp] OR animal[All Fields]))
EBSCO	SU (((drug interaction) OR (drug interactions) OR (drug-drug interaction) OR (drug-drug interactions) OR (drug co-administration)) AND ((acute coronary syndrome) OR (STEMI) OR (NSTEMI) OR (unstable angina pectoris) OR (TI "myocardial infarction")) NOT (review OR meta-analysis OR "in vitro" OR animal)) OR AB (((drug interaction) OR (drug interactions) OR (drug-drug interaction) OR (drug-drug interactions) OR (drug co-administration)) AND ((acute coronary syndrome) OR (STEMI) OR (NSTEMI) OR (unstable angina pectoris) OR (TI "myocardial infarction")) NOT (review OR meta-analysis OR "in vitro" OR animal))
Scopus	((drug AND interaction) OR (drug AND interactions) OR (drug-drug AND interaction) OR (drug-drug AND interactions) OR (drug AND co-administration)) AND ((acute AND coronary AND syndrome) OR (stemi) OR (nstemi) OR (unstable AND angina AND pectoris) OR TITLE ("myocardial infarction")) AND NOT (review OR meta-analysis OR "in vitro" OR animal)
SCIndeks	(ARTAK: ((drug interaction) OR (drug interactions) OR (drug-drug interactions) OR (drug-drug interaction) OR (co-administration) OR (combined therapy)) AND ((acute coronary syndrome) OR (STEMI) OR (NSTEMI) OR (myocardial infarction) OR (unstable angina pectoris)))
Google Scholar	drug interaction drug-drug interactions coadministration "acute coronary syndrome" OR STEMI OR NSTEMI OR "unstable angina" OR "myocardial infarction" -review -"meta-analysis" -animal -"in vitro"

RESULTS

Results of the literature search are shown in Figure 1. A total of 117 articles were included in this systematic review. An overview of drug combinations involved in DDIs in ACS patients and their possible outcomes is provided in Table 2.

DDIs leading to decreased efficacy of antiplatelet drugs

Morphine and antiplatelet drugs. Morphine use in ACS patients was associated with a delayed onset of action of oral antiplatelet agents: clopidogrel (15), prasugrel (15-18) and ticagrelor (16, 17, 19-21) probably due to a delay in drug absorption as a result of inhibition of gastrointestinal motility and gastric emptying. Also, STEMI patients who received morphine with ticagrelor had a less favorable ischemic outcome (22). In addition, morphine co-administration in STEMI patients who received DAPT (aspirin with clopidogrel or ticagrelor), was associated with impaired thrombotic status at presentation, reduced spontaneous myocardial reperfusion (before primary percutaneous coronary intervention (PCI)) and larger infarct size (23).

Acid-suppressive drugs and antiplatelet drugs. Clopidogrel is a prodrug which requires conversion via cytochrome P450 (CYP) system (dominantly by CYP3A4, CYP3A5 and CYP2C19) before the desired clinical effect is achieved (24-35). Drugs that interfere with or are co-metabolized via those enzymes might decrease its antiplatelet effect and increase the risk of thrombotic events (24-35). Proton pump inhibitors (PPIs) are metabolized by CYP enzymes, leading to a potential inhibition of CYP2C19 (24-35).

Figure 1. Selection of studies

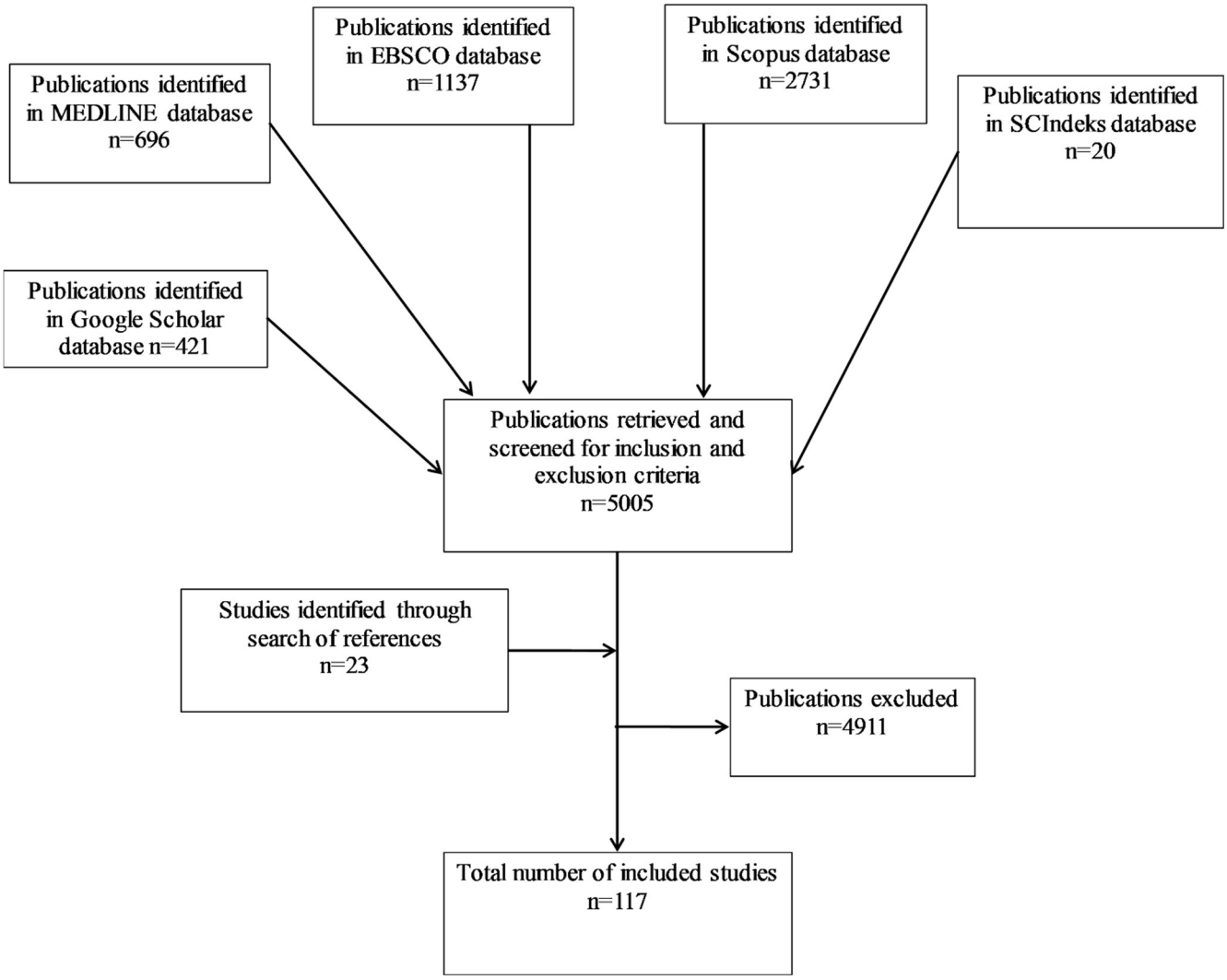

Table 2. An overview of drug combinations involved in DDIs and their possible outcomes in ACS patients

Drug combination	Description
Amiodarone + antiretroviral drugs	Acquired long QT syndrome and torsades de pointes cardiac arrest (122).
Amiodarone + sofosbuvir + daclatasvir	Syncope, extreme bradycardia and cardiac asystole (123).
Anticoagulants + thrombolytics	Increased risk of bleeding (99).
Antiplatelet drugs (combinations)	Increased risk of bleeding (82-89).
Antiplatelet drugs + anticoagulants	Increased risk of bleeding (91-97).
Antiplatelet drugs + anticoagulant + thrombolytic	Increased risk of bleeding (100).
Antiplatelet drugs + morphine	Delayed onset of action of oral antiplatelet drugs (e.g. clopidogrel (15), prasugrel (15-18), ticagrelor (16, 17, 19-21)), less favorable ischemic outcome with ticagrelor (22), impaired thrombotic status at presentation (aspirin with clopidogrel or ticagrelor) (23).
Antiplatelet drugs + NSAID + corticosteroid	Increased risk of bleeding (101)
Antiplatelet drugs + SSRIs	Increased risk of bleeding (102)
Antiplatelet drugs + thrombolytics	Increased risk of bleeding (98, 99).
Antithrombotic therapy + NSAIDs	Increased risk of excess thrombotic events and bleeding (62).

Drug combination	Description
Aspirin + ACE inhibitors	Antagonized effects of ACE inhibitors (125) (no negative interaction in (126-129)).
Aspirin + ibuprofen	Increased rate of recurrent acute myocardial infarction (61) (not confirmed in (63)).
Aspirin + PPIs	Increased risk of an adverse cardiovascular event (59) or enhanced antiplatelet effects of enteric-coated aspirin (60).
Carvedilol + dobutamine	Severe hypotension (118).
Clonidine + duloxetine + atorvastatin	Unexplained high fever (131).
Clopidogrel + ACE inhibitors	Increased risk of bleeding (107).
Clopidogrel + aprotinin	Reduced antiplatelet effect of clopidogrel (64).
Clopidogrel + atorvastatin	Hepatotoxicity (68, 116).
Clopidogrel + calcium channel blockers	Increased risk of ACS re-hospitalization in ACS patients not compliant to guideline-recommended secondary prevention drugs (65) (interaction not confirmed in (66, 67)).
Clopidogrel + cimetidine	Increased risk of re-infarction (28).
Clopidogrel + PPIs	Reduced antiplatelet effects (36-42) (not confirmed for all PPIs in (39, 43-47)) and increased risk of adverse cardiovascular events (24-35, 48, 49) (not confirmed for all PPIs in (40, 43, 48, 50-56)).
Escitalopram + lansoprazole	QT interval prolongation and ventricular fibrillation (117).
Escitalopram + methylene blue	Serotonin syndrome (130).
Metoprolol + cocaine	Exacerbated coronary vasoconstriction, crushing substernal chest pain, pulseless electrical activity and death (119).
Metoprolol + CYP2D6 inhibitors (e.g. paroxetine, terbinafine)	Excessive bradycardia or severe hypotension (120, 121).
Mexiletine + theophylline	Ventricular arrhythmias (124).
Nitroglycerin + heparin	Heparin resistance and attenuated anticoagulant effect (78, 79) (not confirmed in (80, 81)).
Nitroglycerin + rTPA	Reduced thrombolytic efficacy of rTPA (76, 77).
Rivaroxaban + simvastatin	Bleeding (103).
Simvastatin + diltiazem	Rhabdomyolysis (115).
Statins + fibrates	Rhabdomyolysis (113, 114).
Ticagrelor + antiepileptic CYP3A inductors (e.g. phenytoin, carbamazepine, phenobarbital)	Reduced antiplatelet effects of ticagrelor (75).
Ticagrelor + cyclosporine	Increased exposure to both drugs with increased risk of bleeding and increased cyclosporine trough concentration (105, 106).
Ticagrelor + statins (e.g. atorvastatin, simvastatin, rosuvastatin)	Rhabdomyolysis (108-112).
Warfarin + ritonavir	Removal of ritonavir led to the increase in warfarin effect (104).
Abbreviations: ACE - Angiotensin-converting enzyme; NSAID(s) - Non-steroidal anti-inflammatory drug(s); PPI(s) - Proton pump inhibitor(s); rTPA - Recombinant tissue-type plasminogen activator; SSRI(s) - Selective serotonin reuptake inhibitor(s).	

However, results of the clinical studies have shown conflicting results regarding PPIs influence on antiplatelet effects of clopidogrel and risk of adverse cardiovascular events in ACS patients. There are some studies which suggest that pantoprazole (36), esomeprazole (37) and omeprazole (38-40) may reduce antiplatelet effect of clopidogrel, and that PPIs as a group may reduce antiplatelet effects of thienopyridines (clopidogrel or ticlopidine) (41). One study showed that the degree of the interaction between clopidogrel and PPIs is not homogeneous within the class of PPIs and is less marked with pantoprazole than with omeprazole (42). On the other hand, some studies suggested that pantoprazole (39), lansoprazole (43), esomeprazole (44), rabeprazole (44) and omeprazole (45, 46) do not reduce antiplatelet effect of clopidogrel and that pantoprazole does not interfere with antiplatelet effect of $P2Y_{12}$ inhibitors (clopidogrel, ticagrelor, prasugrel) (47). In numerous studies concomitant use of clopidogrel and PPIs as a group (24-35), as well as some of the individual PPIs (omeprazole (48, 49), esomeprazole (49)), was associated with a significant increase in risk of adverse cardiovascular events. In one of these studies, a similar association was observed between cardiovascular events and PPI use during ticagrelor treatment and with other non-PPI gastrointestinal treatment, suggesting that the association between PPI use and adverse events may be due to confounding, with PPI use more of a marker for, than a cause of, higher rates of cardiovascular events (33). Also, in one of the studies use of PPIs did not significantly affect the comparative effectiveness of prasugrel versus clopidogrel (34). In one study risk of rehospitalization among PPI users varied by CYP2C19 genotype, with the greatest increase in risk observed among carriers of the CYP2C19*17 allele (31). However, some studies didn't show significant influence of PPIs as a group (50-54) or individual PPIs (lansoprazole (43, 48),

omeprazole (40, 55, 56), esomeprazole (48), rabeprazole (48), pantoprazole (48)) on risk of adverse cardiovascular events in patients with ACS treated with clopidogrel. Also, in one of the studies pantoprazole did not have significant effects on the incidence of hospital-acquired pneumonia and 30-day mortality in ACS patients treated with clopidogrel (57). Prasugrel antiplatelet effect was also not significantly affected by concomitant administration of PPIs (58). In one study aspirin, was associated with increased risk of an adverse cardiovascular event (recurrent myocardial infarction, stroke, or cardiovascular death) when used concomitantly with PPIs possibly due to modification of intragastric pH which may reduce lipophilicity and bioavailability of aspirin (59). However, another study showed contradictory results: enhanced antiplatelet effects of enteric-coated aspirin in patients with ACS undergoing PCI (60). In this study methacrylic acid enteric-coated preparation of aspirin was used (60). Methacrylic acid is stable in acid solutions which allow the drug to pass intact through the upper gastrointestinal tract (60). However, gastric juice alkalized by the addition of pantoprazole may cause earlier destabilization of the methacrylic acid sheath allowing aspirin to reach the duodenum already in its soluble form, thus assuring its rapid absorption (60). Concomitant use of clopidogrel and cimetidine, H2-receptor antagonist which is a CYP2C19 inhibitor, was associated with increased risk of re-infarction in ACS patients in one study (28).

Non-steroidal anti-inflammatory drugs (NSAIDs) and antiplatelet drugs. Aspirin and ibuprofen share a common docking site in the cyclooxygenase-1 enzyme, so ibuprofen may competitively inhibit aspirin's antiplatelet effect (61). In one study there was a trend towards an increasing rate of recurrent acute myocardial infarction with longer duration of ibuprofen exposure, suggesting that regular, but not intermittent, use of ibuprofen may abrogate the benefits of aspirin in secondary prevention of acute myocardial infarction (61). In another study, among patients receiving antiplatelet therapy after myocardial infarction, the use of NSAIDs was associated with increased risk of excess thrombotic events, even after short-term treatment (62). Aspirin and ibuprofen did not adversely interact in a cohort of elderly patients discharged after myocardial infarction (63).

Clopidogrel and aprotinin. Findings of one study suggested that aprotinin may reduce antiplatelet effect of clopidogrel in ACS patients (64). Aprotinin increased ADP induced platelet aggregation from 84% to 94% corresponding to a median decrease in relative platelet inhibition of >50% (64). This effect was restricted to clopidogrel responders, suggesting that aprotinin interacts with clopidogrel-blocked ADP-receptors, making them available to ADP stimulation (64).

Clopidogrel and calcium channel blockers (CCBs). Some CCBs may inhibit CYP3A4 and may theoretically interfere with its metabolic conversion to active metabolite leading to the reduced clinical efficacy (65). However, results of the clinical studies have shown conflicting results. Some studies involving ACS patients showed that CCBs did not interfere with the clopidogrel clinical efficacy (66, 67). One study showed that different guideline compliance of secondary prevention medications could modify this DDI: concomitant use of CCBs and clopidogrel was significantly associated with increased risk of ACS re-hospitalization in patients not compliant to guideline-recommended secondary prevention drugs (65).

Clopidogrel and statins. Both clopidogrel and some statins (e.g. simvastatin, atorvastatin, lovastatin) are metabolized via CYP3A4 (68). Studies involving ACS patients didn't show reduced clinical efficacy of clopidogrel when it was used concomitantly with statins (43, 66, 69-74).

Ticagrelor and antiepileptic CYP3A inducers. One study showed that co-administration of antiepileptic CYP3A enzyme pathway inducers (including phenytoin, carbamazepine and phenobarbital) may adversely affect the antiplatelet efficacy of ticagrelor as evident by higher $P2Y_{12}$ reaction units (PRU) values, representing suboptimal platelet inhibition in ACS patients (75). Over 1/3 of these patients developed high on-treatment reactivity, suggesting that they may be at risk for ischemic complications (75).

DDIs associated with decreased efficacy of thrombolytics

Nitroglycerin and recombinant tissue-type plasminogen activator (rTPA). Concurrent administration of nitroglycerin with rTPA (alteplase) in patients with acute myocardial infarction was associated with significantly reduced thrombolytic efficacy of rTPA: less frequent flow restoration (76, 77), longer time to reperfusion (76), greater incidence of in-hospital adverse events (76) and a higher incidence of coronary artery reocclusion (76). This DDI may be related to increase in hepatic blood flow in response to nitroglycerin resulting in increased catabolism of rTPA (76).

DDIs associated with decreased efficacy of anticoagulants

Nitroglycerin and heparin. Nitroglycerin induced qualitative antithrombin III abnormality may impair directly the ability of intravenous heparin to produce a state of systemic anticoagulation (78). However, evidence regarding interaction of these two drugs are conflicting as there are studies which suggest that intravenous nitroglycerin may induce heparin resistance and attenuate its anticoagulant effect in ACS patients (78, 79), while some studies did not confirm this finding (80, 81).

DDIs associated with increased risk of bleeding

Antiplatelet drugs, anticoagulants and thrombolytics and their combinations. Most DDIs associated with increased risk of bleeding involving anticoagulants, antiplatelet drugs and thrombolytics are pharmacodynamic. Dual antiplatelet therapy (e.g. aspirin + clopidogrel, aspirin + ticagrelor, aspirin + prasugrel) was associated with increased risk of

bleeding in ACS patients, including increased risk of major bleeding (82-87). Combination of an emerging antiplatelet drug vorapaxar (platelet thrombin receptor antagonist), and glycoprotein (GP) IIb/IIIa receptor inhibitors (88) was associated with increased bleeding risk in ACS patients. On the other hand, its combination with thienopyridines in some cases may lead to increased risk of clinically significant bleeding (89), while one study didn't observe significant interaction between clopidogrel and vorapaxar (90). Combining anticoagulants and antiplatelet drugs may also increase risk of bleeding in ACS patients (e.g. oral anticoagulants and aspirin or DAPT (91-95), thienopyridines and low molecular weight heparin (96), tirofiban and heparin (97)), as well as combinations of thrombolytics and antiplatelet drugs (particularly GP IIb/IIIa receptor inhibitors (98, 99)) or parenteral anticoagulants (99). Combination of thrombolytic (tenecteplase), parenteral anticoagulant (heparin) and DAPT (aspirin and clopidogrel) may also be associated with increased bleeding risk: orbital subperiosteal hematoma occurred in acute myocardial infarction patient on combination of these drugs (100).

Non-steroidal anti-inflammatory drugs (NSAIDs) and antithrombotic therapy. Among patients receiving antithrombotic therapy after myocardial infarction, the use of NSAIDs was associated with increased risk of bleeding (62). The association was observed for all antithrombotic treatment regimens (monotherapy with aspirin, clopidogrel, or a vitamin K antagonist; DAPT with aspirin plus clopidogrel, aspirin plus a vitamin K antagonist, or clopidogrel plus a vitamin K antagonist; or triple therapy including all three drugs) (62). There was no safe therapeutic window for concomitant NSAID use, because even short-term (0-3 days) treatment was associated with increased risk of bleeding (62). Also, one study reported occurrence of melena in a 68-year-old male patient with a history of chronic gastritis and myocardial infarction receiving clopidogrel and aspirin, who had also received a 4-day NSAID treatment for radiculitis and a 2-day parenteral methylprednisolone for drug-induced urticaria prior to hospitalization (101).

Selective serotonin reuptake inhibitors (SSRIs) and antiplatelet drugs. SSRIs use alone has been associated with an increased risk of bleeding (102). Platelets release serotonin at sites of bleeding and vascular damage, but they do not synthesize it and instead acquire it from the blood and store it, so SSRIs inhibition of serotonin transporters is thought to be the cause of increased bleeding risk (102). One study conducted in a large cohort of patients following acute myocardial infarction showed that patients taking a SSRI with aspirin or DAPT were at increased risk of bleeding (102).

Oral anticoagulants and drugs interfering with their metabolism. One case report described a 79-year-old patient on rivaroxaban for cardioembolic strokes and atrial fibrillation who was hospitalized for NSTEMI in the context of severe anemia related to gastrointestinal bleeding (103). It was hypothesized that both genetic and environmental factors contributed to increased susceptibility to rivaroxaban: the homozygous presence of ABCB1 variant alleles (which encode P-glycoprotein) and reduced CYP3A4/5 activity due to DDI with simvastatin which could contributed to decreased rivaroxaban elimination (103). Another study reported increase in international normalized ratio after switching from atazanavir/ritonavir to darunavir/cobicistat in a HIV-infected 72-year-old patient on stable warfarin therapy with history of myocardial infarction (104). Removal of ritonavir in this patient led to the loss of CYP2C9 induction and subsequent increase in warfarin effect manifested by recurrent nosebleeds, which required a 60% reduction in warfarin dose (104).

Ticagrelor and cyclosporine. Co-administration of ticagrelor and cyclosporine may lead to increased exposure to both drugs, because both drugs are substrates and inhibitors of CYP3A4 and P-glycoprotein, as evidenced by two case reports (105, 106). In one case report a 49-year-old patient with a stable renal graft managed with cyclosporine with stable trough blood concentrations for several years was treated with ticagrelor for unstable angina pectoris (105). He complained of several minor bleeding events and his cyclosporine trough concentration increased 7 days after ticagrelor was started (105). The cyclosporine trough concentration in blood decreased within 13 days after ticagrelor was stopped and cyclosporine dose reduced (105). Patient did not have any bleeding events after ticagrelor was stopped, and switched to clopidogrel (105). In another case report a 58-year-old male patient who received cyclosporine for 5 years after renal transplantation developed gum bleeding and life-threatening bloody stool accompanied with the sudden drop of blood pressure 8 days after ticagrelor was added for treating ACS (106). This patient had decline in the hemoglobin level of 5.3 g/dL and received transfusion of 10 units of packed red blood cells (106).

Clopidogrel and ACE inhibitors. One study reported that in vitro coincubation of clopidogrel with ACE inhibitors increased clopidogrel bioactivation, as well as that concomitant treatment with these drugs in patients with first-time myocardial infarction was associated with increased risk of clinically important bleeding (107).

DDIs leading to rhabdomyolysis

Ticagrelor and statins. There are several case reports of rhabdomyolysis (108-112) associated with the concomitant use of ticagrelor and statins in patients who had ACS (three men (108, 111, 112) and two women (109, 110)). Age of the patients varied from 62 years (109) to 78 years (111). Statins involved in the interaction were: simvastatin (20 mg/day) (108), atorvastatin (80 mg/day in two cases (109, 110) and 40 mg/day in one case (112)) and rosuvastatin (40 mg/day) (111). Ticagrelor is metabolized through the enzymes CYP3A4/3A5 and is also a weak inhibitor of CYP3A, so it may increase the serum concentration of statins which are metabolized via CYP3A4 (e.g. simvastatin, atorvastatin) (108). Rosuvastatin is not metabolized by CYP3A4, however a deterioration of the renal function after introduction of ticagrelor led to the accumulation of rosuvastatin which

resulted in rhabdomyolysis (111). In one case amlodipine, a weak inhibitor of CYP3A4 (110), and in another clarithromycin, a potent CYP3A4 inhibitor (112), were also used concomitantly with atorvastatin and ticagrelor, so they may contributed to the increased exposure to these drugs (110, 112). One patient had normal kidney function at all times (108), acute kidney injury developed in three patients (109-111) and one patient had slightly increased urea and creatinine above reference values (112). Duration of concomitant therapy of statins and ticagrelor varied from one (111) to five months (108). All patients recovered from the rhabdomyolysis (108-112).

Fibrates and statins. Combined statin and fibrate therapy may also lead to rhabdomyolysis in patients with ACS (113, 114). Both statins and fibrates have been independently associated with a risk of muscle-related toxicity as monotherapy, and statin-fibrate combination therapy increases this risk further (113, 114). An 81-year-old male patient developed rhabdomyolysis without significant deterioration of renal function after seven days of concomitant use of pravastatin (20 mg/day) and fenofibrate (160 mg/day) (113). Treatment of rhabdomyolysis resulted in resolution of the skeletomuscular symptoms and clinical recovery (113). In another case report 63-year-old male patient developed rhabdomyolysis and acute renal failure after two weeks of combined use of lovastatin (20 mg twice daily) and gemfibrozil (600 mg twice daily) (114). Patient had to be on hemodialysis for 3 months until partial return of renal function occurred (creatinine clearance 22 mL/min) (114).

Statins metabolized via CYP3A4 and CYP3A4 inhibitors. Three-month combined use of simvastatin (80 mg/day) and diltiazem (240 mg/day), a CYP3A4 inhibitor, also resulted in rhabdomyolysis and renal failure in a 75-year-old man who had a history of previous myocardial infarction (115). Patient underwent hemodialysis and received intensive physiotherapy (115). Within three weeks, muscle pain disappeared, and he regained the functional ability of his legs, but he remained hemodialysis dependent (115).

DDIs associated with hepatotoxicity

Clopidogrel and atorvastatin. As previously mentioned, both clopidogrel and atorvastatin are metabolized via CYP3A4 (68). Two case reports described occurrence of hepatotoxicity when clopidogrel was used concomitantly with atorvastatin in ACS patients (68,116). Both patients were male, one was 58 years old (116) and other 61 years old (68). Dose of atorvastatin in the first patient was 20 mg/day (116) and in the second 10 mg/day (68) when hepatotoxicity occurred. One of the patients was also taking diltiazem and prednisone which both may affect CYP3A4 metabolism (116). After withdrawal of atorvastatin, ALT recovered to baseline level in 10 days, and then pravastatin was prescribed (116). Pravastatin, which does not undergo CYP metabolism, did not cause notable elevation of ALT during 2-month follow-up (116).

DDIs associated with adverse effects on cardiovascular system

Escitalopram and lansoprazole. QT interval prolongation and ventricular fibrillation developed in a 46-year-old patient with acute myocardial infarction probably due to enhanced QT interval prolonging effect of escitalopram caused by lansoprazole's inhibition of its metabolism via CYP2C19 (117).

Carvedilol and dobutamine. In a 68-year-old ACS patient 85-90 minutes after carvedilol administration (plasma peak time for carvedilol), blood pressure decreased up to 70/40 mmHg leading to severe hypotension when dobutamine was started and dobutamine dose increased (118). When dobutamine dose was decreased and dopamine started the patient's blood pressure increased (118).

Metoprolol and cocaine. One case report described a 54-year-old patient with a cocaine associated myocardial infarction who received metoprolol nearly two hours after presentation for persistent tachycardia (119). Shortly after, patient complained of crushing substernal chest pain, developed pulseless electrical activity, and could not be resuscitated (119). Beta blockers may exacerbate coronary vasoconstriction in patients on cocaine probably due to unopposed alpha adrenergic agonism which may lead to vasospasm followed by tissue ischemia and infarction (119).

Metoprolol and CYP2D6 inhibitors. Metoprolol is metabolized via CYP2D6 enzyme (120). A pronounced inhibition of metoprolol metabolism by paroxetine (CYP2D6 inhibitor) was observed in one study which included acute myocardial infarction patients, but without serious adverse effects (120). A reduction of metoprolol dose was required in two of 17 patients: one due to excessive bradycardia (<45 beats/min) and another due to severe orthostatic hypotension (120). Also, excessive hypotension (<100 mm Hg) was observed in four patients with initially low systolic blood pressure, but this could not be exclusively assigned to metoprolol action, but rather to combined medications (beta-blockers, mononitrates, ACE inhibitors) (120). Since this condition was well tolerated, a reduction of medication dosages was not required (120). Combination of oral terbinafine, another CYP2D6 inhibitor, and metoprolol also resulted in a clinically significant sinus bradycardia (37 beats/min) in a 63-year-old patient with a history of ACS (121). The heart rate ameliorated first with a decrease in the dose of metoprolol, which was subsequently changed to bisoprolol and the heart rate remained normal (121).

Amiodarone and antiviral drugs. A 53-year-old HIV positive patient with NSTEMI developed acquired long QT syndrome and torsades de pointes cardiac arrest in the context of combined use of amiodarone with antiretroviral agents (abacavir, lamivudine, darunavir and raltegravir) (122). Individually, amiodarone and antiretrovirals may increase QTc interval (122). Also, darunavir is CYP3A4 inhibitor so it may promote amiodarone toxicity via inhibition of its metabolism via CYP3A4 (122). In this patient QTc interval began to

normalize 5 days after discontinuation of amiodarone and antiretroviral agents. In another case report a 61-year-old patient with a history of ACS and hepatitis C virus infection on therapy with amiodarone and atenolol experienced multiple episodes of syncope, extreme bradycardia and cardiac asystole 30 minutes after first sofosbuvir and daclatasvir administration (123). Cardiopulmonary resuscitation and adrenalin restored sinus bradycardia (123). Subsequently, after discontinuation of amiodarone, sofosbuvir and daclatasvir cardiac evaluation was normal (123). Sofosbuvir, daclatasvir and amiodarone interact with P-glycoprotein and CYP3A4, but mechanism of these interactions is unknown (123). Factors that might had contributed to the observed events include concomitant therapy with a beta-blocker (123).

Mexiletine and theophylline. In a 55-year-old patient with a history of two acute myocardial infarctions a DDI between mexiletine and theophylline leading to ventricular arrhythmias on two occasions was reported (124). On the first occasion, mexiletine was added to a steady state theophylline regimen and resulted in a doubling of the theophylline level and an estimated half-life in excess of 12 hours suggesting that theophylline clearance was reduced by 30% to 50 % (124). Since arrhythmia was also noted at therapeutic theophylline concentrations, a pharmacodynamic drug interaction with additive effects, in addition to the pharmacokinetic interaction, may be postulated as both mexiletine and theophylline alone may induce arrhythmia (124).

ACE inhibitors and aspirin. Studies showed conflicting results regarding DDI involving these drugs. Aspirin blocks prostaglandin synthesis and ACE inhibitors tend to increase it, so there is a possibility for their antagonistic interaction (125). One study showed that aspirin antagonized the effect of enalapril on mortality after acute myocardial infarction suggesting that adding enalapril to patients on aspirin may be counteractive to survival (125). Several studies didn't show negative DDI between aspirin and lisinopril (126), captopril (127, 128) or ACE inhibitors in general when used in patients after myocardial infarction (129).

DDIs leading to serotonin syndrome

Escitalopram and methylene blue. One case report described a 62-year-old NSTEMI patient receiving chronic SSRI escitalopram who developed postcardiopulmonary bypass refractory vasoplegia (130). The vasoplegia was successfully treated with methylene blue, but the patient subsequently developed serotonin syndrome with abrupt onset of hyperthermia, upper extremity rigidity, encephalopathy, and cardiovascular instability (130). After discontinuation of all serotonergic medications, the patient slowly recovered with no apparent permanent complications (130). Methylene blue inhibits monoamine oxidase-A (the enzyme responsible for degrading serotonin, dopamine, and norepinephrine) and when given in combination with serotonergic medications it may lead to excess serotonin levels (130).

DDIs leading to drug-induced fever

Clonidine, duloxetine and atorvastatin. One report described a case of drug-induced fever probably associated with clonidine administration in which the higher dose of clonidine alone or in interaction with duloxetine and atorvastatin may have contributed to its development (131). A 66-year-old NSTEMI patient developed fever on the third day after the cardiac catheterization which ranged from 37.2°C to 38.9°C after dose of clonidine was increased to 0.2 mg per os three times a day to optimize blood pressure control (131). Diagnostic examinations did not reveal any source of infection (131). On the sixth day after admission, clonidine was reduced to the baseline dose of 0.1 mg per os three times a day and on the ninth day it was stopped (131). The patient was afebrile on the twelfth day (131). The mechanism by which clonidine might disrupt thermoregulation is still unclear, but both central and peripheral mechanisms may be implicated (131). Serotonergic, noradrenergic and dopaminergic neurotransmitter systems have been implicated in the mediation of drug-induced hyperthermia, so increase in these neurotransmitters may influence thermoregulatory system (131). It is possible that the interaction between the higher dose of clonidine and duloxetine (a serotonin-norepinephrine reuptake inhibitor), might have led to increased core temperature and drug-induced fever (131). Another contributing factor may have been concomitant administration of atorvastatin, CYP inhibitor, which might have inhibited metabolism of duloxetine (131).

DISCUSSION

This review has shown that ACS patients can be exposed to a variety of DDIs with diverse outcomes which include decreased efficacy of antiplatelet drugs, thrombolytics or anticoagulants, increased risk of bleeding, rhabdomyolysis, hepatotoxicity, adverse effects on cardiovascular system (e.g. QT interval prolongation, arrhythmias, excessive bradycardia, severe hypotension), serotonin syndrome and drug-induced fever. Majority of the DDIs involved antiplatelet drugs (e.g. aspirin, clopidogrel and ticagrelor). In addition, evidence of some of the reported DDIs is inconclusive as some of the studies have shown conflicting results (e.g. DDIs of clopidogrel and PPIs or CCBs; aspirin and ibuprofen, ACE inhibitors or PPIs; nitroglycerin and heparin).

DDIs leading to decreased efficacy of antiplatelet drugs have raised concern because of the possibility of increased risk of thrombotic events, but some of the studies evaluating these DDIs did not have consistent results (132). A prime example of such DDI is an interaction of clopidogrel and PPIs for which even several meta-analyses have shown conflicting results (133-135). Meta-analyses of randomized controlled trials endorsed the use of PPI with DAPT for reducing gastrointestinal bleeding without worsening cardiovascular outcomes, while meta-analyses of observational studies showed that PPI significantly increased the risk of all-cause mortality, cardiovascular mortality, myocardial infarction and stroke without reducing gastrointestinal bleeding and this

difference was attributed to differences in design and limitations of each study type (133-135). The European Society of Cardiology (ESC) currently recommends PPI use in combination with DAPT in all coronary artery disease patients as a measure to minimize bleeding risk, but with a note that omeprazole and esomeprazole would appear to have the highest propensity for clinically relevant DDIs, while pantoprazole and rabeprazole have the lowest (136). On the other hand, 2016 American College of Cardiology/American Heart Association (AHA) focused update recommends the concomitant use of PPI with DAPT in patients with a prior history or with a higher risk of gastrointestinal bleeding, but its routine use is not recommended in patients at low risk of gastrointestinal bleeding (137). H2-receptor antagonists are often used as an alternative to PPIs, but this does not appear to be an adequate solution in all cases because they do not have the same efficacy as PPIs in reducing gastrointestinal events and may also act as competitive inhibitors of CYP2C19 (138). Also, there are no particular recommendations regarding clopidogrel and CCBs interaction (12). Regarding interaction of aspirin and ibuprofen, Food and Drug Administration issued a notice that the clinical implication of the interference by ibuprofen on the antiplatelet effect of aspirin is unclear, but that it is potentially important (139). It is stated that it is likely that risk is minimal with occasional use of ibuprofen and that patients who use immediate release aspirin (not enteric coated) and take a single dose of ibuprofen 400 mg should take it at least 30 minutes or longer after aspirin, or more than 8 hours before aspirin to avoid attenuation of aspirin's effect (139). Antiepileptic CYP3A inducers may adversely affect the antiplatelet efficacy of ticagrelor, so risks for DDI and mitigation of $P2Y_{12}$ inhibition must be weighed against discontinuation of the CYP3A inducer or switching to a thienopyridine (75).

A variety of studies reported that morphine may delay and attenuate exposure and action of oral $P2Y_{12}$ receptor inhibitors (clopidogrel, ticagrelor, prasugrel) in ACS patients (15-23). However, current guidelines still continue to recommend morphine as the drug of choice for pain relief in this setting, but with a warning that this DDI may lead to early treatment failure in susceptible individuals (6, 7). Recently published meta-analysis reported that the use of morphine for pain control in the setting of ACS was associated with an increased risk of in-hospital recurrent myocardial infarction and number needed to harm (NNH) was 125 (140). Although the value of NNH might be relatively high, it certainly raises important safety concerns considering high incidence of ACS and widespread use of morphine which translate into a considerable absolute number of events (140). This suggests a need for a cautious use of morphine in the ACS setting until further results of the adequately powered trials are available keeping in mind that the current guidelines recommendations regarding the use of morphine in ACS are mainly based on expert opinion rather than randomized clinical trials (140, 141).

Modern management of ACS includes a mix of antithrombotic therapy and invasive procedures which carry a risk of bleeding with a varying degree (142). DAPT reduces the risk of stent thrombosis, but its duration beyond one year after PCI or myocardial infarction exerts majority of its benefits (136). Bleeding risk with DAPT is proportionally related to its duration, so the decision regarding DAPT duration should be dynamic and reassessed during the course of the initially selected DAPT regimen (136). In ACS patients regardless of the revascularization strategy, the default DAPT duration is 12 months, but 6-month therapy should be considered in high bleeding risk patients, while >12-month duration may be considered in patients who have tolerated DAPT without bleeding complications (136). Patients requiring oral anticoagulant in addition to DAPT should be considered at high risk of bleeding and the indication for oral anticoagulant should be reassessed and treatment continued only if there is a compelling indication (136). The duration of triple therapy should be limited to a maximum of 6 months or omitted after hospital discharge considering the ischemic and bleeding risk (136). In addition, patients may be at increased risk of bleeding due to DDIs with drugs used to treat other comorbidities (e.g. SSRIs, NSAIDs, corticosteroids and drugs which may interfere with metabolism of antithrombotic drugs), so clinicians should be cautions when prescribing new drugs to ACS patients. When bleeding occurs clinicians should weigh the risk related to ongoing bleeding, dose reduction, or temporary discontinuation of antithrombotic drugs, whenever possible, with rapid resumption of the initial antithrombotic therapy, depending on the possibility to treat bleeding and the half-life and reversibility of the effect of that drug (142). Discontinuation of antithrombotic therapy to minimize bleeding place the patient at increased risk of recurrent ischemia and infarction (142).

Concomitant use of statins and fibrates, ticagrelor or drugs which may inhibit metabolism of statins (e.g. diltiazem) may lead to the development of rhabdomyolysis in ACS patients as demonstrated by the several case reports included in this review (108-115). AHA published recommendations for management of clinically significant DDIs of statins (143). Regarding statin-fibrate interaction it is stated that fenofibrate (or fenofibric acid) is the preferred fibrate to use in combination with statins because of a reduced incidence of DDIs compared with statin-gemfibrozil combination therapy (143). However, even combination with fenofibrate was associated with the occurrence of rhabdomyolysis in ACS patients so caution is certainly needed even when fenofibrate is prescribed (113). Regarding ticagrelor-statin interaction some reviews point out that although a theoretical mechanism of this interaction exists, its clinical significance might be limited as ticagrelor is frequently used in ACS patients who simultaneously receive intensive, high-dose statin treatment which may itself lead to rhabdomyolysis (144). Regarding statin-ticagrelor DDIs, AHA stated that co-administration of ticagrelor and atorvastatin results in only a minor increase in statin systemic exposure and the combination is reasonable for appropriate patients (143). Also, combination therapy with ticagrelor and lovastatin or simvastatin may be considered and in this case dose of simvastatin and lovastatin should not exceed 40 mg daily (143). However, as

demonstrated by one of the reported cases the combination of ticagrelor with simvastatin may pose a risk for rhabdomyolysis even with low-dose simvastatin (20 mg/day), so caution is certainly necessary, especially in older populations (108). As ticagrelor-statin interaction might be amplified by co-administration of drugs that are strong or moderate inhibitors of CYP3A close monitoring and dose alteration are also recommended when multiple CYP3A inhibitors are administered with statins metabolized via this pathway (110). In general, close clinical monitoring, particularly in old patients with impaired renal function or when high doses of statins are prescribed with interacting drugs is certainly needed (115).

Patients with ACS may be at particular risk to experience adverse effects on cardiovascular system due to DDIs with possible outcomes including QT interval prolongation, arrhythmias, excessive bradycardia and severe hypotension. So, there is a need for close monitoring of electrocardiogram, QT interval, serum electrolytes and blood pressure in patients receiving a combination of drugs which may expose patients to these adverse effects (122). Negative interaction of ACE inhibitors and aspirin is highly debatable, despite some meta-analyses suggesting an antagonistic interaction between them, but with results not strong enough to contraindicate the aspirin-ACE inhibitor association or to prove clinical relevance of this interaction (145-147). Current ESC guidelines recommend ACE inhibitor use in ACS patients, particularly in those with systolic left ventricular dysfunction or heart failure, hypertension or diabetes (6, 7). Consequences of some of the DDIs may include hepatotoxicity, particularly associated with statin treatment, so it is important to carefully monitor liver function tests in ACS patients, especially if drugs which may inhibit their metabolism are used concomitantly (116). Also, some unexpected consequences may occur as a result of DDIs, such as high fever, so clinicians should be vigilant and keep in mind that some of the adverse outcomes observed in patients may be due to DDIs. In the end, it should be noted that this review summarized DDIs evaluated or reported in ACS patients, so some of the clinically relevant DDIs of drugs that may be used for treatment of ACS or other patient's comorbidities evaluated in other populations could have been omitted. Clinicians who prescribe drugs to ACS patients should always check their prescriptions for potential DDIs and explore their clinical relevance.

CONCLUSION

ACS patients may be exposed to a variety of DDIs with diverse outcomes which include decreased efficacy of antiplatelet drugs, thrombolytics or anticoagulants, increased risk of bleeding, rhabdomyolysis, hepatotoxicity, adverse effects on cardiovascular system (e.g. QT interval prolongation, arrhythmias, excessive bradycardia, severe hypotension), serotonin syndrome and drug-induced fever. Majority of the DDIs involved antiplatelet drugs (e.g. aspirin, clopidogrel and ticagrelor). Evidence of some of the reported DDIs is inconclusive as some of the studies have shown conflicting results. It is essential to evaluate DDIs in the population of patients in which drug is intended to be used, emphasizing the need for additional post-marketing and population-based studies to evaluate the true effects of disease states and other factors on the clinical outcomes of DDIs. Clinicians should be attentive to the potential for DDIs and their associated harm in order to minimize or, if possible, avoid medication-related adverse events in ACS patients.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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ASSESSMENT OF CAUSES OF STRESS IN A PHARMACY STUDENT POPULATION DURING SEMESTER AND EXAM PERIOD

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ABSTRACT

Stress is one of the most significant factors that can influence the academic performance of students. To explore the causes of stress in students, the cross-sectional online survey was conducted during semester (in 2019 year) and during exam (in 2020 year) period at the University of Belgrade - Faculty of Pharmacy. The main results indicated that female gender was the most significant cause of stress during the exam period, and the most frequent stress cause was limited time to prepare exams and/or colloquia. The role of family, friends, and boyfriend/girlfriend showed to be of great importance in stress reduction during the semester and especially during the exam period. The most frequent manifestations of stress were feeling nervous, tired and worried. For stress reduction students usually listened to music and talked with friends. Therefore, student's obligations and their overall living conditions should be observed comprehensively. These results may indicate further actions to decrease stress levels in students, and need for academic environment that may help students to achieve the best academic performance.

Keywords: *Sources of stress, pharmacy students, academic performance.*

INTRODUCTION

Stress is the natural body reaction to various external factors and can be defined as "the nonspecific response of the body to any demand made upon it" (1). Several studies revealed that higher levels of stress in healthcare students' population might lead to anxiety, depression, and higher risk for other diseases (2-4). The stress may intervene with academic performance, behavioral changes, low quality of mental health, lack of empathy and lower decision-making ability (5-7). The stress level in the student population is high because the study period is characterized as a period when making important life decisions, both professional and personal (8-10).

At the beginning of a student life, students are facing a lot of challenges: usually change of place of residence, adaptation to a new living and social environment, development of new learning strategies, adaptation to new methods of learning and studying (11). All of these challenges may lead to feelings of uncertainty and low self-esteem that may cause stress from the beginning of studies career. Stress levels in students may increase during exam periods or when some tasks should be finished in limited time such as colloquia and submission of seminar papers (11).

Master degree of integrated academic programme "Pharmacy", at the University of Belgrade – Faculty of Pharmacy includes 51 courses, 3 professional trainings and defense of master theses. Studies last 5 years (10 semesters) and provides 300 ECTS credits. During this period, each student has to attend 2061 theoretical classes (412.2 on average per year), 1644 practical classes (328.8 on average per year) and 1066 classes of professional trainings (12). Considering that most of the practical classes are performed in the laboratory after theoretical classes, students usually spend most of the day at the faculty facility during semester.

Studies examining stress in health care students have been conducted predominantly in the United States, as well as in other foreign countries such as the United Kingdom, the United Arab Emirates (UAE), China, Malaysia, and India (8, 13-17). These studies indicated different stressors in students: worries regarding the future and expectations of parents (14), health studies, lifestyle, health and academic factors (13, 17) and gender (15). The study conducted in UAE pointed out that there was no difference in stressors and coping strategies related to students' gender (14), opposite to study conducted in India (13). Exposure to stressors could lead to higher risks of several chronical health conditions in students' population (16).

There are several published studies, which included population of Serbian students, that examined stress predictors, burnout (18-20), stress perception and stress levels during COVID-19 (21, 22). Two studies included medical sciences students (19, 20), veterinary students (18), one without specific students type of study (21) and one which included both medical students and academic staff (22). The most common stressors in these studies were: exams (18-20), communication with teaching staff (20), contacts with patients (19) and performing autopsy (19). In two studies the stress levels were higher in female students (19, 21) and one study did not found difference in burnout between students of different gender (18). During the COVID-19 outbreak, new factor that influence stress levels in students was taking care of disabled people (21). Studies on population on pharmacy students had not been previously conducted in Serbia.

THE AIM OD THE PAPER

This study aimed to assess the causes of stress in pharmacy students concerning study year, and periods in school year.

METHODS

The study was conducted as cross sectional study at two points (during semester (December 2019) and during exam period (February 2020)) at the University of Belgrade - Faculty of Pharmacy. All students were invited by e-mail to fill the online survey. Eligible students were those who had active e-mail address, were willing to participate and had internet access. Since the participation in the study was voluntary and all students data was anonymous and protected the approval of the ethics committee was not required.

The survey consisted of items developed by literature search and from discussion with students. The survey was created and conducted online using the Google forms. The completion of online survey was voluntary and anonymous.

The first part of the survey consisted of items regarding the student's age, gender, study year, number of ECTS credits achieved during the studies, student working status and permanent residence. The second part of the survey consisted of multiple choice items that examined causes that may contribute to stress, the self-assessment of overall stress level, the manifestations of stress on students, ability to function under stress, mechanisms for stress reduction and efficiency of those mechanisms. The self-assessment of overall stress level was also estimated by one question with answers rated on 5 point Likert scale (from "no stress" as 1 to "extreme stress" as 5).

Item that examines 13 different causes (living with roommate, living separately from family, financial situation, working/employment during studies, grades, exams/colloquia, scope of teaching, family relationships, friends relationships, health condition, doing sports, relationship with a boyfriend/girlfriend, limited time to prepare for exams) that may contribute to stress were estimated using the 5 point Likert scale where a value of 1 indicates the lowest contribution and 5 the highest contribution of cause to the stress.

The general students' characteristics were analyzed using the descriptive statistics analysis. The chi-squared test of independence has been used to test group differences for categorical data. To test differences between two groups of

interval non-normal distributed variables the Mann Whitney-U test has been used. Association between variables was established using the Spearman correlation. Ordinal logistic regression was used to test association of dependent ordinal variable stress levels (scale from 1 "no stress" to 5 "extreme stress") and variables: gender (male gender as reference category), and 13 examined above mentioned causes of stress (categories from "no stress at all" to "moderately cause" were grouped as a reference category, and categories "causes" and "extremely causes" were grouped as second category). The values of $p<0.05$ have been considered statistically significant in all performed tests.

The results analysis has been conducted using Microsoft Office Excel 2007 and Predictive Analytics SoftWare, version 28 (SPSS Inc. Chicago, Illinois, SAD).

RESULTS

At the first study point (during semester) 402 students accepted to participate in study and 335 students participated at the second point (during exam period). Responses of 3 students at the first and 4 students at the second study point were excluded due to incompleteness. Therefore data of 399 students during semester and 331 students during the exam period were analyzed.

The most prevalent were female students and students at the fifth and third study year (Table 1).

Table 1. General students' characteristics.

Variable	During semester (N = 399)	During exam period (N = 331)	Difference between two students groups
Gender, n (%)			χ^2= 2.1, p > 0.05
Female	366 (91.7)	293 (85.5)	
Male	33 (8.3)	38 (11.5)	
Age in years, average (SD), range	22.14 (1.84), 18-27	22.17 (1.89), 18-32	U= 65416, p > 0.05
Study year, n(%)			χ^2= 5.4, p > 0.05
1 year	15 (3.8)	10 (3.0)	
2 year	70 (17.5)	64 (19.3)	
3 year	98 (24.6)	92 (27.8)	
4 year	84 (21.1)	80 (24.2)	
5 year	132 (33.1)	85 (25.7)	
ECTS, average (SD), range	147 (74.22) 0-330	141.68 (73.68) 0-320	U= 63984.50, p > 0.05
Place of study, n (%)			χ^2= 0.65, p > 0.05
in hometown	121 (30.5)	98 (29.6)	
in other city	276 (69.5)	233 (70.4)	

Using the five point Likert scale, the stress level was rated significantly different during semester and exam period (χ^2= 16.24, $p < 0.05$). The most frequent levels of stress in students during exam period were: higher level of stress (36.25%), moderate stress level (28.70%) and extremely stress (27.19%). During semester these most frequent levels were: higher level of stress (40.35%), moderate stress level (35.59%) and extremely stress (16.04%).

The causes of stress ranked as the highest percentage of extreme stress were limited time for exam preparation and exams/colloquia itself. Causes that contributed to the least grade of stress were: performing sports, working during studies and living with roommate. Causes such as relationships with friends, family members and boyfriend/girlfriend and living with roommate were the least stressful during the exam period (Figure 1). The difference in stress grades during the semester and period of exams was not statistically significant (for all χ^2 statistics, $p > 0.05$).

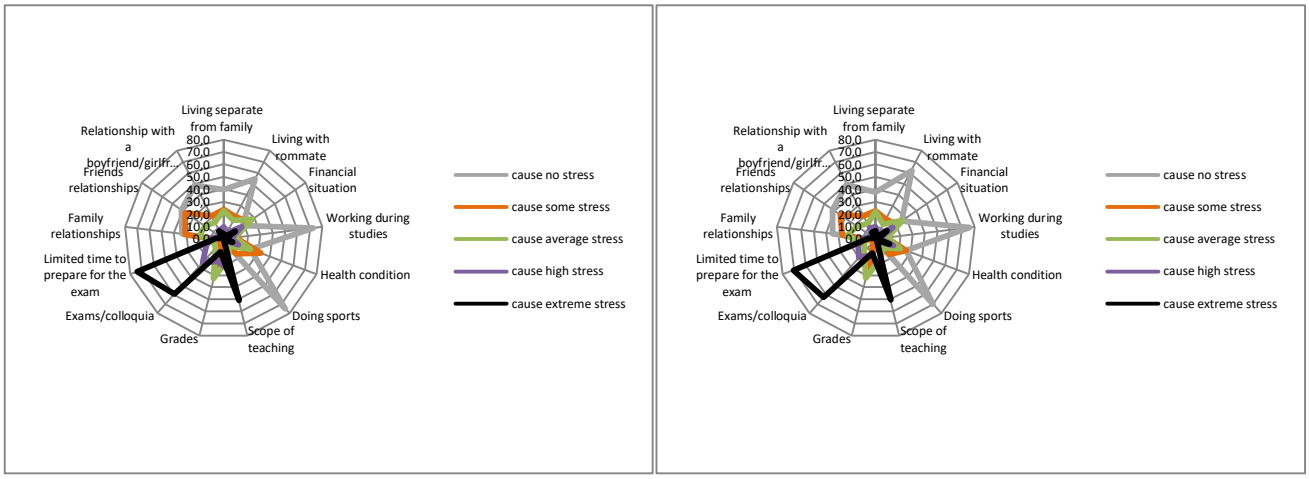

Figure 1. Causes of stress during semester (left) and during exam period (right) (the percentage value of each response level for each cause).

Correlation between students study year and stress grades were low and not statistically significant (ρ=0.03, p>0.05 and ρ= -0.05, p>0.05 during the semester and exam period respectively). Level of stress during semester was in positive correlation with students' age, but the correlation between these variables was not significant during exam period (ρ=0.11, p<0.05 and ρ=0.03, p>0.05, respectively).

During the exam period, female gender was associated with increase in the odds of higher stress grade, with OR=1.56 ((95% CI: 0.90 to 2.21), Wald $\chi2(1) = 21.65$, $p < 0.001$) opposite to semester period when this association was not significant (OR=0.41 (95% CI: -0.25 to 1.08), Wald $\chi2(1) = 1.50$, $p > 0.05$).During semester 57.4% students could perform everyday activities under stress as opposed to 48.6% of students during exam period. This difference was statistically significant ($\chi^2 = 5.56$, $p < 0.05$). Stress had different manifestations in students at two study points(Figure 2), but the overall difference was not statistically significant ($\chi^2 = 11.78$, $p > 0.05$).

Contributions of stress causes were not significantly associated with the increase in stress levels during exam and semester (Table 2).

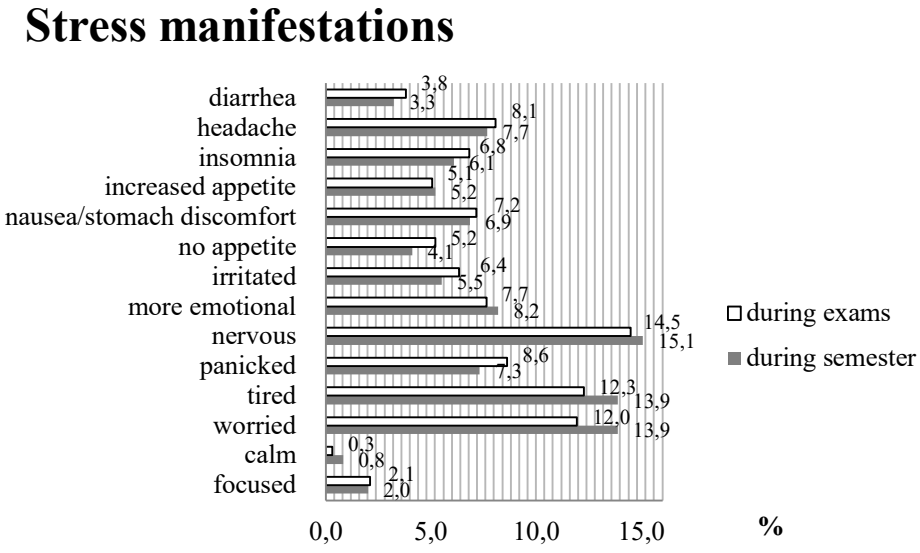

Figure 2. Stress manifestations in students during semester and exam period.

Table 2. Assessment of the impact of stress causes on the stress score, the results of the ordinal logistic regression analysis.

Causes of stress				95% Confidence Interval	
During semester	Sig.	Estimate	Wald $\chi 2$	Lower	Upper
Living with roomate	0.95	-0.01	0.00	-0.31	0.29
Living separate from family	0.15	-0.35	2.10	-0.83	0.12
Financial situation	0.18	-0.21	1.82	-0.52	0.10
Working during studies	0.20	-0.40	1.65	-1.02	0.21
Grades	0.13	-0.33	2.30	-0.77	0.10
Exams/colloquia	0.12	-0.87	2.42	-1.96	0.22
Scope of teaching	0.14	-0.43	2.21	-0.99	0.14
Limited time to prepare for the exam	0.46	-0.23	0.56	-0.85	0.38
Family relationships	0.17	-0.31	1.85	-0.76	0.14
Friends relationships	0.78	-0.06	0.08	-0.51	0.39
Relationship with a boyfriend/girlfriend	0.54	-0.08	0.37	-0.36	0.19
Health condition	0.18	-0.23	1.76	-0.58	0.11
Doing sports	0.99	0.00	0.00	-1.21	1.20
During exam period					
Living with roomate	0.37	0.65	0.81	-0.77	2.08
Living separate from family	0.29	-0.94	1.12	-2.69	0.80
Financial situation	0.41	-0.28	0.69	-0.95	0.39
Working during studies	0.56	0.40	0.34	-0.93	1.73
Grades	0.31	-0.83	1.04	-2.43	0.77
Exams/colloquia	0.30	-1.83	1.07	-5.30	1.63
Scope of teaching	0.43	-0.61	0.64	-2.11	0.89
Limited time to prepare for the exam	0.31	-2.05	1.01	-6.04	1.94
Family relationships	0.50	-0.35	0.46	-1.36	0.66
Friends relationships	0.91	-0.11	0.01	-2.02	1.80
Relationship with a boyfriend/girlfriend	0.41	-0.31	0.67	-1.06	0.44
Health condition	0.31	-0.65	1.05	-1.90	0.60
Doing sports	0.37	0.47	0.79	-0.57	1.51

The most frequent actions that students performed to reduce stress during the semester were: listening to music (23.1%), talking with friends (21.6%), sleeping (16.0%), physical exercising (9.3%), eating (6.8%) and smoking (6.3%). Similar actions were performed during the exams period: talking with friends (26.6%), listening to music (20.2%), sleeping (16.9%), physical exercising (7.6%) and smoking (6.9%). The difference in actions frequencies during semester and exam period was not statistically significant (χ^2= 16.61, $p > 0.05$). On a Likert scale, most students rated the effectiveness of these actions as moderate (51.4% during semester vs. 44.7% during exam period) but the difference in rating was significant (χ^2= 20.66, $p < 0.001$).

DISCUSSION

Pharmacy students at the University of Belgrade are constantly under pressure caused by numerous teaching activities (theoretical and practical) that require spending a lot of time at the facility, usually from the early morning and sometimes until the evening hours. This may lead to social isolation of students. Recommendations to decrease social isolation listed in the paper by Haas et al. are focused on activities to improve social interactions, for example, student-led walking initiatives (23). Similar to these recommendations, students at the University of Belgrade-Faculty of Pharmacy are supported in many activities within the sports society of students, the choir, the group who prepare the students' magazine, and the photographic section (24).

According to the study results by Gerber et al., academic performance and pressure to succeed were the most common sources of stress in pharmacy student population (2). The most frequent stress sources in our study were limited time for preparation of exams and exams/colloquia. In this regard, stress sources were related to students' academic performance.

A study conducted in Romania included enrolled pharmacy students from Romania and international students. The results pointed out that female gender, homesickness and study year may contribute to higher stress levels (25). Students who studied with relatives or old friends had less stress than others (25). In our study, the role of family, friends, and boyfriend/girlfriend showed to be of great importance in stress reduction during semester and especially during the

exam period. A study by Geslani et al. showed that the most effective way for stress reduction is time spent with family members (26). In addition, another study pointed out the significant factor in coping with stress is talking to friends (27).

According to our findings, it was interesting to see that students' employment during studies did not cause a stress in students. We have to emphasize that only 22% (1% as full-time and 21% as part-time employment status) of students during semester and only 25% (2% as full-time and 23% as part-time employment status) during the examination period were employed. Several studies indicated that employment during studies may affect academic performance (28-30). Work stress could also impact person's behavior and health state (16, 31). On the other hand, several studies presented opposite results in favor of students employment. They stated that working habits could positively influence students' performance, responsibility and feeling of success. In addition, salary also had positive impact (32-35). Nevertheless, we should interpret our results with caution as we had very small sample of students (1-2%) who had full-time job and approximately one quarter of students, part-time job.

Similar to our sample, number of female students enrolled at the University of Belgrade – Faculty of Pharmacy is around 6 times higher than enrolled males (according to official, unpublished Faculty report). Several published studies indicated that female students might have higher level of stress than their male colleagues (19, 36, 37). In addition, according to Donaldson et al., who performed a study on a sample of children and adolescents, there was no statistically significant difference in coping strategies in response to stressors related to gender (38). One more study by Gomathi at al. found that, among health-care undergraduate students, there are no statistically significant difference in stressors or coping strategies between students of different gender (14). The results presented in this manuscript pointed out that the female gender was a significant predictor of stress levels, but only during the exam period.

The results of the study by Votta et al. showed that significant predictors of stress were: gender (female students are exposed to stress more than male), age (older students are more receptive to stress compared to younger), year of study (students at the first two years have higher stress level than at higher years), type of enrolled programme (students that enrolled entire master program have more stress than students who enrolled two-stage programme (bachelor and then master) and postgraduate students) and grade point average (stress is more present in students with better achievements) (39). During the semester slightly positive correlation was established between age and stress levels. Correlation between study year and stress levels was not significant at both study points. In addition, our results did not find statistical significantly relation between causes of stress and increase of stress levels during exam and semester.

This study possesses several limitations. Stress could be in association with depression, anxiety and other disorders (40, 41). However, we did not examine overall mental health and did not check whether students suffer from other conditions that could affect the presented results. In addition, stress could be measured using physiological, psychological, or combined methods (41). We have used only self-reported measure which could be considered as limitation. The study had to be repeated with the same students, and their results should be compared. In this regard, students had to choose abbreviated names or initials that they required to report in the repeated survey. In the repeated survey, many students indicated that they could not remember the first chosen abbreviated name. For this reason, the results were analyzed as a repeated study with a new population. As this survey was voluntary, anonymous, and all data was protected the approval of the ethics committee was not required.

To our knowledge, our study is the first that examines stress in pharmacy students in Serbia. The presented results indicated that female gender was significant predictor of stress during the exam period. The most frequent stress sources were limited time to prepare exams and exams/colloquia, and the most frequent manifestations of stress were feeling nervous, tired and worried. For stress reduction students usually listen to music and talk with friends. The information obtained from the study may contribute to understanding of the causes of stress among pharmacy students in Serbia and to decrease stress levels in students. The students' academic obligations need to be more balanced in order to provide better academic atmosphere and better academic achievements.

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COMPARATIVE STUDIES OF CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITY OF *JUNIPERUS COMMUNIS L.* ESSENTIAL OIL FROM DIFFERENT LOCALITIES IN THE REPUBLIC OF SERBIA

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ABSTRACT

This work aims to determine the chemical composition of Juniperus communis (J. communis) essential oil from different localities in the Republic of Serbia and examine its antimicrobial and antioxidative effects together with chemometric analysis and principal component analysis. Essential oils were extracted via hydro distillation from the fruits of J. communis gathered from four different habitats in Serbia. Extraction yields ranged from 1.56 % in the sample from Bavanište to 1.98% in the sample from Mačkat. A total of 23 compounds in the four essential oil samples were identified by Gas Chromatography-Mass Spectrometer (GC-MS). The dominant compounds were α-pinene (32.68-51.10 %), β-phellandrene (6.43-24.77 %), and β-pinene (9.84-14.09 %). Compared with the other ecological factors, precipitation showed a strongly positive correlation (0.871) with essential oil yields. The four samples could be classified into two clusters based on the variance in their components. All the essential oils samples showed bioactivities. Among them, the essential oil from the Mačkat sample showed the best ABTS radical scavenging activity (IC50=237.74 μg/ml), DPPH radical scavenging activity (IC50=308.83 μg/ml) and antimicrobial activities. Consequently, the essential oil extracted from Mačkat has the potential for commercial viability in the food, cosmetic, or medical fields.

Keywords: *Juniperus communis, Essential oil, Chemical composition, Antioxidant activity, Antimicrobial activity.*

INTRODUCTION

Essential oils are natural volatile mixtures of complex compounds that are the result of the secondary metabolism of aromatic plants. Thanks to their different chemical composition, essential oils have several biological activities such as digestive (1), anti-inflammatory (2), antimicrobial, antiviral (3), antioxidant (4), hepatoprotective (5), and anticancer (6). Given that there is a huge increase in the resistance of microorganisms to the use of synthetic antimicrobial agents, the attention of the scientific community has been increasingly focused in recent years on the results of studies that examine the effect of various active metabolites of plant origin, among which essential oils and their components stand out the most.

Juniperus communis L. (*J. communis*), popularly known as juniper, pine, or spruce, is an evergreen shrub or lower tree from the *Cupressaceae* family. This perennial plant is distributed throughout the Northern Hemisphere, in the mountainous areas of Europe (Alps, Pyrenees, Dinarides, Carpathians), Central Asia, North America, and less often in North Africa. In Serbia, it occurs naturally mostly in the mountains of Kosovo and Metohija (Šara, Mokra Gora, Kopaonik, Rogozna) and Southwestern Serbia (Tara, Zlatibor, Golija, Pešter) (7), which especially applies to areas of degraded forests and abandoned agricultural areas. This plant species has a long history of use in folk medicine as a diuretic, anti-inflammatory, antiseptic, stomachic, anti-rheumatic, and antidiabetic, for kidney and bladder inflammation (8-11). It is most important bioactive ingredient is essential oil, which has a wide range of applications (food, cosmetic, pharmaceutical industry, veterinary) and high commercial value. The characteristic composition of the essential oil obtained by steam distillation from ripe, unfermented berries of *J. communis* mostly includes terpene hydrocarbons (monoterpenes up to 85 %): α-pinene (20-50 %), myrcene (1-35 %), sabinene (<20 %), limonene (2-12 %), β-pinene (1-12 %), caryophyllene (<7 %), terpinene-4-ol (0.5-10 %), then sesquiterpenes (up to 27%) and their oxidized derivatives (up to 4 %) (7). The wide distribution of this species, due to the different effects of environmental factors, leads to variability in terms of the chemical composition and biological activity of its metabolites (12). Variations in chemical and structural type are exactly what makes essential oils functionally versatile and thus more interesting to the scientific public. Variability of the chemical composition of *J. communis* essential oil is also confirmed by a wide range of values in the requirements of the European Pharmacopoeia (European Pharmacopoeia 8). Among the activities of juniper essential oil tested so far, the greatest application potential is reflected in its antimicrobial activity against a wide range of bacteria, the effect of which is determined by the chemical nature of the metabolites, its concentration, and the taxonomic properties of microorganisms (13-14). Considering the potentially toxic and carcinogenic effects of synthetic antioxidants in humans and animals their replacement with natural antioxidants is beginning to be justified. Depending on the presence of active components, the essential oil of juniper berries shows positive effects in slowing down the lipid peroxidation of foods of animal origin (15), as well as antiradical activity against the DPPH radicals (16). However, scientific reports emphasizing the potential of juniper essential oil for health purposes, as well as in food production and storage, are rare.

This work aims to determine the chemical composition of *J. communis* oil from different localities in the Republic of Serbia and examine its antimicrobial and antioxidative effects together with chemometric analysis and principal component analysis (PCA), and thus contribute to the results of previous research, considering the qualitative and quantitative differences of *J. communis* essential oils of different geographical origins. The essential oil compositions of the four samples were analyzed using Gas Chromatography-Mass Spectrometer (GC-MS). The samples were then classified by PCA and hierarchical cluster analysis (HCA) to determine their relationships. Furthermore, with this work, we want to clarify in more detail whether the biological potential of *J. communis* essential oil is mainly the result of the activity of the components present in the highest concentrations or whether the investigated antimicrobic and antioxidative effects arise from the synergism of all present molecules.

MATERIAL AND METHODS

Plant material

Drug samples were collected from wild juniper bushes from four different locations in the Republic of Serbia (Kopaonik (**JC1**), Mačkat (**JC2**),), Takovo (**JC3**), and Bavanište (**JC4**)) in October 2019. Table 1 displays the meteorological data for the four habitats from which samples were collected. After collection, the drug was cleaned from the leaves, then properly dried and stored under conditions that do not allow spoilage and contamination (dry environment with low humidity levels; temperature between 10-20°C; dark container; sealed container). The fruits are crushed in an electric mill (IKA® A11 basic) and thus prepared for steam distillation. The preparation of the plant material as well as the isolation of the essential oil were carried out in the laboratory of the Faculty of Medical Sciences of the University of Kragujevac.

Isolation of essential oil

One hundred grams of chopped juniper fruits were placed in a glass balloon and then subjected to hydrodistillation. During two hours, water was heated in a vessel with a flat bottom (steam generator) at a temperature of 100 (±10) °C. The essential oil was separated in a Florentine bottle, where the separation of the two phases - oil and water phase - could be observed. The essential oil was separated by decantation, which was then stored in screw-cap vials with adequate labeling.

Gas-mass analysis of essential oil

Analyzes were performed on an Agilent 7890A gas chromatograph equipped with an Agilent 5975C mass-selective

detector (Agilent Technologies, Santa Clara, CA, USA) and a capillary column (HP5-MS, 30 m × 0.25 mm, 0.25 μm). As the mobile gas phase, helium (He) gas was used with a constant flow rate of 1 ml/min. The injector temperature was set to 230 °C, while the detector temperature was 250 °C. The column temperature was linearly changed in the range from 40 to 220 °C, at a rate of 3 °C/min. The injected volume of the sample (dissolved 1/1 in hexane (Fisher, UK), v/v) is 1.0 μl in a split ratio of 1:50. Mass spectra were recorded at 70 eV in the range m/z 40-450. The identification of the detected compounds was performed by comparing their mass spectra with spectra from the spectral database NIST08 (National Institute of Standards and Technology, Gaithersburg, MD, USA), which contains 192,108 spectra of different compounds. Quantification was performed by the method of normalization of the areas under the peaks, that is, based on the correlation of the areas of the peaks and the percentage representation. Qualitative and quantitative analysis of the chemical composition of the obtained essential oils was carried out in the laboratories of the Institute of Public Health in Kragujevac.

Antimicrobial activity

The antimicrobial activity of the essential oil was tested against nine microorganisms. The experiment involved eight strains of bacteria (five standard strains (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Proteus mirabilis* ATCC 12453, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922) and three isolates (*Staphylococcus aureus, Escherichia coli*, and *Salmonella enterica*)). Also, one yeast standard strain (*Candida albicans* ATCC 10231) was tested. All isolates were a generous gift from the Institute of Public Health, Kragujevac. The other microorganisms were provided from the collection held by the Microbiology Laboratory Faculty of Science, University of Kragujevac.

The bacterial suspensions were prepared using the direct colony method. The turbidity of the initial suspension was adjusted with a densitometer (DEN-1, BioSan, Latvia). When adjusted to the turbidity of the 0.5 McFarland standard, the bacterial suspension contained approximately 10^8 colony-forming units (CFU)/mL, while the yeast suspension contained 10^6 CFU/mL. Ten-fold dilutions of the initial suspension were additionally prepared in sterile 0.85% saline.

Microdilution method

Antimicrobial activity was tested by determining the minimum inhibitory concentrations (MIC) and minimum microbicidal concentrations (MMC) using the microdilution plate method with resazurin (17). The 96-well plates were prepared by dispensing 100 μL of nutrient broth (Mueller–Hinton broth for bacteria and tryptone soy broth for yeast) into each well. A 100 μL aliquot of the stock solution of the tested essential oil, dissolved in Tween 40 at a 1:1 ratio, was added to the first row of the plate. Twofold serial dilutions were then performed using a multichannel pipette. The resulting concentration range for the **JC3** sample was 500 to 0.98 μL/mL, while for the **JC1**, **JC2**, and **JC4** samples, the range was 2000 to 3.91 μL/mL.

The microtiter plates were inoculated with suspensions to achieve final concentrations of 5×10^5 CFU/mL for bacteria and 5×10^3 CFU/mL for fungi. Microbial growth was monitored using resazurin (Alfa Aesar GmbH & Co., KG, Karlsruhe, Germany), a blue dye that turns pink when reduced by viable cells. Plates were incubated at 37°C for 24 hours (bacteria) and 28°C for 48 hours (yeasts). The MIC was the lowest extract concentration preventing the color change of resazurin. MMC was determined as the lowest concentration showing no microbial growth after plating samples onto nutrient agar. Each test included growth control and sterility control. All experiments were performed in duplicate.

Tetracycline and fluconazole were used as positive controls. The antibiotic tetracycline (Pfizer Inc., USA) was dissolved in a nutrient liquid medium, Mueller–Hinton broth (Torlak, Belgrade, Serbia), while the antifungal agent fluconazole (Pfizer Inc., USA) was dissolved in tryptone soya broth (Torlak, Belgrade, Serbia).

Antioxidant activity

Determination of DPPH (1,1-diphenyl-2-picryl-hydrazyl) free radical scavenging activity

The ability to scavenge free radicals was tested using DPPH radical according to the method described by Mishra et al (18). First, a solution of DPPH (Alfa Aesar GmbH & Co., Germany) in methanol (Fisher, UK) was prepared at a concentration of 0.05 mg/ml and stored in a darkened bottle in the refrigerator until the experiments were performed. Then, a series of standard solutions of the tested extracts and standards in methanol was made (ie 1000, 500, 250, 125, 62.5, and 31.25 μg/ml). Test tubes were filled with 200 μl of solutions containing either tested extracts or standards at specified concentrations, along with 2 ml of DPPH solution. After intensive mixing, the mixture was incubated in the dark for half an hour. After incubation, the absorbance of the solution was measured at 517 nm compared to the control. Ascorbic acid (Sigma-Aldrich, USA) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Acros Organics, Belgium) were used as positive controls. The concentration of DPPH radicals was calculated according to the equation: Inhibition capacity of the DPPH radical (%) = 100 * (Ak−Au)/Ak

Where Ak denotes the absorbance of the control (which contains all reagents, except the tested extract or standard), and Au is the absorbance of the sample. Based on the obtained values, a nonlinear calibration curve was constructed, which was used to determine the concentration of the tested sample that inhibits 50% of DPPH radicals (IC_{50}).

Determination of the ability to neutralize ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radicals

The ability to neutralize free radicals was tested using ABTS radicals according to the previously described method by Tabassum et al. (19) with modification. During the preparation of the experiment, the mixture of 7 mM ABTS (Alfa Aesar GmbH & Co., Germany) and 2.45 mM potassium persulfate (Sigma-Aldrich, USA) was incubated at room temperature without the presence of light for 24 hours. This solution was diluted until an absorbance of 0.700±0.02 at 734 nm was achieved. A volume of 300 µl of extract or standard solution was mixed with 600 µl of ABTS solution. This mixture was incubated at room temperature for 30 min. Absorbance was measured at 734 nm. Ascorbic acid and Trolox were used as positive controls. The ABTS radical concentration was calculated according to the equation: Inhibition capacity of the ABTS radical (%) = 100 * (Ak−Au)/Ak

Ak denotes the absorbance of the control (which contains all reagents, except the tested extract or standard), and Au is the absorbance of the sample. Based on the obtained values, a nonlinear calibration curve was constructed, which was used to determine the concentration of the tested sample that inhibits 50% of ABTS radicals (IC_{50}).

Statistical analysis

ANOVA test with α=0.05 was used to determine the differences between the assays. The correlation coefficients between yields and compounds of essential oils and the main ecological factors of collection locations were calculated. PCA was employed to identify the interrelations among the essential oils of *J. communis* obtained from the four places. HCA was performed based on the between-group linkage to classify the four oil samples examined. All statistical analyses were performed using SPSS 21.0.

RESULTS AND DISCUSSION

Essential oil yield

The information from the four samples of *J. communis*, including the collection sites, meteorological data, essential oil yields, and correlation coefficients between four environmental factors and the essential oil yields, is summarized in Table 1. The essential oil extracts from all four samples were colorless. Yields of essential oils (yields, g/g, dry fruit) obtained from the four samples ranged from 1.56 % to 1.98 %. The yield of **JC2** (1.98 %) was higher than the others, followed by **JC1** (1.71 %), **JC3** (1.58 %), and **JC4** (1.56%). Reports suggest that the yields of essential oils in plants can be influenced by altitude, precipitation, temperature, and other ecological factors of their habitats. (20). Compared with the other three ecological factors, precipitation showed a strongly positive correlation (0.871) with essential oil yields, suggesting it could be a dominant environmental factor affecting the essential oil yields of *J. communis* from various regions. Labokas and Ložienė also observed the impact of precipitation on essential oil yield in plants (21).

Table 1. The collection sites, meteorological data, and yields of oils from the four *J. communis* samples and the correlation coefficients between four environmental factors and essential oil yields.

Sample	Place	Average Altitude (m)	Temperature[1] (°C)	Precipitation[1] (mm)	Humidity[1] (%)	essential oil yield (wt%)
JC1	Kopaonik	1710	9.6	966.04	79.83	1.71
JC2	Mačkat	600	9.74	995.26	78.58	1.98
JC3	Takovo	330	11.7	818.82	75.83	1.58
JC4	Bavanište	80	13.38	768.34	69.83	1.56
Correlation coefficients		0.273	-0.756	0.871	0.637	

[1]The annual average meteorological data (2015-2019) of four locations from the Republic Hydrometeorological Service of Serbia (annuals bulletin for Serbia, https://www.hidmet.gov.rs) and "Weather Atlas" (https://www.weather-atlas.com)

[2]Oil yields (wt.%) = Weight (crude oil)/Weight (dry fruit) × 100;

CHEMICAL COMPOSITION OF ESSENTIAL OIL

Examination of the essential oil of juniper fruit from different locations in the Republic of Serbia included the preparation of plant material, isolation of the essential oil by hydrodistillation, phytochemical analysis of the mentioned oils by a combined chromatographic-spectroscopic method (gas chromatography-mass spectrometry) and comparison of the obtained results.

By analyzing the chemical composition of juniper essential oils in samples from Kopaonik, Mačkat, Takovo, and Bavanište, 23 compounds were separated and identified as shown in Table 2.

Examination of the chemical composition of these essential oils samples revealed the presence of several monoterpene and sesquiterpene compounds.

The most abundant compound in all samples is α-pinene (**C2**, 32.68-51.10 %), as shown in previous studies (22,23). β-pinene (**C7**), limonene (**C8**), α-cubebene (**C10**), α-elemene (**C20**), β and α-caryophyllene (**C13, C14**) were identified in all examined oils, but their quantitative representation differed. The content of β-pinene in the oil of fruits originating from higher altitudes (**JC2** and **JC1**) was above 10 %, while its content was below 10 % in the oils obtained from the fruits of areas of lower altitudes (**JC4** and **JC3**). The terpenes sabinene (**C4**, 17.82 %), aristolene (**C22**, 4.28%), and humulene (**C16**, 0.68 %) were identified in the drug oil originating from **JC2**, while their presence was not confirmed in the other oils. In the essential oil originating from **JC3**, the presence of the following compounds was confirmed: bicyclogermacrene (**C19**, 2.58 %), β-elemene (**C12**, 1.41 %), and copaene (**C11**, 1.18 %), which were not found in other oil samples. β-terpinene (**C6**) is a terpene that, with a percentage of 2.23%, was an integral part of only the essential oil obtained from juniper originating from **JC4**.

Table 2. Chemical composition of *J. communis* essential oils from different habitats

NO	Compound	Retention time (min)	Relative abundance (%)			
			JC1	JC2	JC3	JC4
C1	α - thujen	5.392	2.23	1.72	/	1.24
C2	α-pinene	5.559	32.68	41.95	51.10	46.82
C3	β-phellandrene	6.541	24.77	/	6.43	12.27
C4	Sabinene	6.547	/	17.82	/	/
C5	Tricyclene	6.605	2.19	2.57	2.23	/
C6	β-terpinene	6.593	/	/	/	2.32
C7	β-pinene	7.040	14.09	13.92	9.84	9.99
C8	D - limonene	8.005	4.16	3.78	4.86	3.81
C9	Terpinolene	9.660	1.40	1.02	/	1.10
C10	α-cubebene	16.798	0.76	0.80	2.08	0.76
C11	Copaene	17.480	/	/	1.18	/
C12	β - elemene	17.925	/	/	1.41	/
C13	β -caryophyllene	18.583	1.69	2.42	1.39	2.26
C14	α - caryophyllene	19.443	1.22	1.53	1.42	1.31
C15	γ-cadinene	20.130	9.56	6.45	/	/
C16	Humulene	20.488	/	0.68	/	/
C17	γ - elemene	20.494	0.80	/	/	0.65
C18	β - cubebene	20.136	/	/	11.09	4.36
C19	Bicyclogermacrene	22.452	/	/	2.58	/
C20	α - elemene	20.714	0.85	0.71	2.03	0.57
C21	Cadinene	21.164	0.70	0.88	1.18	/
C22	Aristolene	21.927	/	4.28	/	/
C23	Elixene	21.938	2.93	/	/	3.72

The representation of monoterpene secondary metabolites in all four essential oils is significantly higher than the content of sesquiterpenes. It has been shown that altitude has an influence on the composition of essential oils (24). α-pinene (C2), whose representation of over 50 % was the highest in juniper fruit essential oil originating from JC3, may emphasizes the consistency of certain chemical profiles within different geographical regions. There is a higher content of β-pinene (C7) in areas of higher altitude, as well as γ-cadinene (C15) which was identified only in oils from higher

altitudes. This can certainly be explained by the fact that different altitudes have different effects on the amount of precipitation, day and night temperatures, relative humidity, and exposure to wind. At high altitudes, there is greater exposure to sunlight and low temperatures, which in plants leads to changes in morphology and physiology, and therefore to changes in the production and composition of secondary plant metabolites. A noticeable difference is observed in the presence and concentration of other terpenes such as β-phellandrene (C3), sabinene (C4), β-pinene (C7), D-limonene (C8), α-cubebene (C10), and others, which further emphasizes the complexity of the influence of environmental factors on the chemical composition of essential oils. The high prevalence of α-pinene (C2) and β-phellandrene (C3), as the main components in the oil from JC1, corresponds to the qualitative analysis of the essential oil of juniper berries from the central part of Portugal where α-pinene and β-phellandrene were also identified as the two main components of the essential oil (25).

The identification of specific terpenes in different samples, such as sabinene (**C4**), aristolene (**C22**), and humulene (**C16**) identified only in the **JC2**, bicyclogermacrene (**C19**), β-elemene (**C12**) and copaene (**C11**) in the **JC3**, as well as β-terpinene (**C6**) in the **JC4**, indicates specific adaptations of plants to local conditions, which can be useful in identifying the geographical origin of essential oils and potentially useful in situations that require the application of certain chemical components.

Differences in the quantitative and qualitative composition of essential oils, which are the result of various factors such as altitude, geographical location, ripeness of the fruit, age of the plant, and production method, highlight the complexity of factors that affect the chemical profile of the essential oil (26,27). These factors not only affect the quality and characteristics of essential oil but also represent the basis of the individual biological properties of juniper essential oil (15,23). The present compounds have a high biopotential and previous studies have confirmed their various biological activities. This tells us that the essential oil of the juniper fruit is a source of natural active compounds with a wide application potential in the pharmaceutical industry as natural bioactive ingredients, in the cosmetic industry for the development of products with specific therapeutic properties, as well as in the food industry as natural preservatives or flavorings (14,15).

Principal component analysis and hierarchical cluster analysis

Hierarchical cluster analysis (HCA) and principal component analysis (PCA) were used to examine the variability in the chemical composition of four samples of juniper essential oils (**JC1**, **JC2**, **JC3**, and **JC4**). PCA was performed on a total of 23 identified compounds.

Principal component 1 (PC 1) explains 52.09 % of the variance and is strongly positively correlated with the following compounds: D-limonene (**C8**, 0.938), α-cubebene (**C10**, 0.998), copaene (**C11**, 0.999), β- elemene (**C12**, 0.999), β-cubebene (**C18**, 0.934), bicyclogermacrene (**C19**, 0.999) and α-elemene (**C20**, 0.980). All mentioned compounds belong to the group of sesquiterpenes except D-limonene (**C8**) which belongs to monoterpenes.

Principal component 2 (PC 2) explains 30.11 % of the variance and is strongly positively correlated with the following compounds: sabinene (**C4**, 0.893), humulene (**C16**, 0.893), and aristolene (**C22**, 0.893). Sabinene (**C4**) is the only compound that belongs to the monoterpene group, while the other compounds belong to the sesquiterpene group. The arrangement of identified compounds in the context of PC 1 and PC 2 is presented in Figure 1.

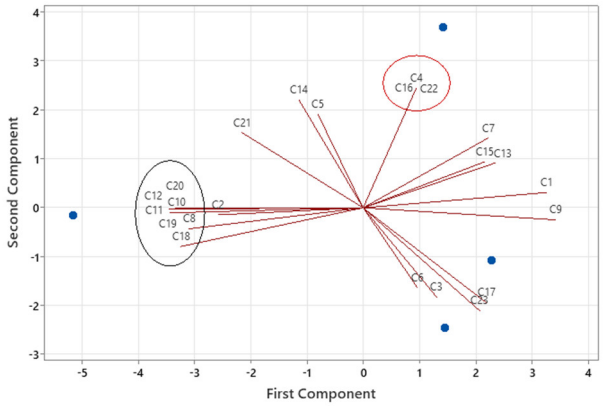

Figure 1. The loadings of each compound for principal components 1 and 2. The components with the highest correlation by component are encircled (PC1 - black line; PC2 - red line)

HCA was conducted using Centroid Linkage and Squared Euclidean Distance. It was shown that there are two clusters, where one cluster consists of essential oil from junipers collected on **JC1**, while the other cluster consists of essential oils obtained from junipers collected at the remaining three locations. Also, it was shown that essential oils from juniper originating from **JC3** and **JC4** are very similar. The HCA dendrogram is presented in Figure 2.

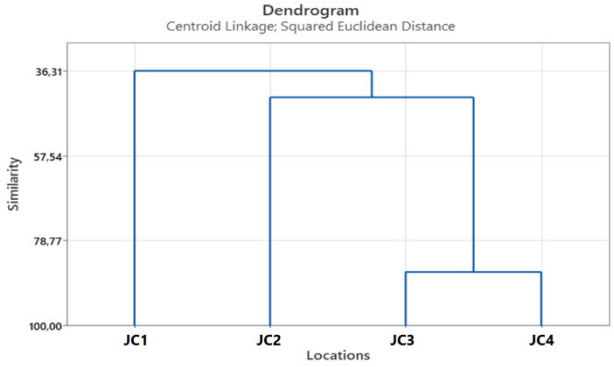

Figure 2. Dendrogram obtained by hierarchical cluster analysis of juniper essential oils from four different locations in the Republic of Serbia.

Antimicrobial activity

The results of *in vitro* testing of the antimicrobial activities of the tested essential oils, tetracycline, and fluconazole are shown in Table 3. The intensity of antimicrobial action varies depending on the microorganism species and the type of essential oil. In general, the tested juniper berry essential oil showed good activity, with the **JC4** sample being the most effective.

The **JC2** sample demonstrated the best antimicrobial effect (MIC ranging from <3.91 µl/ml to 31.25 µl/ml), followed by the **JC4** sample (MIC ranging from <3.91 µl/ml to 62.5 µl/ml). The **JC3** sample exhibited moderate activity (MIC ranging from <3.91 µl/ml to 250 µl/ml), while the **JC1** sample showed a limited and selective effect (MIC ranging from 250 µl/ml to >2000 µl/ml).

The strongest antibacterial effect of the **JC2** sample was observed against *E. coli* ATCC 25922 (MIC and MMC values at <3.91 µl/ml). The **JC4** sample showed the strongest effect on *P. mirabilis* ATCC 12453 and *E. coli* ATCC 25922 (MIC and MMC values at <3.91 µl/ml). The **JC3** sample exhibited stronger activity against *S. aureus* ATCC 25923 (MIC at <3.91 µl/ml; MMC at 7.81 µl/ml) and *P. aeruginosa* ATCC 27853 (MIC at <3.91 µl/ml; MMC at 15.65 µl/ml).

The **JC4** and **JC3** samples also demonstrated the best antifungal effects (MIC at <3.91 µl/ml; MMC at 15.65 µl/ml), followed with **JC2** sample (MIC at <3.91 µL/mL; MMC at 62.50 µL/mL).

The positive antimicrobial effect of the essential oil from the **JC2** sample can be attributed to the high content of α-pinene (**C2**), as such indications have already been presumed (28,29). Sabinene (**C4**), identified only in the essential oil from **JC2**, has already shown effectiveness of inhibiting the growth of certain bacterial strains (30), so we can assume its additive antimicrobial effect in this oil. The same can be said for sesquiterpene such as β-caryophyllene (**C13**), present in all samples with the highest abundance in the oil sample from **JC2**, known for its anti-inflammatory properties, but studies also indicate its role in antimicrobial activity (31,32). Marčetić et al. (33) observed that the qualitative and quantitative composition of essential oils varies based on the substrates where the species were sampled. These variations explain the differing antimicrobial effects of essential oils from the same species collected in different localities. Furthermore, Hyldgaard et al. (34) highlighted that the antimicrobial activity of juniper essential oil is influenced not only by its major constituents but also by the interactions between major and minor constituents, which can lead to additive or synergistic effects.

Compared to Tetracycline, essential oils show significantly weaker antimicrobial activity. This is not surprising, given that synthetic antibiotics are specifically designed to target key pathways in pathogenic microorganisms with high efficiency. However, the importance of essential oils may lie in their application as complementary therapies, especially in the context of the growing problem of antibiotic resistance. Many studies have shown that combining essential oils with a conventional antibiotic can enhance the antimicrobial effect, suggesting a synergistic interaction between these agents (32,35).

Table 3. The antimicrobial activity of *J. communis* essential oils

Tested species	JC1		JC2		JC3		JC4		Tetracycline/ Fluconazole	
	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC
B. subtilis ATCC 6633	500	500	15.63	62.50	125	1000	62.50	62.50	1.95	15.63
S. aureus	1000	>2000	<3.91	31.25	125	500	7.81	31.25	0.98	15.62
S. aureus ATCC 25923	500	1000	15.63	31.25	<3.91	7.81	7.81	62.50	0.22	3.75
P. aeruginosa ATCC 27853	500	500	7.81	62.50	<3.91	15.65	7.81	250	62.5	125
P. mirabilis ATCC 12453	500	1000	31.25	31.25	62.50	125	<3.91	<3.91	125	125
E. coli	>2000	>2000	7.81	31.25	31.25	62.50	<3.91	15.65	15.63	31.25
E. coli ATCC 25922	500	1000	<3.91	<3.91	7.81	15.63	<3.91	<3.91	15.63	31.25
S. enterica	>2000	>2000	7.81	62.50	250	500	<3.91	15.65	15.63	31.25
C. albicans ATCC 10231	250	1000	<3.91	62.50	<3.91	15.65	<3.91	15.65	31.25	1000

MIC-minimum inhibitory concentrations; MMC-Minimum microbicidal concentration; Values are expressed in µl/ml for essential oils and in µg/ml for tetracycline and fluconazole

Antioxidant activity

Antioxidant activities of the essential oils were analyzed using free radical scavenging (DPPH and ABTS assays and the results are presented in Table 4.

Table 4. The antioxidant activity of the *J. communis* essential oils

Samples/ Standards	DPPH scavenging activity	ABTS scavenging activity
	IC_{50} (µg/ml)	
JC1	> 1000	896.11 ± 7.02
JC2	308.83 ± 3.85	237.74 ± 4.61
JC3	> 1000	> 1000
JC4	966.89 ± 10.83	793.66 ± 16.23
Ascorbic Acid	9.08±1.96	8.28±0.24
Trolox	14.26±3.81	12.40±0.40

*DPPH-1,1-diphenyl-2-picryl-hydrazyl free radical; ABTS-2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical; IC_{50}- concentration of the tested sample that inhibits 50% of radicals;

Essential oil from **JC2** demonstrates a relatively low IC_{50} value for both tests (308.83 µg/ml for DPPH and 237.74 µg/ml for ABTS), which indicates a significant ability to neutralize free radicals. **JC1** and **JC3** are characterized by IC_{50} values greater than 1000 µg/ml for the DPPH test and high but measurable values for the ABTS test. Essential oil from **JC4** sample with IC_{50} values of 966.89 µg/ml for DPPH and 793.66 µg/ml for ABTS, shows moderate antioxidant activity. α-Pinene (**C2**), present in high concentrations in all essential oils, is known for its antioxidant activity. Studies have shown that α-pinene effectively neutralizes free radicals and contributes to protection against oxidative stress (36, 37). It is present in the largest amount of essential oil **JC2**, which also showed the best antioxidant activity. β-Pinene (**C7**) is present in all tested samples. α-pinene and β-pinene belong to the group of monoterpene hydrocarbons, so their antioxidant effect can probably be explained by the presence of methylene groups in these molecules (37, 38). β-Phelandrene (**C3**) is present in significant amounts in three essential oil samples (24.77% **JC1**, 12.27% **JC4**, 6.43% **JC3**), and its antioxidant activity has already been confirmed (37). The absence of β-phellandrene in the **LC2** may indicate that the combination of different terpenes, and not the presence of a single compound, determines the overall antioxidant activity. D-Limonene (**C8**), in a previous study, showed its antioxidant activity even at low doses (39), and was also present in all oil samples tested. Its consistent presence may contribute to the baseline level of antioxidant activity in all oils tested, but variations in antioxidant activity suggest that the presence of other compounds plays a key role. β-Caryophyllene (**C13**), a sesquiterpene with observed antioxidant properties (40), although in small amounts, was present in all oil samples tested. The superior antioxidant activity of **JC2** can be attributed to the synergistic effects of its high α-pinene (**C2**) concentrations, the presence of D-limonene (**C8**), and the combination of β-caryophyllene (**C13**) and α-caryophyllene (**C14**), which together form a complex mix of antioxidant active compounds. **JC1** and **JC4** with moderate to weak antioxidant activity, show that even the presence of high concentrations of potentially active components (such as α-pinene in **JC1**) is not sufficient for high antioxidant efficiency without appropriate synergy between the components. Essential oil **JC3** with the weakest antioxidant activity, indicates that the absence of key antioxidant terpenes and the potential presence of compounds that do not significantly contribute to antioxidant activity, or even inhibit the activity of other components, may result in lower overall efficacy. It is very difficult to attribute the antioxidant activity of essential oils to only one active principle, considering that essential oils are mixtures of different chemical components. It is important to emphasize that sometimes the biological activity of the oil is not only influenced by the most abundant active principles but that the less abundant components can also have a positive effect on the activity.

CONCLUSION

The current study compared the yields, compositions, and bioactivities of essential oils extracted from *J. communis* plants collected from distinct areas in Serbia. The research revealed that precipitation in the habitat was the most influential ecological factor affecting the essential oil yields of the samples. A total of 23 components, rich in monoterpenes and sesquiterpenoids, were identified in the essential oils from the four samples. The chemical variation among the essential oils from the four samples could be strongly influenced by the altitude of the habitats. The four samples could be divided into two clusters according to the variability of their components. The study assessed the antioxidant and antimicrobial activities of essential oils extracted from the four samples. The results suggested that the essential oils from *J. communis* possess promising potential for applications across several industries, such as food, cosmetics, and pharmacy, owing to

their inherent dual antioxidant and antimicrobial properties. The sample from Mačkat (**JC2**) exhibited superior performance in DPPH, ABTS, and antimicrobial assays, coupled with the highest yield. This implies that *J. communis* sourced from this location shows the greatest potential for further industrial utilization.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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EFFECT OF HYPERBARIC OXYGENATION ON THE SEVERITY OF EXPERIMENTAL AUTOIMMUNE MYOCARDITIS IN GAL-3 DEFICIENT MICE

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ABSTRACT

Myocarditis is an inflammatory heart disease, which is characterized by the presence of a cellular infiltrate in the myocardial interstitium, along with the degeneration and necrosis of cardiomyocytes. Depending on the predominate immune mechanism in the disease, Gal-3 may either attenuate or enhance the development of inflammation. Treatment with hyperbaric oxygenation (HBO) is considered a promising adjunctive therapy for cardiovascular disease due to increasing evidence of its beneficial effect on myocardial function. The potential effects of HBO treatment on myocarditis in animal models have not been investigated. The aim of this study was to delineate the impact of HBO on both the clinical course and histochemical characteristics of EAM. EAM was induced in Gal-3-deficient mice on the C57BL/6J background by immunization with myosin peptide MyHCα$_{334-352}$. The EAM group treated with HBO characteristically showed a significant improvement in FS compared to the untreated EAM group, as well as a reduction in LVIDd and LVIDs. Gal-3KO mice developed more severe myocarditis, characterized by accumulation of mononuclear cells and single mononuclear cells between cardiomyocytes, than animals treated with HBO. Additionally, EAM mice receiving HBO treatment showed a lower degree of degeneration and necrosis compared to the untreated EAM group. A significant reduction in fibrosis was noted in Gal-3KO mice with EAM after HBO treatment compared to the untreated group of EAM mice. The results showed that HBO treatment can improve cardiac function, reduce cardiac inflammatory infiltration, myocardial necrosis, and fibrosis, which could alleviate cardiac remodeling, dilated cardiomyopathy, and subsequent development of heart failure.

Keywords: *Experimental autoimmune myocarditis, Galectin-3, HBO, rats.*

INTRODUCTION

Myocarditis is an inflammatory heart disease, which is characterized by the presence of a cellular infiltrate in the myocardial interstitium, along with the degeneration and necrosis of cardiomyocytes. Clinical presentation can vary widely, from an asymptomatic disease to a severe condition that can lead to a fatal outcome. Autoimmune processes are increasingly recognized as important mechanisms in myocarditis initiation and development (1,2). Autoimmune myocarditis, also known as giant cell myocarditis, is a rapidly progressive form of myocarditis that frequently results in chronic inflammation and subsequent life-threatening complications (3). The experimental autoimmune myocarditis (EAM) animal model is a suitable platform for testing novel treatments and elucidating the potential therapeutic value of interventions in myocarditis management (4).

Hyperbaric oxygenation (HBO) has emerged as a promising and cost-effective therapeutic modality for counteracting inflammation. Notably, the interplay between hyperoxia and hyperbaric pressure modulates inflammation by targeting oxygen and pressure sensitive genes (5). Preclinical studies have demonstrated that HBO treatment significantly reduces levels of inflammatory cytokines such as intraleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) in animal models of ischemia or injury (6-17). Additionally, HBO treatment is considered as a promising adjunctive therapy for cardiovascular diseases due to growing evidence of its beneficial effects on myocardial function. Cardioprotective effects of HBO therapy has been demonstrated in streptozotocin-induced diabetic rat (18). Furthermore, clinical study conducted by *Leitman et al*. has shown that prolonged HBO treatment can improve ventricular function and myocardial performance (19).

Galectin 3 (Gal-3), a galactosidase binding lectin, plays multiple roles in immune responses and can be constitutively or inducibly expressed in different cell types. The expression of this protein can be induced by various inflammatory stimuli. Depending on the predominate immune mechanism in the disease, Gal-3 may either attenuate or enhance the development of inflammation (20-22). For example, as a toll-like receptor 4 ligand, Gal-3 has been shown to enhance inflammation, and its depletion has neuroprotective and anti-inflammatory effects in LPS-induced inflammation (23). Furthermore, HBO has been shown to regulate the expression of Gal-3 and TLR-4 genes. In a rat model of neuroinflammation treated with HBO, inhibition of these inflammatory genes expressions was observed (15).

The role of Gal-3 in cardiovascular diseases is complex and multifaceted. While some studies have shown that depletion of Gal-3 enhances the severity of myocarditis and type 2 cardiac inflammation in EAM mouse models (24), other studies have demonstrated that inhibition of Gal-3 slows the progression of myocardial inflammation, impedes myocardial fibrogenesis, and improves cardiac function (25-30). Additionally, inhibition of Gal-3 has been shown to reduce the size of infarcts and decrease tissue injury in models of myocardial ischemia/reperfusion injury both in vivo and in vitro (31). Gal-3 has been identified as a mediator of myocardial fibrosis, promoting fibroblast proliferation and heterogeneous deposition of collagen types, ultimately leading to impaired cardiac function (28-30, 32-33).

The current therapeutic options for myocarditis are primarily focused on symptom management, highlighting the need for novel and effective treatment strategies. Hyperbaric oxygen therapy is an attractive candidate due to its cardioprotective and anti-inflammatory effects. However, the potential effects of HBO treatment on myocarditis in animal models have not been investigated. Thus, this study aimed to evaluate the impact of HBO on both the clinical course and histochemical characteristics of EAM in Galectin 3 deficient mice.

MATERIALS AND METHODS

Experimental Animals and Animal Care

Animals used in this study were male Gal-3-deficient mice on the C57BL/6J background (Gal-3KO), aged between 6-8 weeks. They were originally obtained from the University of California Davis (Davis, CA; by courtesy of D.K.Hsu and F.T.Liu). To create Gal-3KO mice, the Gal-3 gene was targeted for disruption in C57BL/6J embryonic stem cells, resulting in the generation of mice homozygous for the disrupted Gal-3 gene (34). The genotypes of the Gal-3KO mice were confirmed by PCR. The mice were housed in the animal facilities of the Faculty of Medical Sciences, University of Kragujevac, Serbia, and maintained under standard laboratory conditions with ad libitum feeding. All animal experiments were approved by the Ethical Committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia (01-2630). Experiments were in accordance with the European Union Directive for the welfare of laboratory animals (No. 2010/63/EU).

Initially, mice were randomly divided into 3 groups (6 animals per group) according to the applied protocol as follows:

- Healthy control Gal-3KO mice (CTRL)
- Gal-3KO mice with induced EAM (EAM)
- Gal-3KO mice with induced EAM and HBO treatment (EAM+HBO)

Induction of Experimental Autoimmune Myocarditis

Myocarditogenic peptide ($MyHC\alpha_{334–352}$, Shanghai ShineGene Molecular Biotech) was used to induce EAM. The suspension was prepared by dissolving 100 μg $MyHC\alpha_{334–352}$ peptide in 100 μL of PBS and was emulsified with 100 μL of complete Freund's adjuvant (CFA) (Sigma-Aldrich, Germany) that contained 500μg Mycobacterium tuberculosis (strain H37 RA; Difco Laboratories, Detroit, MI). Subcutaneous injection of 200 μl of the prepared suspension was given to mice in the hind flanks on day 0 and day 7. In addition, each treated mouse was intraperitoneally injected with 200 ng of pertussis toxin (List Biological Laboratories,

Campbell, USA) dissolved in 100 µL of distilled water on the same day and again after 48 hours. Mice were sacrificed on day 21 following immunization, and their hearts were collected for further analyses.

HBO Treatment

Mice were exposed to 100% oxygen using a hyperbaric pressure chamber (HYB-C 300, Maribor, Slovenia). Hyperbaric oxygenation was administered daily for 60 minutes at a pressure of 2 ATA, starting from the 2nd day of EAM induction until the 21st day. To avoid the effects of diurnal rhythm variations, hyperbaric oxygenation was always initiated at the same time.

Echocardiographic Analyses

The cardiac function of the animals was assessed *in vivo* on the 21st day using echocardiography before they were sacrificed. Prior to the procedure, the animals were anesthetized with a mixture of ketamine (75 mg/kg) and xylazine (5 mg/kg) injected intraperitoneally. Echocardiograms were obtained using a Hewlett-Packard Sonos 5500 (Andover, MA, USA) sector scanner equipped with a 15.0 MHz phased-array transducer (16). M-mode images were acquired from the parasternal long-axis view in 2-dimensional mode with a perpendicular M-mode cursor positioned on the interventricular septum and posterior wall of the left ventricle (LV) at the papillary muscle level. M-mode measurements included interventricular septal wall thickness at end-diastole (IVSd), LV internal dimension at end-diastole (LVIDd), LV posterior wall thickness at end-diastole (LVPWd), interventricular septal wall thickness at end-systole (IVSs), LV internal diameter at end-systole (LVIDs), and LV posterior wall thickness at end-systole (LVPWs). Fractional shortening percentage (FS%) was calculated using the M-mode LV diameters and the equation: ((LVIDd − LVIDs) / LVIDd) × 100%.

Histological assessment of EAM

Mouse hearts were harvested, fixed in 10% buffered formalin overnight, and then embedded in paraffin. The paraffin-embedded samples were sliced into 5 µm thick sections in a base-to-apex direction and stained using the hematoxylin and eosin (H&E) method. The histological assessment involved blinded microscopy by two independent investigators. The scoring system included quantification of the localization, intensity, and nature of the inflammatory infiltration, as well as the degree of degeneration and necrosis of cardiomyocytes and myocardial fibrosis.

The localization, intensity, and nature of inflammatory infiltration were quantified as follows: localization was graded as 0 (disease-free), 1 (apex), 2 (lateral wall), or 3 (septum); intensity was graded as 0 (no infiltrates in the visual field), 1 (less than 5 individual mononuclear cells in contact with the sarcolemma in the visual field), 2 (5-20 single mononuclear cells in contact with the sarcolemma in the visual field), or 3 (more than 20 individual mononuclear cells in contact with the sarcolemma in the visual field); nature was graded as 0 (no inflammatory infiltrates), 1 (small foci of inflammatory cells along the membrane), 2 (inflammatory cells grouped into confluent aggregates), or 3 (mononuclear cells diffusely scattered in the myocardium). Evaluation of the degree of degeneration and necrosis of cardiomyocytes was quantified using the following score: 0 (disease-free), 1 (discrete, unicellular), 2 (low-grade, >10% of the observed field), 3 (moderate, 10-50% of the observed field), or 4 (severe, pronounced, >50% of the observed field). Fibrosis was scored using the following system: 0 (absent or discrete, very rare connective cells), 1 (low-grade, individual connective cells), 2 (moderate, between 1 and 3), or 3 (pronounced, numerous partly grouped connective cells). The images were captured using a light microscope (Olympus) equipped with a digital camera. Scores were calculated for each tissue clip and compared with the control.

Statistical Analyses

The Statistical Package for Social Sciences v23.0 (SPSS Inc.) was used to perform the statistical analysis. Depending on the normality of the data, the differences between groups were determined using either the non-parametric Kruskal–Wallis H and Mann–Whitney U tests or parametric One-Way ANOVA and Independent Samples T-test. The data are presented as mean ± SEM and a significance level of $P < 0.05$ was considered statistically significant.

RESULTS

The improvement of fractional shortening percentage in Gal3-KO EAM mice was observed after the HBO treatment.

Echocardiography was performed to evaluate the effect of HBO treatment on cardiac function in mice with MyHC$\alpha_{334-352}$-induced myocarditis 21 days after immunization. A significant impairment of fractional shortening (FS) was found in EAM mice compared to the healthy CTRL group. However, a marked improvement of FS was observed in the group of EAM mice after HBO treatment compared to the non-treated EAM group. Additionally, LVIDd and LVIDs were significantly reduced in Gal-3KO mice that received HBO treatment compared to the non-treated EAM mice (Table 1).

Table 1. Effects of HBO treatment on echocardiographic parameters: interventricular septal wall thickness at end systole and end-diastole (IVSs and IVSd), left ventricular internal diameter at end-systole and end-diastole (LVIDs and LVIDs), left ventricular posterior wall thickness at endsystole and end-diastole (LVPWs and LVPWd), and fractional shortening (FS). CTRL: healthy control Gal-3KO mice; EAM: Gal-3KO mice with induced EAM; EAM+HBO: Gal-3KO mice with induced EAM and HBO treatment. Values are presented as means ± mean standard error (SE). Kruskal–Wallis H; Mann–Whitney U tests $^{*}p< 0.05$ indicates statistical significant differences between groups #compared to CTRL, ∗compared to EAM.

	CTRL	EAM	EAM + HBO
IVSd (cm)	0.064±0.052	0.093±0.101	0.100±0.032
LVIDd (cm)	0.249±0.177	0.210±0.134	0.203±0.024*
LVPWd (cm)	0.058±0.062	0.086±0.110	0.092±0.016
IVSs (cm)	0.145±0.034	0.141±0.013	0.161±0.039
LVIDs (cm)	0.090±0.037	0.137±0.017	0.097±0.047*
LVPWs (cm)	0.103±0.023	0.080±0.024	0.107±0.020
FS (%)	63.9±2.241	43.6±5.51#	52.5±4.84 *

The severity of EAM was reduced in the hearts of Gal-3KO EAM mice after HBO treatment.

To assess the impact of HBO treatment on the severity of EAM in mice immunized with MyHC$\alpha_{334-352}$, a histological score was calculated for each experimental group. This score represented the sum of the localization, intensity, and nature of inflammation. The histological analysis showed a significantly higher score in the EAM and EAM+HBO groups compared to the healthy CTRL group. Severe myocarditis was detected in the hearts of immunized mice, characterized by the accumulation of mononuclear cells and single mononuclear cells between cardiomyocytes. Nonetheless, lower histological score and smaller inflammatory infiltrates were found in animals that received HBO treatment (Figure 1A and Figure 2, respectively).

Cardiac muscle degeneration and necrosis decreased in Gal-3KO EAM mice after HBO treatment.

Further investigation focused on assessing cardiac muscle degeneration and necrosis after HBO treatment. The results showed a significant increase of necrosis in both the EAM and EAM+HBO groups compared to healthy CTRL controls. However, EAM mice that received HBO treatment exhibited a lower degree of degeneration and necrosis compared to the non-treated EAM group, but without a statistically significant difference (Figure 1B).

Myocardial fibrosis decreased in Gal-3KO mice with EAM after HBO treatment.

The histological assessment of the HBO treatment effect proceeded with the evaluation of myocardial fibrosis in all experimental groups. A significantly higher level of fibrosis was observed in the EAM and EAM+HBO groups compared to healthy CTRL group. However, it was found that there was a significant reduction in myocardial fibrosis in Gal-3KO mice with EAM after HBO treatment compared to the untreated group of EAM mice (Figure 1C).

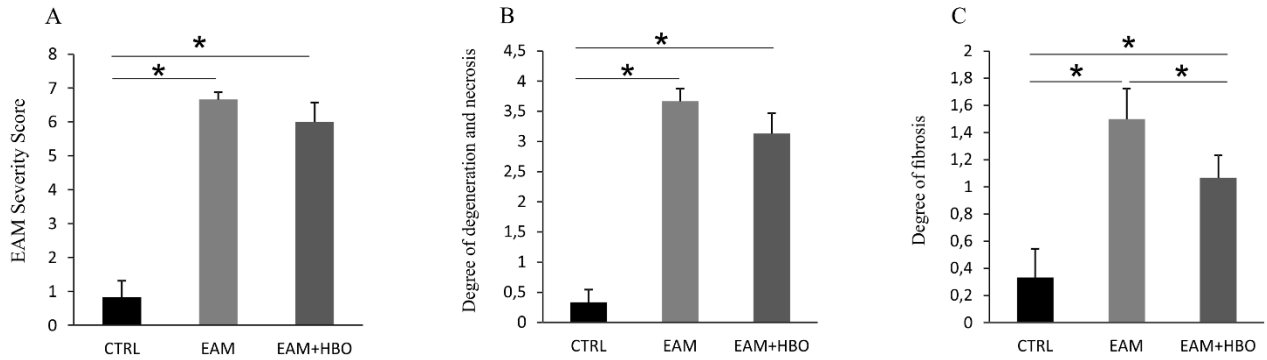

Figure 1. Effects of HBO treatment on EAM severity score (A), degree of degeneration and necrosis of cardiomyocytes (B), and degree of fibrosis (C). CTRL: healthy control Gal-3KO mice; EAM: Gal-3KO mice with induced EAM; EAM+HBO: Gal-3KO mice with induced EAM and HBO treatment. Values are presented as means ± mean standard error (SE). Independent Samples t test; One-Way ANOVA *$p< 0.05$ indicates statistical significant differences between groups.

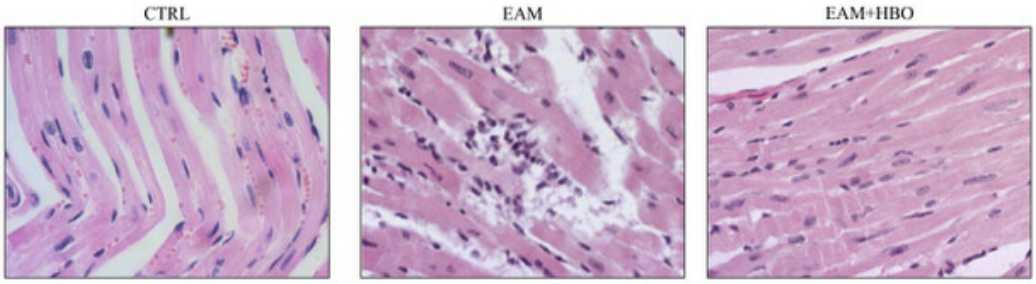

Figure 2. Representative images of hematoxylin/eosin (H&E) staining of paraffin embedded heart tissue sections. CTRL: healthy control Gal-3KO mice; EAM: Gal-3KO mice with induced EAM; EAM+HBO: Gal-3KO mice with induced EAM and HBO treatment. Original magnification 40×.

DISCUSSION

The results of present study indicated beneficial effects of HBO treatment in MyHC$\alpha_{334-352}$-induced autoimmune myocarditis in Gal-3-deficient mice on the C57BL/6J background. We have shown a significant improvement in FS, reduction in LVIDd and LVIDs, as well as a reduction in the severity of EAM, degeneration and necrosis of cardiomyocytes after HBO treatment. Moreover, hearts of Gal-3KO mice treated with HBO had significantly less myocardial fibrosis compared with untreated EAM group.

Myocarditis causes inflammation of heart tissue, leading to the infiltration of inflammatory cells, myocardial necrosis, and replacement fibrosis. These changes ultimately result in impaired cardiac function, dilated cardiomyopathy, and heart failure (1,2). Currently, symptomatic treatment and heart transplantation are the only therapeutic options available for myocarditis. Unfortunately, there is no known therapeutic strategy that effectively halts or reverses the disease's progression (35). However, HBO therapy presents an ideal candidate for potential myocarditis treatment due to its cardioprotective and anti-inflammatory effects, both of which are crucial for addressing the disease's underlying mechanisms.

HBO is considered a promising adjunctive therapy for cardiovascular diseases due to growing evidence of its cardioprotective effects. A recent study demonstrated that prolonged HBO treatment leads to an increase in both left and right ventricular systolic function, particularly in the apical segments, and is associated with better cardiac performance in asymptomatic patients (18). HBO therapy as an adjunct treatment in patients with acute myocardial infarction demonstrated a favourable effect on both cardiac function and the remodelling process, as evidenced by studies (36, 37). Additionally, preconditioning rats with HBO has been demonstrated to alleviate injury to the ischemic myocardium, while both hyperbaria and hyperoxia have been shown to significantly reduce infarct size and attenuate ischemia-reperfusion injury (38, 39). Moreover, a study has shown that HBO treatment increases the recovery of cardiac function and reduces infarct size in rat hearts that have undergone transplantation with mesenchymal stem cells (40). It has been shown that the cardioprotective effect, conferred by the combined exposure of hyperoxia and hyperbaria, is directly dependent on oxygen availability and mediated via the nitric oxide signalling pathway. (41). In our study, we confirmed the cardioprotective effects of HBO therapy in EAM mice. Hemodynamic measurements showed that the EAM group experienced a significant reduction in FS. However, HBO treatment resulted in a marked improvement in myocardial function, as evidenced by the significant improvement in FS. Additionally, we observed wall thinning and reduced myocardial fibrosis in the HBO-treated group. Hence, it is plausible to suggest that HBO treatment may offer a means of preventing or impeding the development of left ventricular remodelling and further progression of myocarditis.

The strong anti-inflammatory properties of HBO therapy have been demonstrated in various animal models of diseases. It has been shown that HBO treatment reduces the levels of inflammatory cytokines, IL-1α and TNF- α in these models (6-18). For example, HBO treatment was shown to markedly lower cardiac TNF-α levels and mitigate myocardial damage-associated inflammation (16). Moreover, recent study has reported decreased cardiac TNF-α levels in both high-fat-fed and aged rats following HBO intervention (17). Our findings are in alignment with previous studies, which provide evidence for the potent anti-inflammatory capabilities of HBO therapy. The histopathological confirmation of EAM induction was based on the distinct and severe inflammatory infiltration and augmented necrosis in the cardiac tissues. Notably, the application of HBO therapy resulted in a reduction in EAM severity and a decrease in necrosis levels.

Previously published studies have indicated that the anti-inflammatory effect of hyperbaric oxygen may involve the inhibition of the Galactin-3-dependent Toll-like receptor 4 pathway in a rat model of neuroinflammation (15). However, our findings suggest that the anti-inflammatory mechanism of HBO in autoimmune myocarditis suggest to be Gal-3 independent, as the effects were observed in Gal-3-deficient mice. While Gal-3 inhibition has been demonstrated to reduce myocardial fibrogenesis (28-30, 32-33) and improve fractional shortening (31), our results demonstrate that Gal-3 deficient mice treated with HBO had reduced fibrosis and improved FS. Therefore, HBO could be a promising alternative therapeutic approach alone or in combination with Gal-3 inhibitors for reducing fibrosis and improving cardiac function in cardiovascular diseases.

CONCLUSION

In summary, this study has demonstrated the potential effectiveness of hyperbaric oxygen as an adjunctive therapy for autoimmune myocarditis. Our findings suggest that HBO treatment can improve cardiac function, reduce cardiac inflammatory infiltration, myocardial necrosis, and fibrosis, thereby could mitigate heart remodelling, dilated cardiomyopathy, and the subsequent development of heart failure. These results represent the initial empirical evidence supporting the therapeutic value of HBO in the context of cardiac autoimmunity. Nevertheless, further research endeavours and additional experiments are necessary to elucidate the precise mechanisms underlying the beneficial effects of HBO in experimental autoimmune myocarditis pathology.

ETHICS APPROVAL

All research procedures were carried out in strict accordance with the European Union Directive for the welfare of labora-tory animals (No. 2010/63/EU) and approved by the Ethics Committee for welfare of experimental animals, Faculty of Medical Sciences University of Kragujevac.

CONFLICT OF INTEREST

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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INFLUENCE OF DIRECT PULP CAPPING WITH CALCIUM HYDROXIDE AND MINERAL TRIOXIDE AGGREGATE ON SYSTEMIC OXIDATIVE STRESS IN RATS

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ABSTRACT

Direct pulp capping is a procedure where materials are placed on exposed vital pulp tissue in order to stimulate reparative dentinogenesis and preserve pulp vitality. Carious pulp exposure and direct pulp capping are usually accompanied by pulp inflammation which can affect pro- and anti-oxidative systems locally or systemically. Therefore, this study aimed to investigate the potential influence of direct capping of inflamed rat dental pulp with calcium hydroxide (CH) and mineral trioxide aggregate (MTA) on parameters of systemic oxidative status.

Dental pulps of the first maxillary molars of Albino rats (n=32) were exposed and capped with either CH (n=8), MTA (n=8) or were left untreated (n=8). Animals with healthy pulp were used as a healthy control (n=8). After four weeks, animals were euthanized and blood samples were collected for biochemical analysis of parameters of systemic oxidative stress by spectrophotometric method.

Untreated control had the significantly higher ($p < 0.05$) values of pro-oxidative parameters and lower ($p < 0.05$) values of anti-oxidative parameters (superoxide dismutase and reduced glutathione) compared to healthy control. CH and MTA groups showed reduced values of pro-oxidative parameters compared to untreated control and values of anti-oxidative parameters comparable to healthy control.

Pulp exposure led to disbalance in systemic oxidative parameters while direct pulp capping with calcium hydroxide and mineral trioxide aggregate restored the levels of systemic oxidative parameters to that of animals with healthy dental pulp. These results indicate the importance of direct pulp capping and the potential influence of untreated inflamed pulp on systemic health.

Keywords: *Direct pulp capping, calcium hydroxide, mineral trioxide aggregate, oxidative stress.*

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INTRODUCTION

The dental pulp tissue is located within dentinal walls, however pulp exposure can occur as a result of dental caries or trauma or can be induced iatrogenically during caries excavation or tooth preparation (1,2). Following traumatic and iatrogenic pulp exposure, dentin and adjacent odontoblast cells are destroyed causing reversible inflammation that, if left untreated, can cause irreversible damage to pulp tissue. This initial inflammation is even more evident in cases of carious pulp exposure (1,3). Direct pulp capping is defined as covering of exposed vital pulp tissue with bioactive material in order to stimulate formation of reparative dentin and preserve pulp vitality (4). Besides the size of pulp exposure, the presence of microorganisms or the patient's age, the type of material used for direct pulp capping affects the success rate of the capping procedure (5). Over time, different materials have been proposed and developed for direct pulp capping (calcium hydroxide (CH), zinc oxide eugenol cement, mineral trioxide aggregate (MTA), enamel matrix derivative, resin-modified glass-ionomer, hydroxyapatite etc.)(2).

CH has been considered the "gold standard" material in direct pulp capping for a long period mainly due to its good properties that include antimicrobial activity, promotion of mineralization, and the formation of a reparative dentin bridge (6). Successful direct pulp capping with CH has been attributed to local rise in pH that induces bactericidal effects and sterile local necrosis of the exposed pulpal tissue. This necrosis produces a mild inflammation that promotes the differentiation and migration of immune and odontoblast-like cells, stimulating the repair processes in dental pulp (7). However, many limitations in the use of CH were recognized including poor sealing ability, low mechanical properties, high resorption, and induction of dentin bridge formation with tunnel defects which may be a pathway for the spread of microorganisms (8). Several bioactive materials, such as MTA, have been proposed to overcome these disadvantages, These materials demonstrated high biocompatibility, excellent sealing properties, and homogenous dentin bridge formation (8). Placing MTA on tissue leads to hydration of cement, formation of calcium silicate hydrate and release of calcium hydroxide, which further dissociates into OH^- and Ca_2^+ ions (9). Ionic exchange with dental pulp tissue makes MTA a bioactive material that acts antimicrobial, regulates the production of pro-inflammatory cytokines, and stimulates the differentiation and activity of odontoblast-like cells (10). Although MTA has not been shown to cause superficial necrosis as calcium hydroxide, it similarly produces high pH environment which together with ion release stimulates acute and mild inflammation that favors the onset of reparative processes in the pulp (11). Both CH, and MTA showed high success rates in direct pulp capping of healthy pulp tissue at around 90% (8). However, in cases of carious pulp exposure which is often accompanied with reversible pulp inflammation, these rates are significantly lower dropping to below 60%, as shown for CH in previous study (12).

Upon inflammation, immune cells, such as neutrophils, are drawn to the site of pulp exposure in response to injury and microbial insults (13). By propagating the inflammatory process in the pulp, neutrophils have been shown to secrete reactive oxygen species (ROS) and reactive nitrogen species (RNS) (14). The formation of ROS and RNS is a normal part of cellular metabolism, however, if they are present in high concentrations or due to insufficient activity of anti-oxidative systems, they cause oxidative stress and can be an important mediator in cellular damage (15). This oxidative disbalance can be reflected systemically and can affect other tissues and organs (16). Dental materials are shown to affect oxidative status having both pro- and anti-oxidative effects depending on the material (17), despite that, effects of direct pulp capping on oxidative parameters in inflamed exposed pulp has not been studied to date.

Since carious pulp exposure and direct pulp capping are accompanied by pulp inflammation and given the complexity of reparative dentinogenesis, we postulated that pro- and anti-oxidative systems can be affected by direct pulp caping procedure. Therefore, we aimed to investigate the potential influence of direct pulp capping in inflamed rat dental pulp with CH and MTA on parameters of systemic oxidative status.

MATERIAL AND METHODS

Animals and ethical considerations

All experiments were approved by the Animal Ethics Committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia (approval number 01-16176/3). The experimental procedures were performed in accordance with the European Union Directive for the Welfare of Laboratory Animals (No. 2010/63/EU), the Directive of the Council of the European Communities (86/609 / EEC), the principles of good laboratory practice and guidelines for animal research.

Eight-week- old male Wistar rats, average weight 250 ± 50 g, maintained in the vivarium of the Faculty of Medical Sciences, University of Kragujevac were used in study. Animals were exposed to a standard diet with unrestricted access to food and water (*ad libitum*) at normal room temperature (22-25 °C) and day/night cycles of 12 hours.

The sample size (n= 8) for each group was calculated based on the study of a similar design (18) using G-Power software (v3.1.9.7; Faculty of Mathematics and Natural Sciences, Dusseldorf, Germany) considering the power of 0.80 and probability of alpha error 0.05 for the T-test conditions for two independent samples.

Experimental pulp exposure and direct pulp capping

Prior to experimental procedure, animals (n=24) were anesthetized by intraperitoneal injection of ketamine hydrochloride (80 mg/kg) and xylazine (10 mg/kg). The cavities were prepared on occlusal surfaces of the left first maxillary molars using sterile round carbide burs (FG.TC 1/2; Micro

Diamond Technologies, Afula, Israel). After reaching a preparation depth at around 1 mm, the dental pulps were exposed using a sterile endodontic K-file size ISO 30/02 (KFS03021; Dentsply Sirona, New York, USA). The cavities were left exposed next 48 hours to induce reversible pulp inflammation as previously described (19).

After 48 hours, the animals were re-anesthetized as previously described. The cavities were washed with sterile saline solution, dried with sterile paper points and disinfected with a paper point soaked in 2% sodium hypochlorite solution. The appropriate capping material was placed in each cavity directly on exposed pulp tissue. The cavities were restored with light-curing glass-ionomer cement (Fuji II LC; GC Europe, Leuven, Belgium) (19).

After the capping procedure, rats were separated in cages depending on the applied material, and divided into four groups as follows:

1. CH (Dycal; Dentsply Sirona, New York, USA) (n=8): calcium hydroxide was prepared according to the manufacturer's instructions and placed on exposed pulps of the left maxillary molars;
2. MTA (MTA Biorep; ITENA Clinical, Paris, France) (n=8): MTA was prepared according to the manufacturer's instructions and placed on exposed pulps of the left maxillary molars;
3. Untreated control (UC) (n=8): the exposed pulp of the left maxillary molars was covered with a collagen membrane (BioMend; Zimmer Biomet Dental, FL, USA) soaked with a drop of sterile distilled water.
4. Healthy control (HC) (n=8): Experimental animals whose pulps were not exposed or capped.

After a four-week follow-up period, the animals were sacrificed followed by dissection of left maxillae and blood sample collection. The experimental animals were exposed to short-term ether anesthesia before sacrifice. The sacrifice was performed by cervical dislocation. Blood samples were collected from jugular vein and placed into test tubes containing sodium citrate anticoagulant. Blood samples were processed and stored immediately. Blood was centrifuged to separate plasma and red blood cells (RBCs).

Pathohistological evaluation

After dissection, the left maxillae were fixed in 4% paraformaldehyde (Thermo Fisher Scientific, Waltham, MA, USA) for 24h. The samples were decalcified in 10% EDTA/phosphate-buffered saline solution (Sigma-Aldrich, St Louis, MO, USA) for three weeks and embedded in paraffin. Serial 5µm sections were cut using a rotary microtome system. Haematoxylin-eosin (H&E) stained sections were examined under DM4000B light microscope with DMC295 camera and Qwin V3 software (Leica Microsystems, Wetzlar, Germany). Histological scores of pulp inflammation and mineralization were evaluated using grading system that was previously reported (19) and presented in Table 1.

Evaluation of Systemic Oxidative Status

The concentration of pro-oxidative markers (nitrites (NO_2^-), hydrogen peroxide (H_2O_2), and superoxide anion (O_2^-)) and index of lipid peroxidation measured as thiobarbituric acid reactive substances (TBARS) were evaluated in plasma samples. The parameters of the anti-oxidative system including the level of reduced glutathione (GSH), and the activities of catalase (CAT) and superoxide dismutase (SOD) were measured in the lysate of erythrocytes. All oxidative parameters were evaluated spectrophotometrically using methods previously reported and described briefly.

Determination of the Index of Lipid Peroxidation

Index of lipid peroxidation was assessed by measuring TBARS using 1% thiobarbituric acid (Sigma-Aldrich) in 0.05 NaOH (Sigma-Aldrich), incubated with plasma at 100°C for 15 minute and determined spectrophotometrically (λ=530 nm) (20)

Nitrite Determination

The level of NO_2^- was determined as an index of nitric oxide (NO) production with Griess's reagent (20). A total of 200 µl of plasma was precipitated with 100 µl of 3 N perchloric acid (Thermo Fisher Scientific) and 400 µl 20mM ethylenediaminetetraacetic acid (Sigma-Aldrich), left on ice for 15 min, and centrifuged at 6000 rpm for 15 min. After pouring of the supernatant, 220 µl of potassium carbonate (Thermo Fisher Scientific) was added. To the 200 µl of final mixture, 250 µl Griess's reagent (Abcam, Cambridge, UK) and 125 µl of previously prepared buffer for NO were added followed by incubation at room temperature for 15 min before measuring. The levels were measured spectrophotometrically (λ= 550nm).

Superoxide Anion Radical Determination

The 50 µl of plasma sample and 950 µl of assay mixture was mixed. The assay mixture contained 50 mM TRIS-HCl (tris (hydroxymethyl) aminomethane-hydrochloric acid) buffer (pH = 8.6) (Sigma-Aldrich), 0.1 mM ethylenediaminetetraacetic acid (Sigma-Aldrich), 0.1 mg/ml gelatin (Sigma-Aldrich) and 0.1 mM nitro blue tetrazolium (Sigma-Aldrich). The level of O_2^- was measured three times every 60 seconds at a wavelength of λ=530 nm (20).

Hydrogen Peroxide Determination

H_2O_2 was measured as a level of Phenol red oxidation by hydrogen peroxide catalyzed by horseradish peroxidase (20). A total of 800 µl of Phenol red solution (MP Biomedicals, Solon, OH, USA) and 10 µl of rhubarb peroxidase (Sigma-Aldrich) were added to 200 µl of plasma sample. After incubation at room temperature for 10 minutes, absorbance was measured at λ=610 nm.

Table 1. Evaluation criteria for rat pulp tissue response based on inflammation and mineralized tissue formation

	Score	Definition
Pulp inflammation		
	0	Absent or very few inflammatory cells
	1	Mild: inflammatory cells only next to dentin bridge or area of pulp exposure
	2	Moderate: inflammatory cells are observed in the part of coronal pulp tissue
	3	Severe: all coronal pulp tissue is infiltrated or necrotic
Pulp mineralization		
	0	No trace of mineralization in the pulp
	1	Increased deposition of hard tissue along the surface of the exposed pulp tissue
	2	Extensive mineral deposition in the coronal pulp tissue

Determination of Reduced Glutathione

The level of GSH was determined on the basis of GSH oxidation with 5.5-dithio-bis- 6.2-nitrobenzoic acid in erythrocyte lysate (20). A mixture was prepared containing 50 µl of sample, 200 µl of 0.1% ethylenediaminetetraacetic acid solution (Sigma-Aldrich) and 385 µl of previously prepared precipitation buffer. This mixture was incubated on ice for 15 minutes and then centrifuged at 4000 rpm for 10 minutes till extract was formed. Afterwards, 300 µl of extract, 750 µl of disodium hydrogen phosphate (Sigma-Aldrich) and 100 µl of 5,5-dithio-bis-6,2-nitrobenzoic acid (Sigma-Aldrich) were added to the new tubes and incubated at room temperature for 10 minutes. The absorbance was measured at λ= 412 nm.

Determination of Catalase activity

CAT activity was determined in a previously diluted erythrocyte lysate (20). A total of 100 µl of erythrocyte lysate was diluted in 10 ml of distilled water and 100 µl of 70% ethanol alcohol (Reahem, Novi Sad, Serbia). A 100 µl of previously prepared dilution was added to 50 µl of previously prepared CAT buffer with 1 ml of 10mM H_2O_2 (Sigma-Aldrich). Measurements were repeated 6 times for each sample. Absorbance was measured in quartz cuvettes at λ=230 nm.

Determination of Superoxide Dismutase

SOD was measured in a mixture containing 100 µl of erythrocyte lysate and 1 ml of carbonate buffer (Thermo Fisher Scientific). After vortexing the mixture, a 100 µl of adrenaline (Sigma-Aldrich) was added. Absorption measurements were performed in duplicate at λ=470 nm (20).

Statistical analysis

Statistical data processing was performed in the statistical software IMB SPSS Statistics for Windows v23.0 (IBM Corp., Armonk, NY, USA). The Shapiro – Wilk normality test was used to assess the normality of the data distribution. Depending on the normality of the distribution, the Nonparametric Kruskal-Wallis H and Mann-Whitney U tests or the parametric One-Way ANOVA and Independent Samples T-test were used. Results are presented as mean ± standard deviation (SD). A value of $p < 0.05$ was set as an indicator of statistical significance.

RESULTS

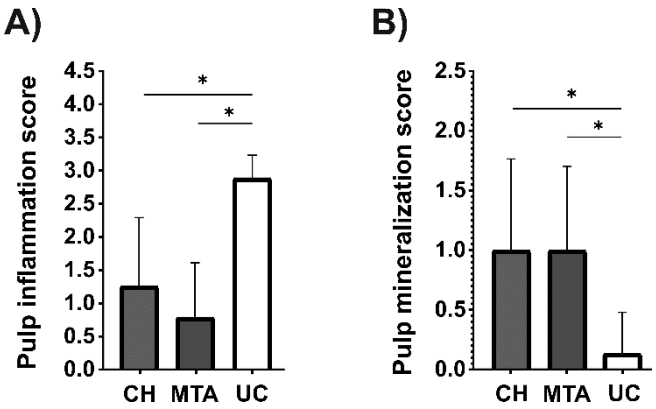
Pulp inflammation and mineralization

Contrary to the sound dental pulp tissue on H&E sections of HC group, UC group presented high level of tissue disorganization and necrosis. H&E sections of UC group presented severe inflammation accompanied by loss of odontoblast layer, inflammatory cell infiltrate and cellular necrosis with absence of pulp mineralization in exposed coronary pulp. CH and MTA groups mostly presented no or mild inflammation with restored odontoblast-like cell layer, mineralized bridge formation and absence of inflammatory cell infiltrate in coronary pulp under the site of pulp exposure. Evaluation of pathohistological scores is presented on Figure 1.

The highest value of pulp inflammation score (associated with the greatest inflammation) was observed in UC group which was significantly higher ($p < 0.05$) compared to CH and MTA groups (Figure 1A). Pulp mineralization score values showed no significant difference ($p > 0.05$) between CH and MTA groups, however, both groups presented signi-

ficantly higher values (p <0.05) (indicating high level of mineralization) compared to UC group (Figure 1B).

Figure 1. Pulp inflammation and pulp mineralization evaluation scores



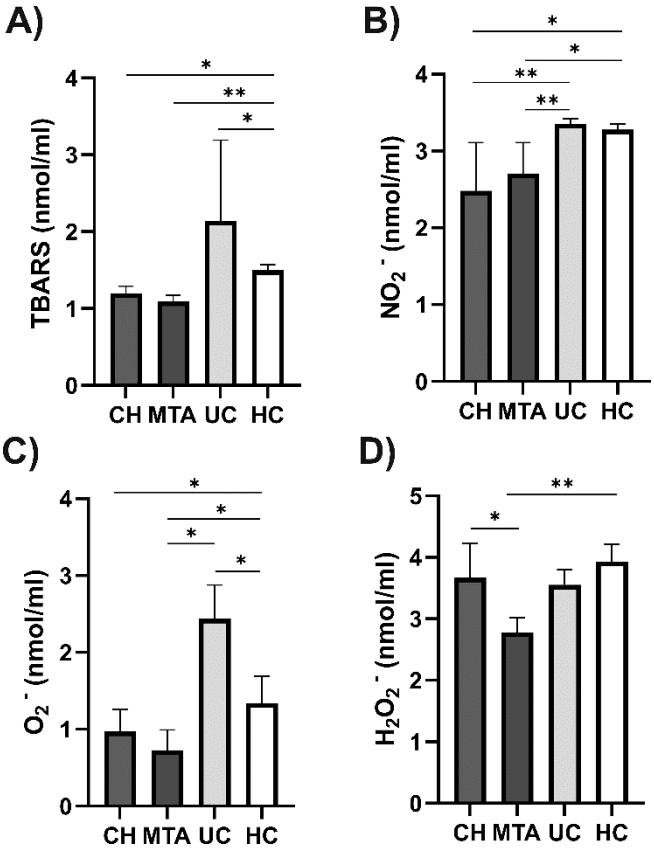
(A) Pulp inflammation scores between CH, MTA and UC groups (B) Pulp mineralization scores between CH, MTA and UC groups. The results are presented as mean ± SD of 8 animals per group. * statistically significant difference (p <0.05) (Kruskal-Wallis post-hoc test);

Effects of direct pulp capping on pro-oxidative markers

The measured values of pro-oxidative markers (TBARS, NO_2^-, O_2^-, and H_2O_2) were compared between the experimental groups (CH, MTA, UC and HC) and presented in Figure 2.

The value of TBARS was the highest in the UC group, which was significantly higher compared to HC group (p <0.05). Although, CH and MTA groups had mean values of TBARS similar to healthy control (HC) group, statistical analysis showed significant difference between CH and HC groups (p <0.05), as well as MTA and HC groups (p <0.001) (Figure 2A). The NO_2^- value was also the highest in UC group. NO_2^- values in CH and MTA groups were significantly lower compared to the UC group (p <0.001), as well as to HC group (p <0.05) (Figure 2B). UC group presented the highest mean value of O_2^- which was significantly higher (p <0.05) compared to CH, MTA, and HC groups (Figure 2C). Both CH and MTA groups showed significantly lower (p <0.05) mean O_2^- values compared to HC group. MTA group also presented the lowest mean value of H_2O_2 which was statistically significant compared to CH (p <0.05) and HC groups (p <0.001). No statistically significant difference (p >0.05) was found between UC and other groups (Figure 2D).

Figure 2. Systemic pro-oxidative parameters



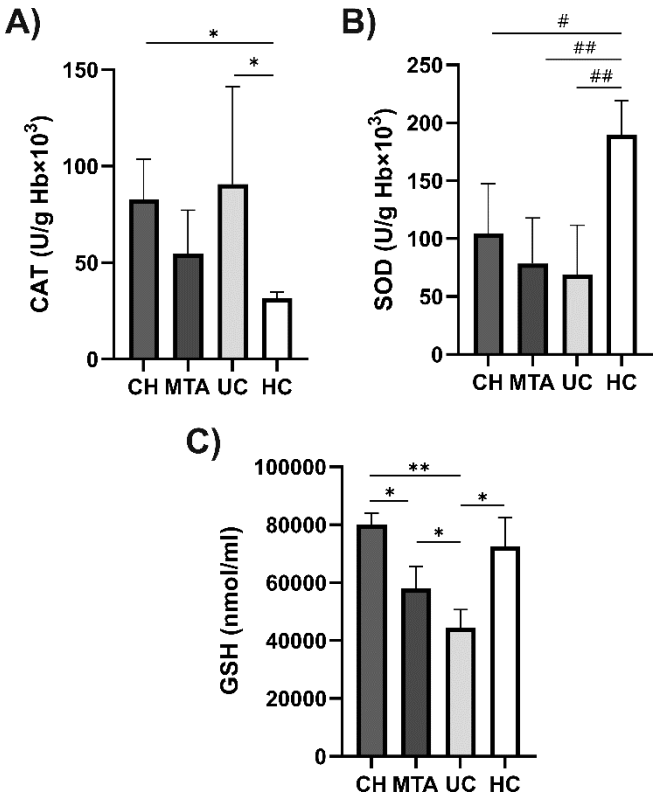
(A) Level of TBARS in systemic circulation of CH, MTA, UC and HC groups (B) Level of NO_2^- in systemic circulation CH, MTA, UC and HC group (C) Level of O_2^- in systemic circulation CH, MTA, UC and HC groups (D) Level of $H_2O_2^-$ in systemic circulation CH, MTA, UC and HC groups. The results are presented as mean ± SD of 8 animals per group. * statistically significant difference (p <0.05) (Kruskal-Wallis post-hoc test); ** statistically significant difference (p <0.001) (Kruskal-Wallis post-hoc test)

Effects of direct pulp capping on anti-oxidative markers

The measured values of anti-oxidative parameters (CAT, SOD, and GSH) were compared between the experimental groups (CH, MTA, UC and HC) and presented in Figure 3.

The mean values of CAT were higher in all groups compared to the HC group. However, a statistically significant difference (p <0.05) was presented between CH and HC and UC and HC groups (Figure 3A). The values for CAT were not statistically significantly different between MTA and HC groups (p >0.05). UC group showed significantly lower (p <0.001) SOD mean value compared to HC group, as well as MTA and CH groups (p <0.05) (Figure 3B). The UC group also showed significantly lower (p <0.05) level of GSH compared to the HC group (Figure 3C). CH and MTA groups showed no statistical difference to HC group (p >0.05), while having significantly higher values (p <0.05) compared to UC group (Figure 3C).

Figure 3. Systemic anti-oxidative parameters



(A) Level of CAT in systemic circulation of CH, MTA, UC and HC groups (B) Level of SOD in systemic circulation CH, MTA, UC and HC groups (C) Level of GDH in systemic circulation CH, MTA, UC and HC groups; * statistically significant difference (p <0.05) (Kruskal-Wallis post-hoc test); # statistically significant difference (p <0.05) (Oneway ANOVA/Turkey post-hoc test); ## statistically significant difference (p <0.001) (Oneway ANOVA/Turkey post-hoc test)

DISCUSSION

In this study for the first time, we analyzed the influence of direct pulp capping of inflamed pulp on systemic parameters of oxidative stress. The goal of direct pulp capping is the healing of exposed dental pulp by the formation of dentin bridge. As shown in our study, direct pulp capping with CH and MTA resulted in mineralized bridge formation presented with higher pulp mineralization scores to UC group (Figure 1B). As in any other wound healing, healing of exposed dental pulp passes through hemostasis, inflammation, cell proliferation, angiogenesis, and eventually tissue remodeling (21,22). Conditions associated with oxidative stress such as inflammation, hypoxia, or the effects of systemic diseases often occur in dental pulp (23). Carious lesion as well as pulp exposure during caries excavation often led to reversible pulp inflammation. During inflammation, immune cells including macrophages and neutrophils produce cytokines as well as ROS in order to destroy pathogens (24,25). In order to cope with ROS production, the cells of the organism possess anti-oxidant systems that include enzymes such as catalase, superoxide dismutase, hydrogen peroxidase and the system of glutathione peroxidase and reductase, among others (17,23,25,26). However, inability of anti-oxidant systems to cope with overproduction of ROS can led to disorders of local oxidative status that can negatively affect surrounding healthy cells causing oxidative stress and leading to irreversible pulp disorders. This can also be reflected systemically, causing the effects of oxidative stress in other organs as well (16,27). One of the properties of direct pulp capping materials is reducing inflammation and preventing the occurrence of irreversible damage to the pulp (28). Thus, we hypothesized that direct pulp capping could affect systemic oxidative parameters in rats with inflamed dental pulp.

Previous study reported that pulp tissue had high level of inflammation 28 days after direct capping of reversibly inflamed rat pulp with only collagen carrier, unlike capping with bioactive materials (29). This coincides with the results of our study, where UC group capped with collagen membrane presented high level of pulp inflammation (Figure 1A). As expected, UC group showed a significant increase in systemic pro-oxidative parameters, NO_2^- and O_2^- (Figure 2B and C) compared to groups capped with bioactive materials (CH and MTA). Initially increased ROS production is considered beneficial for wound healing, due to its antimicrobial effect and stimulation of angiogenesis. On the other hand, prolonged and increased ROS production is associated with delayed healing and chronic wounds (22). In the current study, direct pulp capping with CH and MTA reduced the systemic values of the prooxidative parameters opposed to untreated control (Figure 2). These result are in agreement with reduced inflammation in rat pulp capped with different formulations of CH and MTA shown previously and in our study (30).

To avoid oxidative damage, antioxidant system composed mainly of a group of enzymes responsible for the control of ROS is triggered in state of inflammation (31). However, previous studies showed contradictory results on levels of anti-oxidant enzymes in healthy and inflamed dental pulp (32–34). Grossi et al. (32) reported that low levels of SOD could indicate high susceptibility to inflammation, which is in accordance to our results with significantly low levels of SOD in UC compared to HC group (Figure 3B). On the contrary, Topcu et al. (33) reported that inflamed dental pulp tissue have had significantly higher activity of SOD compered to healthy pulp (33). Regarding CAT, previous study showed very low anti-oxidative activity in healthy human pulp tissue (34), similar to our results with lowest level of CAT in healthy control (Figure 3A). Dental materials with proven cytotoxicity, such as dental resins, were shown to produce depletion of GSH in dental pulp cells inducing tissue inflammation and cellular damage (35). Unlike them, CH and MTA were shown to have anti-inflammatory effect and low cytotoxicity when used in direct pulp capping (10,36) In line with that, use of these materials for direct pulp capping showed higher levels of antioxidative parameter GSH compared to UC group in present study (Figure 3C).

Interestingly, some pro-oxidative parameters in CH and MTA groups were presented at lower levels even when compared to healthy control (Figure 2). This divergence in results, as well as others, can be explained as the consequence of different stages of inflammation in pulp tissue that undergoes dentine reparation. Increased levels of ROS in a sample should not be deem as a certain sign of inflammation, because it has been proven that restoring the redox balance in organism takes time (37). One of the limitations of this study was that the pulp inflammation and parameters of oxidative stress were assessed only once at 28 days after pulp capping procedure. Further studies should evaluate and compare these parameters in different time intervals. The disbalances in several parameters could also be a result of disturbances in other tissues and organs. Although all animals were subjected to same procedures and conditions, health of other organs and tissues was not assessed in our study, which could be considered as another limitation. Importantly, prolonged disbalance between the production and elimination of ROS could increase the risk of developing different diseases. It has been shown that an increased production of ROS linked to oral diseases such as chronic periapical lesions could have consequences on systemic health (24,38).

CONCLUSION

In conclusion, our results indicate that pulp exposure leads to disbalance of systemic oxidative parameters reflected in rise of pro-oxidative and fall of anti-oxidative markers. Additionally, direct pulp capping with calcium hydroxide and mineral trioxide aggregate restored the levels of systemic oxidative parameters to that of animals with healthy dental pulp thus revealing the importance of direct pulp capping and the potential influence of untreated inflamed pulp on systemic health. Therefore, we can conclude from these findings that through adequate pulp therapy, oxidative balance can be restored, thereby avoiding the risk of dentally induced consequences on systemic health.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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PD-1 BLOCKAGE FACILITATES CYTOTOXIC T AND NK CELLS TUMORICIDAL PHENOTYPE IN A MURINE BREAST CARCINOMA

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ABSTRACT

In breast cancer therapy, as the leading cause of death in women, besides chemo-radiotherapy, immunotherapy has been increasingly used. PD-1/PD-L1 axis blockade primarily acts on T lymphocytes, the main effectors of acquired immune response. NK cells, which are part of the innate immune response, also play a role in the anti-tumor response through the blockade of this signaling pathway. The study was conducted to examine the effects of anti-PD-1 therapy on NK and T cells in mouse breast cancer. Female BALB/c mice were used, divided into two groups, one with induced breast cancer and one treated with anti-PD-1 antibody. Breast cancer cell line was used to induce the cancer, and the anti-PD-1 antibody was applied intraperitoneally. Cell populations in spleen and tumor microenvironment were examined using flow cytometry. Data were statistically analyzed using SPSS. The percentage of NK cells expressing FasL, NKG2D, and IFN-γ is significantly higher in spleen and tumor-infiltrating NK cells upon anti-PD-1 therapy, while the expression of inhibitory markers Foxp3 and IL-10 in regulatory NK cells is significantly lower. The percentage of T lymphocytes expressing CD107a and IL-17 is significantly higher in the spleen, while a higher number of T lymphocytes expressing CD69 is present in the tumor microenvironment. The study suggests that anti-PD-1 therapy can activate NK and T cells, and improve anti-tumor immune response in breast cancer. Further research is needed to understand the interplay between these cells during PD-1 blockage.

Keywords: *PD-1, NK cells, cytotoxic T cells, mammary carcinoma.*

INTRODUCTION

Breast cancer is the most common malignant tumor globally and the first cause of death in the female population. Breast cancer is the leading cause of global cancer incidence in 2020, with an estimated 2.3 million new cases, and representing 11.7% of all cancer cases. The number of patients is constantly increasing throughout the world, especially in developed countries (1). GLOBOCAN 2020 data for Serbia show that in 2020, there were 6.724 newly discovered cases of breast cancer in our country, while the number of deaths was 2.342 (1, 2).

Chemoradiotherapy as the main form of treatment for advanced breast cancer has its limitations due to the manifestation of side effects and inadequate response to the drug (3-5). In recent years, treatments of breast cancer have been expanded with immunotherapy (6). Immunotherapy with so-called immune check point inhibitors, is used to alleviate the response of patients and promote the antitumor effect of T cells, and has become an important strategy in the treatment of many malignant diseases (6, 7).

The programmed cell death 1 molecule (programmed cell death 1, PD-1, CD279) was first discovered in 1992 in the LsD9 mouse hematopoietic progenitor cell lines and 2B4-11 mouse T-cell hybridoma in the stages of apoptosis. At that time, the PD-1 molecule was considered to be involved in apoptosis, hence its name (8). PD-1, primarily expressed on activated T cells' surface, but also by NK (natural killer) and NKT (natural killer T) cells (9, 10), can combine with its ligand PD-L1 (programmed cell death 1 ligand), which presents expression on tumor cells and immune cells, to deprive T cells of killing ability (7). However, PD-L1-to-PD-1 binding can suppress the function of lymphocytes and reduce the release of cytokines. It can also promote the apoptosis of lymphocytes. Therefore, the function of $CD8^+$ T cells is inhibited, which may lead to the immune escape of tumors. PD-1/PD-L1 inhibitors are capable of blocking PD-L1-to-PD-1 binding and reducing the immune escape phenomenon of tumors (7).

Ligands for the PD-1 molecule are PD-1L, expressed on macrophages, activated T and B lymphocytes, dendritic cells and some epithelial cells, especially in inflammation, and PD-2L expressed on macrophages, peritoneal B lymphocytes and activated dendritic cells (11–13). In addition to the cells of the immune system, PD-1L is also expressed on tumor cells, which is associated with a worse prognosis (14).

By blocking the PD-1/PD-1L axis, the antitumor response of the immune system is enhanced (15). Through the increased expression of PD-1L on tumor cells, the activation of primarily T lymphocytes is inhibited. In this way, the tumor avoids the antitumor immune response (16). The main effector cells in antitumor immunity are cytotoxic T lymphocytes and helper T lymphocytes (17, 18). They achieve their antitumor role through the production of cytotoxic substances containing granzyme and perforin and through the induction of apoptosis through FasL. Cytotoxic and helper lymphocytes also secrete large amounts of cytokines into the tumor microenvironment, especially IFN-γ, which is responsible for the expression of ligands for PD-1 (19, 20). Cells of innate immunity, NK cells, also play an important role in the antitumor immune response. The degree of tumor tissue infiltration by these cells is a good prognostic indicator (21). They achieve an antitumor effect by different mechanisms, stimulate the migration of T lymphocytes into tumor tissue and directly eliminate tumor cells (22). They also play a role in preventing metastases by eliminating circulating tumor cells (23).

The role of the PD-1/PD-1L axis in tumor biology is well known. However, the data are very modest when it comes to the effect of PD1 blockade on T lymphocytes and NK. Our data suggest that these cells may also be important for more effective anti-PD1 therapy in malignancy, and may contribute to an overall effective immune response to breast cancer, as anti-PD1 therapy induces phenotypic changes in T lymphocytes and NKT cells. In addition, research in animal models has shown that blockade of the PD-1/PD-1L axis significantly delays palpable tumor onset and slows primary tumor growth in experimental breast cancer models.

MATERIAL AND METHODS

The study was conducted, in the period from 2019 to 2021, at the Center for Molecular Medicine and Stem Cell Research of the Faculty of Medical Sciences, University of Kragujevac, as well as at the Institute for Medical Research of the Faculty of Medicine of the Military Medical Academy in Belgrade.

Experimental animals

For the research study, we used syngeneic female BALB/c (wild type, WT) mice, aged 6 to 8 weeks. During the experiments, the mice were kept in the vivarim, and in accordance with standard laboratory conditions (22 ± 2 °C, relative humidity 51 ± 5% and a 12-hour light-dark cycle). The animals had access to a source of food and water throughout the duration of the experiment.

Experimental animals are divided into experimental groups:

1) female BALB/c mice with breast cancer induced;
2) female BALB/c mice induced with breast cancer and treated with anti PD-1 antibody;

Tumor induction

Weakly immunogenic breast cancer cell lines (4T1) compatible with the BALB/c genetic background were purchased from ATCC (American Type Culture Collection, ATCC, USA). An earlier protocol (24) was used. A dose of $5^{x}10^{3}$ 4T1 cells was used, which was determined based on the results of a series of pre-experiments that preceded this research study.

Monitoring of palpable tumor appearance and tumor growth was followed by previously described methods (25).

Administration of anti PD-1 antibodies

Mouse anti PD-1 antibody was purchased from BioXcell (clone number: RMP1-14). The antibody was applied intraperitoneally to BALB/c mice on the third, sixth, ninth and eleventh days, starting from the day of tumor induction, in a dose of 150 μg dissolved in 150 μl of PBS (Phosphate-buffered saline). Mice that did not receive anti PD-1 antibody received intraperitoneally 150 μl of PBS on the same days.

Examination of cell populations in the spleen and tumor microenvironment - flow cytometry

Single-cell suspensions of spleen cells were obtained by mechanical dispersion, while single-cell suspensions of primary tumors were obtained by an enzymatic digestion process, as previously described (25). Fluorochrome-labeled anti-mouse antibodies specific for CD3, CD49, CD69, NKG2D (natural killer group 2D) and isotype controls (BD Pharmingen, NJ/Invitrogen, Carlsbad, CA) were used in the research. For intracellular staining, which was performed according to an established protocol (25), anti-mouse antibodies specific for CD107a, Foxp3 (forkhead box P3), IFN-γ, IL-10 (Interleukin - IL), IL-17 were used (BD Pharmingen/ BioLegend/eBiosciences). From 20,000 to 100,000 cells per sample were analyzed by flow cytometry. Flow cytometry was performed on a FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA) and data were analyzed using FlowJo software (Tree Star).

Ethical statement

The research was approved by the competent Ethical Commission for the welfare of laboratory animals of the Faculty of Medical Sciences of the University of Kragujevac (approval number: 01-12188 dated 26.10.2018). All research procedures were conducted in compliance with the Helsinki Declaration and the Principle of Good Laboratory Practice.

Statistical data processing

All obtained data were statistically processed using the software program (SPSS v.23), using Student's t test, Mann-Whitney U test, ANOVA or Kruskal–Wallis tests as needed. The difference in the appearance of the primary tumor was analyzed using the Kalpan-Meier curve and log-rank analysis.

RESULTS

Effect of PDL/PD-1 blockade on NK cells

The percentage of FasL-expressing $CD3^-CD4b^+$ NK cells was significantly higher in spleens of BALB/c mice on anti PD1 therapy compared to BALB/c mice without therapy ($p<0.05$; Figure 1a). The percentage of $CD3^-CD4b^+$ NK cells that were expressing NKG2D was significantly higher in the spleen of anti PD-1 treated BALB/c mice ($p<0.05$; Figure 1b). Anti PD-1 therapy significantly increased IFN-γ (interferon-gama) expression in splenic $CD3^-CD4b^+$ NK cells BALB/c mice compared to WT mice without therapy ($p<0.05$; Figure 1c). The percentage of $CD3^-CD4b^+$ NK cells producing IL-10 was significantly lower in WT mice on PD-1 therapy ($p<0.05$; Figure 1d). In the tumor microenvironment, anti PD-1 therapy significantly increased accumulation of $CD3^-CD4b^+$ NK cells that were expressing NKG2D ($p<0.05$; Figure 2a). Also, the percentage of FoxP3-expressing $CD3^-CD4b^+$ NK cells was significantly lower in tumors from WT mice on anti-PD-1 therapy compared to WT mice without therapy ($p<0.05$; Figure 2b).

Effect of PDL/PD-1 blockade on T lymphocytes

Application of anti-PD-1 therapy has significantly increased expression of CD107a molecule in splenic $CD3^+CD49b^-$ T lymphocytes ($p<0.05$; Figure 3a). Also, anti PD-1 therapy has significantly increased production of IL-17 in splenic $CD3^+CD49b^-$ T lymphocytes ($p<0.05$; Figure 3b). In the tumor microenvironment, the application of anti-PD-1 therapy has significantly increased percentage representation of $CD69^+$ $CD3^+CD49b^-$ T lymphocytes ($p<0.05$; Figure 4a). Anti-PD-1 therapy has significantly decreased percentage representation of $IL10^+$ $CD3^+CD49b^-$ T lymphocytes in compared to the experimental group without therapy ($p<0.05$; Figure 4b).

DISCUSSION

Immunotherapy of breast carcinoma is currently evolving filed, with usage of immune check-point inhibitors being in the limelight of scientific research (26). Anti-PD-1 therapy is still emerging as one of the leading therapeutics in breast cancer, with it being approved in triple-negative breast cancer (27). Effects of PD-1 blockage are mainly conveyed through facilitation of effector cells, such as T and NK cells (28). The roles of T and NK cells in immune response to breast carcinoma are intertwined. T cells eradicate tumor cells via presentation of tumor antigens, and NK cells additionally eradicate every transformed cell, regardless of tumor antigen presentation (29). In addition, activated NK cells can further on stimulate effector T cells. Anti-PD-1 therapy stimulates both types of cells, making overall immune response more efficient. To illustrate the exact effect behind T and NK cells activation during anti- PD-1 therapy, we conducted this study to analyze the phenotype changes of T and NK cells during immune response to breast carcinoma.

We firstly analyzed NK cells in both, spleen and tumor microenvironment. As mentioned above, NK cells have important role in eradicating all tumors cells, but chronic inflammation that takes place constantly in the tumor microenvironment, could induce inefficient regulatory phenotype of NK cells (30). On the other hand, some recent studies show that increased percentage of NK cells within tumor microenvironment are related to increased survival (31) Our results revealed that administration of anti-PD-1 antibodies increases the expression of activating markers such as FasL,

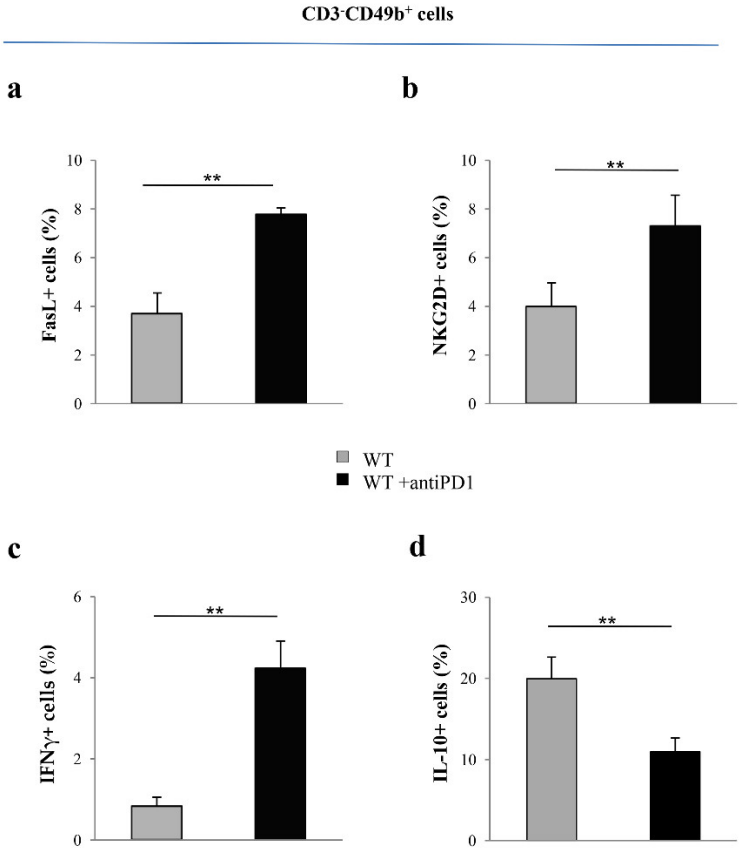
NKG2D and IFN-γ in both, splenic and tumor-infiltrating NK cells (Fig 1-2). These results are in line with previously published data (28). On the other hand, anti- PD-1 therapy significantly decreased the expression of inhibitory markers such as Foxp3 and IL-10 in NK cells (Fig 1-2). Foxp3 and IL-10 are thought to be markers of a regulatory NK cells phenotype (32). The application of anti- PD-1 therapy therefore efficiently reduces phenotype switch of NK cells towards regulatory one within spleen, and more importantly, within tumor environment.

The important role of T cells in antitumor immunity is well established. When it comes to T cells, anti PD-1 therapy increases the percentage of splenic $CD107a^+$ and $IL\text{-}17^+$ T cells (Fig 3-4). Our results are in line with previously published studies (33, 34). CD107a/LAMP-1 (Eng. *Lysosome-Associated Membrane Protein 1*) is one of the main glycoproteins in the membrane of cytolytic granules and also a marker of degranulation of NK cells and CTLs (35). CD107a expression correlates with cytokine secretion and cytotoxic function of NK cells. IL-17 is a marker of increased inflammatory response (36).

Within tumor microenvironment, after application of anti-PD-1 antibody, there is an increased percentage of $CD69^+$ T lymphocytes, and, on the other hand, there is decreased level of immunosuppressive IL-10 (Fig 3-4). The C-type lectin receptor CD69 represents an early activating marker and may play a direct role in mediating cytotoxicity against tumor target cells (37). These results are in line with previously published studies (38, 39). When it comes to IL-10, its increased expression could be indicative of exhaustion in T cells, skewing T cells towards immunosuppressive phenotype (40). As anti PD-1 blockage lowers IL-10 expression in tumor microenvironment, tumor-infiltrating T cells during anti PD-1 therapy are evidently more active conveyers of anti-tumor immunity. These phenotype changes of T cells in spleen and tumor microenvironment altogether illustrate a more active T cell, leading to overall more effective anti-tumor response.

In conclusion, our presented results indicate that anti- PD-1 therapy activates both, NK and T cells, producing a more improved anti-tumor immune response to breast carcinoma, which could potentially lead to better prognosis. More studies are required to further elucidate the interplay between NK and T cells during PD-1 blockage in breast carcinoma.

Figure 1. The percentage of FasL+ cells was significantly higher in spleens of BALB/c mice on anti PD1 therapy compared to BALB/c mice without therapy (1a).



The percentage of cells that were expressing NKG2D was significantly higher in the spleen of anti PD-1 treated BALB/c mice (1b). Increased IFN-γ expression in NK cells BALB/c mice compared to WT mice without therapy (1c). The percentage of NK cells producing IL-10 was significantly lower in WT mice on PD-1 therapy (1d). Statistical significance is defined using Student's t test.

Figure 2. Increased accumulation of $CD3^{-}CD4b^{+}$ NK cells that were expressing NKG2D (2a) and significantly lower percentage of FoxP3-expressing $CD3^{-}CD4b^{+}$ NK cells in tumors from WT mice with anti-PD-1 therapy compared to WT mice without therapy ($p<0.05$; Figure 2b). Statistical significance is defined using Student's t test.

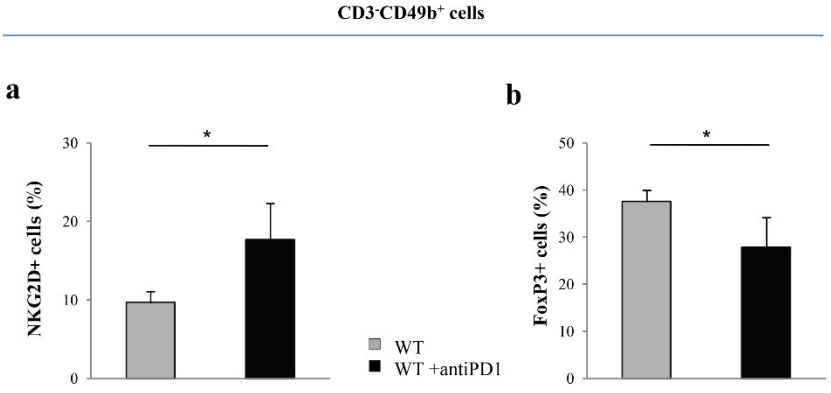


Figure 3. Significantly increased expression of CD107a molecule in splenic CD3+CD49b- T lymphocytes (3a) and significantly increased production of IL-17 in splenic CD3+CD49b- T lymphocytes (3b) in WT mice with anti-PD-1 therapy compared to WT mice without therapy. Statistical significance is defined using Student's t test.

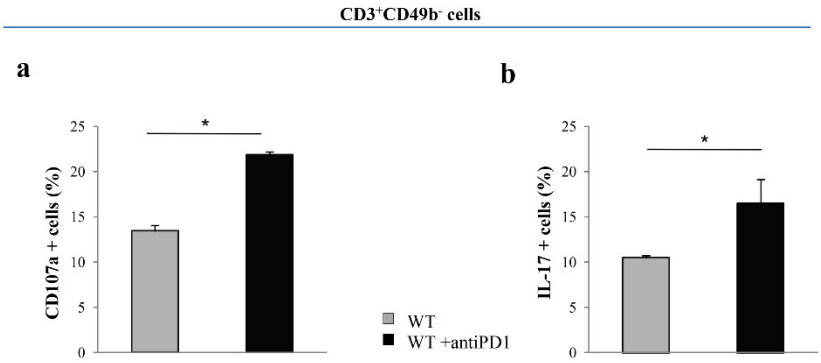
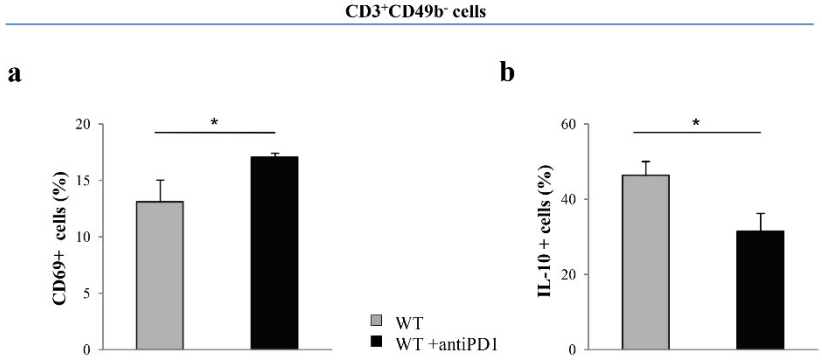


Figure 4. Significantly increased percentage representation of $CD69^{+}$ $CD3^{+}CD49b^{-}$ T lymphocytes (4a) and significantly decreased percentage representation of $IL10^{+}$ $CD3^{+}CD49b^{-}$ T lymphocytes (4b) in the tumor microenvironment of WT mice with anti-PD-1 therapy compared to the experimental group without therapy. Statistical significance is defined using Student's t test.



DECLARATION OF INTEREST

The authors declare that they have no competing interests.

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THE QUALITY OF LIFE OF PATIENTS WITH COLORECTAL CARCINOMA AND STOMA

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ABSTRACT

Surgical treatment and formation of a stoma can be lifesaving for colorectal cancer patients. However, the quality of life is often impaired in patients with stoma. The goal of this study is to determine the quality of life of patients with stoma and cancer, and the relationship between the quality of life and characteristics of these patients. The study was conducted from 2018 to 2020 and included a total of 64 patients of both sexes with colorectal cancer and temporary or permanent stoma. The European Organization for Research and Treatment of Cancer with Quality of Life Questionnaire (EORTC QLQC29-30) and the anonymous WHO Quality of Life Questionnaire SF-36 were used for self-assessment of quality of life. Our study included 39 (61.0%) men and 25 (39.0%) women. 24 patients (37.5%) had colostomy, 14 patients (21.9%) had ileostomy, and 26 patients (40.6%) underwent surgery for resection of colorectal cancer without stoma. A significant number of women were in the group of patients with a permanent stoma ($p = 0.01$). There was no statistically significant difference in the assessment of general health ($p = 0.680$) and quality of life ($p = 0.721$) during the past month in relation to gender. Patients without a stoma rated their general health better compared to those with stoma and the difference reached statistical significance ($p = 0.035$). There was no statistically significant difference in the assessment of quality of life between the group of patients with stoma and without stoma, as well as between the patients of different age groups. Patients with stoma rated their general health as worse, but not their quality of life.

Keywords: *Quality of life, colorectal cancer, stoma.*

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INTRODUCTION

Colorectal cancer is one of the biggest health problems in the world and a major public health issue with a trend of steady increase in new cases. (1) After the operation, patients get a stoma and their self-confidence and quality of life decreases greatly. (2) It is important that patients accept life with a stoma and adapt to the new situation, as well to acquire the skills and procedures that they will need throughout their lives. (3, 4) They need adequate and quality education from doctors and nurses and the help of family and friends who are with them at all times, helping them in the recovery period and everyday life. (5, 6, 7, 8) After colorectal cancer surgery, the lives of these patients are challenging as they need to adjust to life with cancer and a stoma. The concept of quality of life refers to the patient's subjective experience and feelings about the experience and the existence of specific determinants and conditions. (9) Initially, the feeling of joy and satisfaction with life with a stoma, inner peace and well-being and a life without burdens, especially without fear and uncertainty, which can often completely discourage the patient and thus impair their quality of life, is important. Such a relaxed life and a life without subconscious burden, without mental disorders and depression, with happiness, are the main emotions that describe the quality of life. The patient with a malignant disease and the consequences it bears feels and lives worse and it doesn't depend solely on the severity of the disease but also on the patient's view on their disease, improvement of their laboratory results, disease evaluation and health-related quality of life. (10, 11)

Historically, definitions and measurements of quality of life have changed. The World Health Organization Quality of Life Group (WHOQOL Group) defines quality of life in the context of the culture in which the patient lives and in relation to personal goals, expectations and concerns. (12, 13) Quality of life is viewed under the influence of physical health, mental well-being, degree of independence, connection with other people. Quality of life is a subjective concept and a subjective assessment of life as a whole or satisfaction with the degree of functioning. (14)

A stoma is formed when a part of the bowel is brought to the anterior abdominal wall. That visible part of the bowel called a colostomy, can be permanent or temporary, and causes many challenges in terms of quality of life and everyday functioning. Stoma localization depends on the localization of the cancer. The formed stoma temporarily excludes the distal intestine from the passage until the diseased part of the intestine recovers or is a permanent opening that serves to drain feces and gas. It is important that the stoma is positioned in such a way that it is accessible to the patient's sight and touch. (15) Healthy stoma is red or pink, round or oval, 2-5cm in diameter, has no nerve endings and therefore is not sensitive to touch. (16, 17) After the operation, the doctor informs the patient and his family if additional treatment is needed, such as radiation and chemotherapy, and about controls. The quality of life of a patient with a stoma largely depends on how much he and his family have accepted the procedures important for stoma care and the way of life with a stoma. (4, 18) Patients may have comorbid conditions that affect their ability to manage stoma care and may interfere with the patient's coordination and stoma management function. If the patient is incapable to take care of himself, the spouse and family are involved. The patient and family should be explained the importance of surgery and informed about the changes that occur after surgery.

Stoma brings changes in lifestyle with inevitable mental crises. Throughout the process of treatment and recovery, the influence and help of the family are important from a psychosocial point of view, especially to establish better patient stability. (5, 6, 7) After surgery and stoma formation, it is difficult to get used to the new situation that affects the patient, and in a broader sense, their family members. As per good quality of life, emotional experiences such as anger, helplessness, anxiety should be considered and faced and eventually 'feeling good' must grow into 'being good'.

Nowadays, the quality of life of patients with stoma is an integral part of the evaluation of therapeutic and rehabilitation procedures. The detail of personal factors of quality of life is very relevant in the field of health care of patients with malignant disease and stoma. Definitions of quality of life differ as do the ways in which they are assessed. One of the definitions of quality of life describes it as general well-being influenced by objective indicators and a large share of subjective perception, and assessments of emotional, social and material well-being, development of purposeful activity. (9) In recent years, efforts to improve the quality of life of patients with stoma, as one of the important goals of treatment, have encouraged doctors to undertake more research to identify different dimensions of quality of life and ways to improve them. Making good decisions to control complications of the disease, treatment and improvement of the quality of life is a very important goal in taking care of patients with stoma. (15) After surgery, many cancer and stoma patients experience greater stress, worry, and physical problems as a result of skin irritation (76%), stoma pouch leaks (62%), unpleasant odor (59%), and decreased pleasant activities (54%) and depression (53%). (19) In such circumstances, it is worth assessing the quality of life in assessing the outcome of various therapeutic and rehabilitation procedures and their ultimate impact on the quality of life of patients with stoma.

As previously noted, each patient experiences and accepts the stoma differently, so the quality of life after primary resection of colorectal cancer and stoma formation is individual and depends on the patient. Most patients take their lifestyle seriously and adhere to regular doctor's appointments, while some often neglect this and think that surgery has solved the problem. Of course, after the operation there are various negative effects that affect the social and mental, but also the physical component of the patient's life after getting a stoma. Patients are not accustomed to a different body appearance that comes with wearing a stoma and there is also a fear of

rejection by family and friends. They do not want to show themselves in public because they are afraid of judgement and prejudice of others and think that everyone will notice the stoma pouch under their clothes. (20) Due to that, many patients withdraw and do not want to contact with the social environment. (19, 21) In addition, they are not familiar with or educated about the balanced and adequate diet, necessary for physical activity and maintaining a high quality of life. Prevention and treatment of long-term complications in patients with stoma is also a well-known factor affecting the quality of life (22) Knowing the factors that contribute to stoma complications will help identify patients at higher risk. In addition, early detection enables timely interventions so that conservative measures can be effectively implemented (23).

The goal of this study is to determine the quality of life of patients with stoma and cancer, and the relationship between the quality of life of these patients and age, gender, education, work and marital status, type of complications and life expectancy. In addition, determining the effect of how well-informed the patient is on the quality of life with a stoma.

METHODS

The study was conducted in 2018-2020 and included a total of 64 patients of both sexes with colorectal cancer and the temporary or permanent stoma. The inclusion criteria were having a stoma for at least six months, the absence of other diseases and adequate physical and mental ability. The study was conducted using the anonymous WHO Quality of Life Questionnaire SF-36, which included questions on demographic data and patient satisfaction, and tested the difference in results between patients operated on for colorectal cancer with or without temporary or permanent stoma. The EORTC QLQC29-30 (European Organization for Research and Treatment of Cancer with Quality of Life Questionnaire) and SF-36 (tm) Health Survey questionnaires were used for self-assessment of quality of life.

Categorical data are presented in absolute and relative frequencies, and numerical data are described by median and interquartile range limits. Due to deviations from the normal distribution, differences in numerical variables by gender and marital status were tested using the Mann-Whitney U test, and differences in numerical variables according to age groups, complications, and colostomy duration were tested using the Kruskal-Wallis test. All p values are two-sided.

RESULTS

The study included 64 patients, of whom 39 (61.0%) were men and 25 (39.0%) women. The mean age of patients was 62.55 ± 9.365. The largest number of patients has completed high school 29 (45.3%), 31 are retired (48.5%), and 45 are married (70.0%). Of the total sample, 38 patients had a stoma, which is more than half of the sample, 59.5% to be exact.

Table 1. Patient characteristics

Patient characteristics	n (%)
Total	64(100)
Gender	
Male	39 (61.0)
Female	25 (39.0)
Age	
<30	0
31-50	17 (10.9)
51-70	36 (71.9)
>70	11 (17.2)
Education	
No formal education	0
Primary school	18 (28.1%)
High school	29 (45.3%)
College	11 (17.2%)
University and more	6 (9.4%)
Employment status	
Unemployed	8 (12.5%)
Employed	25 (39.0%)
Retired	31 (48.5%)
Marital status	
Unmarried	6 (9.5%)
Married	45 (70.0%)
Divorced	6 (9.5%)
Widowed	7 (11.0%)
Stoma	
Without	26 (40.5%)
Temporary	29 (45.5%)
Permanent	9 (14.0%)
Duration from stoma creation (months) (median-interquartile range)	48 (12 - 102)

Difficulties adjusting to life with a stoma make the patient's current problem, which is associated with fear, feeling of helplessness and loss of control. All this is accompanied by daily pressures, which are affected by daily challenges, loss of self-confidence and the problem of intimacy, long-term fears and worries that often lead to low self-esteem and loss of socialization.

Table 2. Patients in terms of pain and fatigue

Pain	n (%)				
	Not at all	A little	Quite a bit	Very much	Total
Have you had pain?	30 (46.8)	23 (36.0)	9 (14.1)	2 (3.1)	64 (100)
Did pain interfere with your daily activities?	26 (40.6)	25 (39.1)	12 (18.8)	1 (1.5)	64 (100)
Fatigue	n (%)				
	Not at all	A little	Quite a bit	Very much	Total
Did you need to rest?	20 (31.4)	28 (43.7)	14 (21.7)	2 (3.1)	64 (100)
Have you felt weak?	27 (42.2)	28 (43.7)	7 (11.0)	2 (3.1)	64 (100)
Were you tired?	22 (34.8)	27 (42.6)	12 (18.8)	3 (4.0)	64 (100)

We assessed feelings of pain and fatigue in our study. Most patients in our study reported feeling no pain (46.8 %) while 36.0% of them had mild pain, and 18.8% of patients stated that the pain interfered with their daily activities. Most patients (42.6%) felt a little tired and needed rest.

Table 3. Patient age and years with stoma by gender

	Median (interquartile range)			p*
	Male	Female	Total	
Patient age	61 (53 - 65)	57 (44 - 70)	60 (49 - 69)	0.53
Years with stoma	4.5 (3 - 8)	2 (1 - 4)	3.5 (2 - 7)	**< 0.001**

*Mann Whitney U test

The mean age of patients with stoma was 60 (median 60, interquartile range 49-69), ranging from 62.55 ± 9.365 years. In terms of living with a stoma, patients had a stoma for about 3 years in average (median 3.5, interquartile range 2-7) while men had a stoma for a significantly longer time (median 4.5 interquartile range 3-8) Mann Whitney U test, $p < 0.001$.

Table 4. Distribution of patients by stoma characteristics

	Gender			
	Male	Female	Total	p*
Type of stoma				
Colostomy	15 (35.8)	9 (41.0)	24 (37.5)	0.10
Ileostomy	9 (21.4)	5 (22.7)	14 (21.9)	
No stoma	18 (42.8)	8 (36.3)	26 (40.6)	
Stoma by duration				
Permanent	4 (17.4)	5 (33.4)	9 (23.7)	**0.01**
Temporary	19 (82.6)	10 (66.6)	29 (76.3)	
Complications				
Skin changes	4 (20.0)	4 (22.2)	8 (21.0)	
Stoma prolapse	3 (15.0)	1 (5.5)	4 (10.5)	**0.001**
Stoma hernia	1 (5.0)	2 (11.0)	3 (7.9)	
No complications	12 (60.0)	11(61.3)	23 (60.6)	
Taking care of the stoma by themselves				
No	4 (17.4)	2 (13.6)	6 (15.8)	0.80
Yes	19 (82.6)	13 (86.4)	32 (84.2)	
Total	23 (100)	15 (100)	38 (100)	

* Fisher's exact test; Chi-squared test

24 patients (37.5%) had colostomy, 14 patients (21.9%) had ileostomy, and 26 patients (40.6%) underwent surgery for resection of colorectal cancer without stoma. Of the total number of patients with a permanent stoma, there was a significant number of women (Chi-squared test, p = 0.01). Prior to surgery, 21 patients (55.3%) had a marked stoma site, with no significant gender difference. Of the total number of patients (64) in our study, there were significantly more men with colorectal cancer. Reported complications were changes in the peristomal skin in 21.0% of patients which was significantly more reported in women. 60.6% of patients had no complications, and stoma prolapse was more pronounced in men (Fisher's exact test, p = 0.001). Of the total number of patients with stoma, 32 patients (84.2%) performed stoma care independently, without significant gender difference.

Patients generally rated their health and overall quality of life as *good* during the past month, only a small number of patients rated it as *unsatisfactory*.

Patients with stoma most often rated their general health with a four (31.3%). The most frequently rounded answer on the quality of life table was answer a five (28.1%), and the mean value of both particles was four.

Table 5. Patients and living with a stoma

	n (%)				
	Not at all	A little	Quite a bit	Very much	Total
Are you generally satisfied with the information you received about life with a stoma?	1 (1.7)	10 (16.1)	41 (63.4)	12 (18.8)	64 (100.0)
How satisfied are you with the information you received after the surgery?	4 (6.3)	17 (26.7)	31 (48.5)	12 (18.5)	64 (100.0)
How satisfied are you with the information you received from the surgeons who operated on you?	5 (7.9)	16 (25.1)	27 (42.3)	16 (25.0)	64 (100.0)
How satisfied are you with the information you received from the ward nurse?	4 (6.3)	14 (21.9)	30 (47.6)	16 (25.0)	64 (100.0)
Are you satisfied with the information about the stoma you gathered on the Internet?	14 (21.9)	11 (17.2)	23 (36.0)	16 (25.0)	64 (100.0)
Are you satisfied with the information about the stoma you received from other media?	16 (25.3)	14 (22.0)	26 (40.7)	8 (12.5)	64 (100.0)
Are you satisfied with the information about the stoma that you received from other stoma patients?	6 (9.4)	13 (12.4)	32 (50.0)	13 (28.6)	64 (100.0)
How satisfied are you with the information about the stoma you received from the other people?	17 (26.6)	16 (25)	24 (37.5)	7 (10.9)	64 (100.0)
Needed information before surgery	5 (7.9)	8 (12.5)	20 (32.4)	31 (11.2)	64 (100.0)
Needed information after surgery	2 (3.2)	3 (4.7)	23 (35.8)	34 (56.3)	64 (100.0)
Willingness to agree to surgery and stoma formation	6 (9.5)	10 (16.1)	33 (29.5)	15 (44.99)	64 (100.0)
Changed negative attitudes about stoma after the surgery	5 (7.9)	8 (12.6)	27 (42.2)	24 (37.3)	64 (100.0)
Would you recommend surgery and a stoma to the patient with your diagnosis?	1 (1.6)	7 (11.0)	16 (25.0)	40 (62.4)	64 (100.0)

Patients mostly agree with the statement that they need information before and after the surgery, and that they are quite satisfied with the information they received about life with a stoma. They mostly rated satisfaction with the information they received from the surgeons who operated on them with *quite a bit* and *very much* (67.3%) and would recommend surgery and a stoma to patients with their diagnosis in 87.4% of cases. A total of 26.6% patients operated on with a stoma were not satisfied with the information about the stoma they received from other people.

Table 6. Patients by quality of life self-assessment

	n (%)				
	Not at all	A little	Quite a bit	Very much	Total
Did you urinate uncontrollably?	47(73.5)	10(16.0)	4(6.3)	3(4.2)	64(100.0)
Have you had painful urination?	48(75.0)	11(17.2)	4(6.3)	1(1.5)	64(100.0)
Have you had abdominal pain?	39(61.0)	14(22.0)	9(14.2)	2(2.8)	64(100.0)
Have you lost hair due to therapy?	51(79.6)	5(7.9)	4(6.3)	4(6.3)	64(100.0)
Have you had any problems with the sense of taste?	45(70.4)	14(22.0)	3(4.8)	2(2.8)	64(100.0)
Were you worried about your health?	30(47.3)	10(15.7)	12(18.5)	12(18.5)	64(100.0)
Were you worried about your weight?	29(45.4)	15(23.4)	10(16.1)	10(16.1)	64(100.0)
Were you dissatisfied with your appearance?	52(46.8)	27(24.3)	17(15.3)	8(12.3)	64(100.0)
Have you had stool leakage from your stoma?	31(48.5)	22(34.4)	8(12.5)	3(4.6)	64(100.0)
Have you had painful skin around the stoma?	40(62.3)	17(26.7)	6(9.4)	1(1.6)	64(100.0)
Did you change your pouches more often during the day?	31(48.5)	18(28.2)	12(18.8)	3(4.5)	64(100.0)
Did the stoma make you feel uncomfortable?	29(45.4)	18(28.2)	9(14.1)	8(12.5)	64(100.0)
Have you had problems caring for a stoma?	32(50.0)	19(29.8)	8(12.5)	5(7.79)	64(100.0)

Many patients were concerned about their health (18.5%), their weight (16.1%), 17 (15.3%) said they were very dissatisfied with their appearance, only 29.8% had little trouble taking care for the stoma, 35 patients felt uncomfortable (54.8%), 25 patients experienced abdominal pain (39%) and 7 patients (10.5%) uncontrolled and painful urination.

We also tested the gender differences in assessed parameters. There was no statistically significant difference in the assessment of general health ($p = 0.680$) and quality of life ($p = 0.721$) during the past month in relation to gender. Although women rated their physical (6.5 (4.7 - 7) compared to 5.9 (4.6 - 7.2)) and spiritual well-being (5.3 (4 – 7.3) compared to 4.9 (4.1 - 6)) (median (interquartile range)) slightly better than men, no statistically significant difference by gender was found in relation to physical, social, mental and spiritual well-being as well as the overall scale.

Then we compared general health and quality of life during the past month between the patients with and without stoma and found a statistically significant difference in "General health during the past month" between these groups of patients ($p = 0.035$). Patients without a stoma rated their health better. There was no statistically significant difference in the assessment of quality of life between the group of patients with stoma and without stoma.

Table 7. Assessments of subscales and whole scales of quality of life of patients with colostomy

	Median (interquartile range)	Minimum - maximum
Physical well-being	5.9 (4.6 – 7.2)	1.5 – 9.3
Social well-being	5.3 (4.1 – 6.1)	1.9 – 8.6
Mental well-being	5.3 (4.1 – 6.1	3.4 - 7.5
Spiritual well-being	5.0 (4.1 – 6.0)	0.9 – 7.4
Overall scale	5.4 (4.7 – 6.0)	3.0 – 7.4

Patients rated physical well-being with the highest score in average 5.9 (interquartile range 4.6-7.2), ranging from 1.5-9.3 and spiritual well-being with the lowest score, median 5.0 (interquartile range 4.1-6.0) ranging from 0.9-7.4.

Table 8. Assessments of subscales and whole scales of quality of life of patients with colostomy by duration of living with colostomy

	Median (interquartile range) of duration of living with stoma				p*
	Up to 3 years (N=25)	3-10 years (N=29)	11 and more years (N=10)	Total (N=64)	
Physical well-being	6.1 (4.8 – 7.4)	5.4 (4.3 – 6.7)	7 (5.5 – 7.8)	5,9 (4.6 – 7.2)	0.255
Mental well-being	5.2 (4.4 – 5.7)	5.2 (4.8 – 5.8)	5.5 (5.2 – 5.8)	5.2 (4.8 – 5.8)	0.413
Social well - being	5.1 (4.2 – 6.1)	4.8 (3.2 – 5.3)	5.9 (4.8 – 6.8)	5.0 (4.1 – 6.0)	0.110
Spiritual well-being	4.7 (4.1 - 6)	5.4 (4.3 – 6.1)	4.6 (3.6 – 7.3)	5.3 (4.1 – 6.1)	0.657
Overall scale	5.3 (4.7 – 6.2)	5.3 (4.4 – 5.6)	5.6 (5.3 – 6.2)	5.4 (4.7 – 6.0)	0.489

*Kruskal-Wallis test

There was no statistically significant difference in the assessment of the quality of life of patients with colostomy in relation to the time elapsed since the implantation of colostomy.

Next, we grouped patients according to age into three groups: the youngest patients (31 – 50-year-olds), the middle-aged group (51 – 70-year-olds) and the oldest patients (70 years old and older), in order to test the differences in their quality of life assessments.

Assessment of physical well-being revealed that the youngest patients reported fatigue and sleep disorders as their biggest problems, patients from the middle-aged group peristomal skin changes while the biggest physical issues in the oldest group of patients were sleep disorders and physical strength. No statistically significant difference in the tested aspects of physical well-being was found between the mentioned age groups.

The youngest group of patients rated their mental well-being with the lowest score, and their biggest fear was recurrence of the disease. The biggest mental issue in the middle-aged group was dissatisfaction with their appearance, while the oldest patients were dissatisfied with their life and its quality. We found no statistically significant difference in the tested aspects of mental well-being between the mentioned age groups.

In terms of social well-being, in patients aged 31-50 years stoma mostly limits them in intimate activities and makes it more difficult to travel due to stoma care. The biggest problems of patients aged 51-70 years are family stress due to stoma, restrictions in intimate activities and travel, and worsening of their financial situation due to the treatment of the disease. Worsening of the financial situation has reached statistical significance in the age group of 51-70 years (Kruskal-Wallis test, $p = 0.017$). In the oldest age group of 71 and over, the biggest problem is lack of support from family and friends and difficulties in stoma care.

On the scale of spiritual well-being, the youngest patients assessed the claim that the support they receive by going to church meets their needs and that the stoma has brought some value to their lives. In the same category, patients aged 51-70 rated the statement that they have reasons to live and that going to church meets their needs with the lowest score. They were the most hopeful group of patients, but the difference did not reach statistical significance. Lack of reasons to live was the biggest issue in the oldest age group of patients in terms of their spiritual well-being.

DISCUSION

The study included 64 patients, 39 male (61.0%) and 25 female (39.0%), of whom 45 (70.0%) were married. Most of them were aged 51-70 years (71.9%), while the the least numerous age group was 31-50 years (10.9%), and no patients younger than 30 years old were in the study. The mean age of patients was 62.55 ± 9.365 years. Most patients included in the study have a high school degree (45.3%), while the smallest number of them has a university degree or more (17.2%). There were no patients without any formal education. In terms of employment status, most patients were retired (48.5%), and the least unemployed (12.5%). 26 patients (41.0%) underwent surgery without stoma formation, and 38 patients (59.0%) had stoma, of whom 9 had permanent stoma (23.7%), while 29 patients had temporary stoma (76.3%).

The literature shows that patients with stoma face many challenges in terms of their quality of life. (19, 24, 25, 26) The findings of our study similarly highlighted a number of

problems and challenges regarding the quality of life. The impact of stoma on patients' quality of life was explored in many dimensions. The stoma-related physical problems, including irritated parastomal skin and unpleasant odor, noted in our study, were also reported by other researchers (19, 27, 28).

While the patient is in the hospital, the biggest problem is the psychological adjustment to the new condition. Of course, there are many other problems after leaving the hospital, such as leakage of liquid stool under the stoma disc, problems with putting on or changing the stoma pouch, odor, stoma size, "sound" of gas in the stoma pouch, stoma visibility, and hernias. After leaving the hospital, the adjustment begins, a period characterized by a high level of uncertainty and insecurity. (8, 19, 20) During the recovery period after the formation of the stoma, patients try to get involved in everyday life. The goal of the procedures in the recovery period is to help the patient take control and get involved in life as much as possible and allowed, to continue to live at the same pace as before. After a certain period, 6 months after colorectal cancer surgery and stoma formation, the priorities change leaving consequences on the patient and reducing his quality of life.

The formation of a temporary or permanent stoma reduces the patient's quality of life regardless of the diagnosis. The authors state that in these cancer patients, the embarrassment associated with ostomy outweighs all other patient concerns that is even greater than the concerns regarding stoma formation itself. (26) The impact of stoma on physical, mental, social and spiritual well-being is not unexpected, but it is little described in the literature. Research shows that quality of life is increasingly recognized as an important measure of the outcome of survival after major surgical interventions. (29) For these purposes, quality of life is seen as a multidimensional concept that defines the level of well-being and satisfaction with one's life because the patient's life is affected by the treatment of the disease. Research shows that the stoma has a great impact on the patient's quality of life and patient's everyday life as well (24). The patient is happy to see his quality of life and lifestyle change after the formation of a stoma.

Many studies have shown a reduction in the quality of life in patients with stoma (2, 19, 24, 25, 26). Dissatisfaction with preoperative preparation and postoperative care, negative thoughts and beliefs associated with stoma contribute to the reduction of quality of life. Conversely, stoma gives many patients hope, prolongs their life, gives them ability to act and continue life plans (25). Adaptation to a new life situation lasts from several months to two years and is influenced by several factors that include the level of medical knowledge and skills that the patient possesses (4, 30). Quality of life is an important measure for a patient with stoma. Assessing the quality of life of patients with stoma will lead to a better understanding and improvement of life (19, 24). The impact of psychosocial needs on quality of life has been researched, but a small number of studies suggest interventions that would address problems patients with stoma face that reduce their quality of life. In the hardships of life, the patient feels and lives worse due to the presence of malignant disease or its consequences. And his quality of life depends not only on how sick the patient is, but also on how the patient experiences his illness. Nowadays, the therapeutic effects of the treatment of patients should be evaluated not only based on improved laboratory findings and clinical status of the patient, but also considering the improvement of the quality of their lives (9). Difficulties adjusting to life with a stoma make the patient's current problem, which is associated with fear, feeling of helplessness and loss of control. All this is accompanied by daily challenges, loss of self-confidence and the problem of intimacy, long-term fears and worries that often lead to low self-esteem and loss of socialization (21, 31).

Results of our study show that the patients have no problem in terms of privacy during stoma care. For most patients, their condition has been a burden to the family, some of them state that the stoma interfered with their intimate life or limits them in sports and recreation. Dabirian et al. noted that patients reported changes of skin around the stoma, sleep problems, unpleasant odor and gas (32). The results of our study show that, in the context of spiritual well-being, most patients have the reasons to live, and some are completely uncertain about their future. In contrast, some authors state that the minority of patients have a reason to live, they are insecure about their future while others have emotional support and inner peace and are hopeful for further life and healing. They highlighted family problems as the patient’s biggest problem, as well as troubles in recreational activities and the disturbancies in intimate relationships, the feeling of isolation from society and difficulties adaptating to the new situation (19, 24).

Most patients agreed that they needed information before and after the operation, that they were very satisfied with the information they received from the surgeon, the ward nurse, and that they would recommend surgery and a stoma to any patient with the same diagnosis. For the patient to consciously accept the operation and life with the stoma, it is certainly necessary for the operating surgeon to talk to the patient before performing the procedure. The patient should be told what the reason is for performing such an intervention and what he gains from it. How and where the stoma will be formed, the functioning and maintenance of the stoma, and what kind of life awaits him after the treatment should be explained to the patient (22, 33). A good relationship between the patient and the nurse who takes care of the stoma is considered key to patient’s successful adaptation the to life with the stoma. This relationship promotes continuity of stoma care, and psychosocial adjustment, increases patients' ability to develop practical stoma care skills, and reduces the risk of rehospitalization (15). Healthcare professionals should always strive to improve the quality of life of patients with stoma, as their knowledge and skills can help improve the patient’s quality of life before and after surgery.

Problems of emotional functioning are more or less pronounced as more or less patients are irritable or depressed. Some studies have shown that 70% of patients with stoma experience dissatisfaction and depression. The results of our research show that stoma affects the quality of life, however, patients rated their general health and overall quality of life during the past month mostly well, only a small part of them rated it as unsatisfactory (19, 24).

Our results show that there is a significant difference only when comparing "General health during the past month" (Mann-Whitney U test $p = 0.035$) with respect to the presence of stoma. Patients who have a stoma rated their general health as worse than those without a stoma. Likewise, the assessment of general health as worse did not affect the assessment of quality of life, i.e., the item „Quality of life during the past month" did not show a statistically significant difference between groups (Mann-Whitney U $p = 0.492$). Considering the functional scales, there were no significant difference in relation to the stoma and sex, except in one, which is the assessment of general health (QL). Patients with and without stoma, patients of both sexes haven't shown any significant difference in physical, social and emotional functioning. Ostomy type may influence patients' general health, as colostomy carriers perceive their general health as better than ileostomy carriers (34). Nevertheless, colostomy and ileostomy have similar impact on patients' life quality (21, 35, 36).

The results of our study show that in the context of mental well-being, most patients are afraid of recurrence of the disease. It was difficult for them to adjust to the stoma, they feel useless, the stoma makes them feel uncomfortable, they find it difficult to look at and take care of the stoma, and they are dissatisfied with their lives (5, 13). However, our findings can still be useful to physicians and nurses in creating a supportive environment to improve the quality of life of patients with stoma. Using a quality of life questionnaire gives stoma patients the opportunity to express their concerns about quality of life issues. Due to the qualitative approach in this study, we could not show a correlation between demographic characteristics and quality of life.

CONCLUSION

The analysis of the results of our research has shown that the patients with stoma rated their general health as worse, but not their quality of life. The quality of life of a patient with stoma after cancer surgery largely depends on how much the patient is willing to cooperate in treatment, regular examinations and adherence to guidelines for stoma care, depending on the level at which the patient adopted certain procedures for taking care of stoma and accepted life with it. Nowadays, most patients lead an orderly and fulfilled life, continue to work as before, and partially perform social activities, lead a normal family life, normal communication and socializing with friends, and engage in recreation and sports. Contemporary assessment of the therapeutic effects of the treatment of patients should include not only the evaluation of improved laboratory findings and clinical status of the patient, but also the improvement of the quality of their lives.

DECLARATION OF INTEREST

The authors declare that they have no competing interests.

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ANALYSIS OF THE STRESS OF HEALTH WORKERS OF PSYCHIATRY CLINIC AND DERMATOVENEROLOGY CLINIC

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ABSTRACT

Stress at a workplace represents stress caused by the job of a person, which occurs when the demands of the workplace are not in accordance with the needs of health workers. Two basic types of pathological stress at workplace are: burnout syndrome at work and technostress. The set goals were to assess the level of stress among health workers employed by the Psychiatric Clinic and the Dermatovenerology Clinic, and compare the results obtained, and identify a group of health workers exposed to the highest level of stress within the studied healthcare institutions. A total of 93 respondents were included in the study, of which 43 were from the Dermatovenerology Clinic and 50 from the Psychiatric Clinic. In the process of collecting data from respondents, the questionnaire used is modified version of "The Workplace Stress Scale". The most important is to note that there is no correlation between night-shift work and stress level, as well as gender, or level of education, even though many studies in the world indicate a connection between these factors - which is marked as an increased risk of stress. Starting from the goals of the work, it is concluded that employees at the Dermatovenerology Clinic are more exposed to stress than employees in the Psychiatric Clinic. The level of education, gender, age, shift work does not affect the level of stress. The factor that most affects the level of stress is personal income and a short deadline for executing the given actions.

Keywords: *Stress, professional stress, health workers.*

INTRODUCTION

Professional stress represents the stress caused by or related to a job that a person performs, that is, for special events, conditions or requirements that come to light when performing a certain professional activity. Work-related stress can also be defined as a harmful physical or emotional response that occurs when job demands are not in line with the abilities, resources or needs of workers (1).

Increased exposure to professional stress is typical for certain occupations, such as: medical nurses/technicians (2), police officers (3), firefighters (4), field medical emergency service (5), air traffic controllers (6), social workers (7), operators of telephone exchanges (8) and dentists (9).

In addition to directly endangering health, stress also directly affects the work performance and the psychological state of individuals. Stress can be associated with an increased incidence of chronic non-communicable diseases (high blood pressure, heart disease, diabetes, etc.) and in general, reducing the organism's resistance to disease. There is an opinion that a fall in performance is resulting from a higher level of stress, while lower and moderate levels of stress produce a better performance. However, even in such situations, one should be careful, as the very low level of individual stress can result to unbalance, which can result in a decrease in performance (10).

The objectives were to assess the level of stress among health workers employed at the Psychiatric Clinic and the Clinic for Dermatovenerology, and to compare the results obtained, and identify a group of health workers exposed to the highest level of stress within the studied healthcare institutions. Also, one of the goals was the analysis of the factors that dominantly affect the occurrence of stress in the number of persons employed in these clinics.

METHODS

Respondents

Study was conducted in accordance with the accepted ethical standards for research practice (guidelines of the Declaration of Helsinki of 1975, as revised in 1983). The Ethics Committee of the Clinical Center of Serbia approved this research (No 30/14). In a cross-sectional study, 93 respondents were included in the survey, of which 43 were from the “Vladimir Vujić” Psychiatry Clinic of the Clinical Center of Serbia (hereinafter KPKCS) and 50 from the Clinic for Dermatovenerology of the Clinical Center of Serbia (hereinafter KDKCS). Of the total number of respondents, 28% were male and 72% female. The average age of all 93 respondents was about 42 years (minimum 20, maximum 58 years). Respondents are classified into two groups, based on the institution in which they are employed.

Questionnaire

In the process of collecting data from respondents, a specially designed questionnaire was used, which the respondents themselves filled in and for whom they gave written approval. The questionnaire used is a modified version of the questionnaire "The Workplace Stress Scale" (Figure 1), which was compiled by experts from the American Institute for Stress (AIS). It is available in electronic form and no permission is required for its use.

Statistical analysis

In the statistical processing of the data, appropriate descriptive statistical methods were used: measures of central tendency - arithmetic mean, variability measures - standard deviation and absolute numbers. To calculate the statistical significance of the difference, we used a Chi-square test, a t-test for comparing the 2 groups, and an ANOVA test for comparing three or more groups. In cases where the layout within the groups was not normal, Mann-Whitney U test and the Kruskal-Walis test were used according to the above analogy.

RESULTS

Of all respondents involved in the survey, 76.3% worked only in daily shifts and 23.7% in switching shifts work. Respondents from KDKCS and KPKCS most often worked in daily shifts (76.7% to 76.0%, respectively). There was no statistically significant difference in the frequency of shift work between the investigated groups ($p = 0.933$) (Table 1).

Of all respondents involved in the survey, 38.7% had a high school degree, 24.7% had a college degree, 25.8% had a university degree, and a doctorate had 10.8%. Respondents from KDKCS and KPKCS most often had high school degree (39.5% to 38.0%, respectively). There was no statistically significant difference in the frequency of education between the examined groups ($p = 0,169$).

Being able to adequately use their knowledge and skills at work, respondents from KDKCS mostly answered “often” (48.8%), and from KPKCS “sometimes” (38.0%). There is a statistically significant difference in the degree of the answers to the question “I can adequately use my knowledge and skills at work between the investigated groups” ($p = 0.014$). That the superiors are friendly towards them at work, respondents from KDKCS mostly answered “very often” (41.9%), and from KPKCS “sometimes” (56.0%). There is a statistically significant difference in the degree of the answers to the question: The superiors at work behave friendly to me? between the examined groups ($p <0.001$). For interpersonal relationships evaluated as good/supportive of work colleagues, respondents from KDKCS mostly answered “very often” (37.2%), and from KPKCS “sometimes” (48.0%). There is a statistically significant difference in the degree of the answers to the question: I estimate human

relationships as good/I have the support of colleagues I work with? between the examined groups (p = 0.017) (Table 2).

The average stress value of all respondents involved in the study is 31.4 ± 4.2. The lowest value is 14 and the highest is 41. The average value of the total stress of the respondents from KDKCS is 32.8 ± 4.8, while in the respondents from KPKCS 30.2 ± 3.1.

There is a statistically significant difference in the average stress scores between subjects relative to the department (p = 0.002) (Table 3).

Table 1. Distribution of respondents according to working hours

Working time	KDKCS		KPKCS		Total		p value
	N	%	N	%	N	%	
Daily work	33	76.7	38	76.0	71	76.3	p = 0.933
Shift work	10	23.3	12	24.0	22	23.7	
Total	43	100.0	50	100.0	93	100.0	

Table 2. Distribution of respondents according to the questions

Questions / Answer	KDKCS		KPKCS		Total		p value
	N	%	N	%	N	%	
I can adequately use my knowledge and skills at work?							
Never	3	7.0	4	8.0	7	7.5	p = 0.014*
Rarely	0	0.0	0	0.0	0	0.0	
Sometimes	8	18.6	19	38.0	27	29.0	
Often	21	48.8	18	36.0	39	41.9	
Very often	9	20.9	4	8.0	13	14.0	
The superiors at work behave friendly to me?							
Never	1	2.3	1	2.0	2	2.2	p <0.001*
Rarely	1	2.3	2	4.0	3	3.2	
Sometimes	7	16.3	28	56.0	35	37.6	
Often	16	37.2	12	24.0	28	30.1	
Very often	18	41.9	7	14.0	25	26.9	
I estimate the interpersonal relationships as good / I have the support of colleagues I work with?							
Never	1	2.3	0	0.0	1	1.1	p = 0.017*
Rarely	2	4.7	5	10.0	7	7.5	
Sometimes	12	27.9	24	48.0	36	38.7	
Often	12	27.9	13	26.0	25	26.9	
Very often	16	37.2	8	16.0	24	25.8	

* statistically significant

Table 3. Total stress score

Total stress score	N	x̄	sd	med	min	Max	p value
KDKCS	43	32.8	4.8	33.0	14.0	41.0	p = 0.002*
KPKCS	50	30.2	3.1	30.0	25.0	40.0	
Total	93	31.4	4.2	31.0	14.0	41.0	

* statistically significant

Figure 1. Modified version of the questionnaire "The Workplace Stress Scale“

Questions	
1	I perceive the situation at work as unpleasant?
2	I think my job has a negative impact on my physical and mental health?
3	I have a lot to do at work for an unrealistic short time?
4	I hardly express my opinions and feelings about the work to the superiors?
5	The pressure that I feel at work is transferred to my family and/or my private life?
6	I have full control over my work duties?
7	I am sufficiently rewarded for my commitment at work?
8	I can adequately use my knowledge and skills at work?
9	The superiors at work behave friendly to me?
10	I estimate human relationships as good/I have the support of colleagues I work with?

Answers				
Never	Rarely	Sometimes	Often	Very often

DISCUSSION

Our research has shown that gender differences do not play a major role when it comes to the impact of stress, but neither do day or night shifts. Of all respondents involved in research, 76.3% worked only in daily shifts and 23.7% in switching shift work. Respondents from KDKCS and KPKCS mostly worked in daily shifts. The study did not find a statistically significant relationship between the frequency of shift (night) work and exposure to stress.

For example, in contrast to the results of our research, a 2010 study dealing with vascular stress in younger medical workers in the night shift showed a significant rise of blood pressure by 9.7 mmHg, during the night shift compared to a daily shift. During the day off, after a night shift, blood pressure falls, but does not return to the baseline level (11).

The problem of nightshift work and the growing demands of the global economy are, of course, a source of stress (12). According to recent data from the US Employment Bureau, about 15 million Americans work in the night shift. This applies especially to medical workers, police officers, pilots, and the time of globalization also requires the night work of IT professionals.

It would have been expected that, due to the nature of the work, people employed at the Psychiatric Clinic were under greater stress than those employed at the Clinic of Dermatovenerology, however, the research showed otherwise. These results confirm the thesis that the nature of the work is not the key, but it is the individual impression of a person, which largely depends on interaction with its surroundings**.** For example, in our research, there is a statistically significant difference in the degree of the answers to the question: I evaluate interpersonal relationships as good / I have the support of colleagues I work with? between the investigated, i.e. persons working at the Psychiatric Clinic consider interpersonal relationships significantly better than those employed at the Clinic for Dermatovenerology.

Interpersonal relations refer to the relationship between associates or management and employees. These relationships are a natural part of the work environment and are sometimes pleasant and creative, and sometimes they are a source of tension and frustration. There are other relationships at work such as a relationship with a patient, clients, other professionals and the like. An important aspect is how the administration relates to strategies and procedures that directly affect employees. Social support is probably the most widely examined dimension of interpersonal relationships at work (13). Essentially, this is about the instrumental support (a person is given the means of work and all necessary information) and emotional support that includes support, backup, personal feedback and appreciation (14).

In our research, we have come up with similar data as in a study published by Cox et al. (15): namely, there is a statistically significant difference in the degree of the answers to the question: The superiors at work behave friendly to me? between the examined groups, i.e. they evaluated the relationship of superior staff as significantly better at the Psychiatric Clinic.

In the context of a fair attitude towards employees, a fair attitude is important, how decisions are made (whether reasonable or not, according to employees' estimates), how decisions are made about rewards and punishments. Of all this depends the commitment to the work, motivation and psycho-physical health of employees (16).

That they can adequately use their knowledge and skills at work, respondents from KDKCS mostly answered “often” (48.8%), and “sometimes” from KPKCS (38.0%). There is a statistically significant difference in the degree of the answer to the question: I can adequately use my knowledge and skills at work? between the examinees. In this case, it can be spoken of a question that is indicative of the degree of stress, and this is what NIOSH research is about, too. Exclusion or social isolation can be considered as part of social support and reflects the social climate and normative relationships in an organization. The relation of administration to employees is very important, because it implies appreciation, sharing of information and adequate distribution of tasks (in accordance with the professional and other possibilities of the individual) (13).

It can be concluded that the occurrence of stress depends on many factors, which primarily involve social interactions, social support, interpersonal relationships, the way of conflict resolution, fairness in decision-making and job distribution, while in the second plan are, according to the results of our research - weight of work, gender differences or shift work. There is also a high prevalence of stress, anxiety, and depression among healthcare professionals caring for patients with COVID-19. Therefore, measures are needed to reduce these disorders in staff treating patients with COVID-19, which would increase the productivity of medical workers during pandemic (17).

In any case, due to the insufficient distinction between these concepts (phenomena), it is necessary to undertake a more comprehensive research that will include all these factors.

CONCLUSION

It would have been expected that, due to the nature of the work, people employed at the Psychiatric Clinic were under greater stress than those employed at the Department of Dermatovenerology, however, the research showed the opposite. These results confirm the thesis that the nature of the work is not the key, but it’s the individual impression of a person, which largely depends on interactions with its environment.

The research did not confirm that the main sources of work stress among employees in the analyzed institutions are dissatisfaction with material rewards and excessive demands at work. It can be concluded that the occurrence of stress depends on many factors which primarily involve social interactions, social support, interpersonal relationships, the way of resolving conflicts, fairness in decision-making and job distribution, while in the second plan are, according to the results of our research - the weight of work, income, gender differences or shift work.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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THE IMPACT OF INCREASE IN THE VERTICAL DIMENSION OF OCCLUSION ON NOCICEPTION IN RATS - A PRELIMINARY REPORT

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ABBREVIATIONS

VDO - vertical dimension of occlusion
TMJ - temporomandibular joint
CNS - central nervous system
HPA - hypothalamic-pituitary-adrenal

ABSTRACT

Since the change in vertical dimension of occlusion (VDO) is extremely important in prosthetic dentistry, the aim of the study was to examine the effect of VDO increase on nociception parameters in rodent experimental model. The study was performed on seven experimental groups (6 animals per group) on male Wistar-albino rats: sham; 0.6/3, 0.9/3, and 1.2/3 groups where VDO was increased by 0.6, 0.9, and 1.2 mm (respectively), for three days; 0.6/20, 0.9/20, and 1.2/20 groups where VDO was increased by 0.6, 0.9, and 1.2 mm (respectively), for twenty days. The VDO raising protocols were performed as follows: on a day 1, following anaesthesia, a two-phase impression was taken with addition silicones; on a day 3, the cementing process for both maxillary incisors and inside crowns preparation was performed, and cementing zirconium crowns, manufactured using CAD-CAM technology, were applied. The behavioural testing (the tail flick and hot plate test) was performed on day 3 and 20. The results obtained in the tail flick test suggest that the raise in VDO in the early phase induced increased sensitivity to pain in a stepwise manner, while this hyperalgesic effect was diminished in a time-dependent manner. The stepwise increase in VDO also resulted in significant decline in the pain tolerance with the higher VDO (0.9 and 1.2 mm) in the hot plate test that persisted after twenty days in 1.2/20 group. It seems that VDO elevation is sufficient to produce hyperalgesic effect in this experimental model, which may be attenuated in time-dependent manner.

Keywords: *vertical dimension of occlusion-VDO, behavior, nociception, adaptation, rat.*

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INTRODUCTION

Rodents are a species with a very specific dentition, which is characterized by the presence of two groups of teeth, namely incisors and molars, and between them there is a long, empty space called a diastema. Incisors are the most representative dental feature of this order (1). The specificity of incisors in rodents is reflected in the fact that these teeth grow and wear continuously throughout life (2). These teeth are highly specialized for gnawing - a model of chewing of great importance for rodents. Thanks to this kind of dentition and attachment position of the masseteric muscle, rodents, especially rats, can bite strongly. Gnawing is also important from the behavioral aspect and plays a vital role in maintaining rats' health as well as preventing the development of the malocclusions (3).

When the teeth of the upper and the lower jaws are in contact, they have a role in maintaining a stable vertical dimension of occlusion (VDO). The term VDO applies to the distance of two marked anatomical points in the maximal intercuspal position (4). Sometimes, changes in VDO can be related to the therapeutic procedure in the rehabilitation of worn teeth or a phase within orthodontic therapy, while more often occurs as a result of insufficiently planned therapy (5). The change in VDO is extremely important from the clinical point of view. When adaptive abilities are exceeded, this change can lead to the side effects on the structures of the orofacial system that affects patient functionality, comfort and aesthetics (6). Occlusal interferences in the form of increasing VDO are common in clinical practice, and associated with the development of chronic stress (7) which results in pain and overload of the masticatory muscles and temporomandibular joints (TMJ), as well as central nervous system (CNS) (8).

Recurrent episodes or chronic stress have been reported to cause functional alterations in the brain that lead to disturbances in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis (9), and the relationship between physiological responses to stress and behavioral changes is very complex and changes in this relationship may be related to HPA axis adaptation (10). This is related to the fact that the brain reacts physiologically and behaviorally to every change in order to adapt to a given situation. Therefore, chronic stressors, such as VDO increase, may lead to significant behavioral changes, manifested by nervous mood disorders, including anxiety, depression, poor sleep, as well as eating disorders. Those behavioral alterations patterns are very comparable to those described in post-traumatic stress disorder (11). It is well known that prolonged and/or repeated stress may cause HPA axis disturbance resulting in the permanent elevation of glucocorticoid levels (12), with the significant impact on hippocampal function (13, 14). Since the VDO increase may be analyzed both through acute and prolonged response to specific stimuli, it is worth to notice that literature data, among behavioral alterations also offer an evidence for the nociception alterations. Namely, hypoalgesic effect is usually reported as an early response to acute stress (15), while the prolonged exposure to stressors results in hyperalgesic effect (16). Typical signs of recognizing chronic pain are its chronic presence, hyperalgesia, and allodynia (17).

Both experimental and clinical studies showed that altered VDO which are not extreme and are well planned have very good degree of adaptation (18), while the extreme alterations in VDO can cause enormous changes in the masticatory muscles and the TMJ. It is usually considered that the adaptation to the new VDO will take place in the period between 6 weeks and 3 months (19), while the minimal adaptation period for altered VDO is 4 weeks (20).

Due to the lack of data for the impact of VDO changes on certain behavioral patterns in rodents, such as pain tolerance, the aim of the study was to examine the effect of VDO increase of different dimensions and duration on nociception parameters.

MATERIAL AND METHOD

Animals and treatment

This study involved 42 male Wistar albino rats (8-10 weeks old, 180-220 g body weight), which were purchased from the Military Medical Academy, Serbia. The animals were housed in transparent cages (three animals per cage), under standard environmental conditions (temperature 23±1 °C, humidity 50±5 %, light/dark cycle 12/12h). All animals were allowed food and tap water intake ad libitum. All protocols lasted for 20 days. The rats were randomly assigned into seven equal groups, as follows:

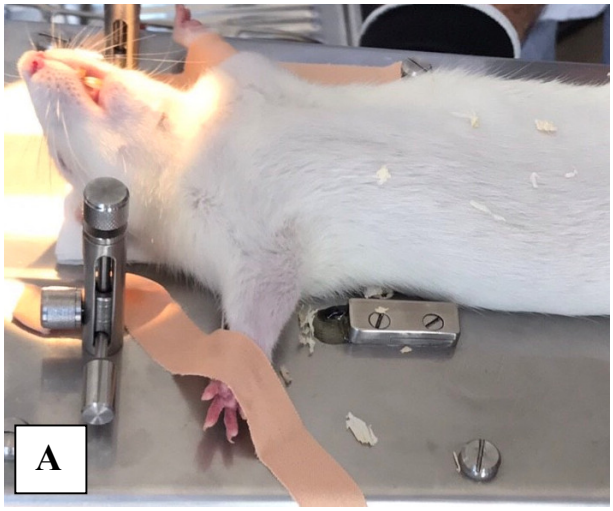
Sham group;

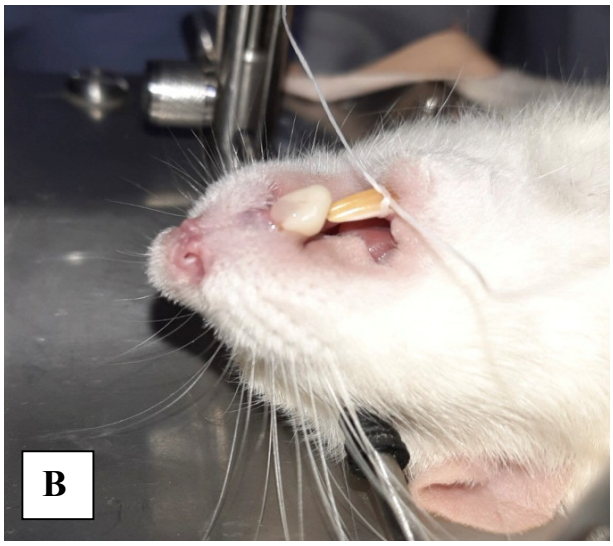
1) 0.6/3 group, where VDO was increased by 0.6 mm, for three days;
2) 0.6/20 group, where VDO was increased by 0.6 mm, for twenty days;
3) 0.9/3 group, where VDO was increased by 0.9 mm, for three days;
4) 0.9/20 group, where VDO was increased by 0.9 mm, for twenty days;
5) 1.2/3 group, where VDO was increased by 1.2 mm, for three days;
6) 1.2/20 group, where VDO was increased by 1.2 mm, for twenty days.

The VDO raising protocols were performed according to the following algorithm: on a day 1, the animals were anesthetized (combination of ketamine 10 mg/kg and xylazine 5 mg/kg, i.p., Figure 1A) and a two-phase impression was taken with addition silicones (Hydrorise putty and light body, Zhermack, Italy). On a day 3, the cementing process for both incisors and inside crowns preparation and cleaning was performed, and then cementing zirconium crowns (Figure 1B) on both maxillary incisors were applied (Multilink Automix, Ivoclar, Vivadent, Liechtenstein), according to previously

described procedure (21), also following anaesthetic procedure. The zirconium crowns were manufactured using CAD-CAM technology based on the working model, to achieve the VDO alteration for 0.6, 0.9 and 1.2 mm. The animals in the control group underwent a sham operation in which the maximum jaw opening was maintained for 5 minutes under the anaesthesia protocol in order to mimic the impact of pharmacological procedures performed in the experimental groups. The zero point for experimental protocol duration was established by the momentum of zirconium crowns application in each experimental group, and the behavioural testing was performed on day 3 and 20 in predefined groups for all three VDO.

Figure 1. Anaesthesia and immobilization (A), an outcome of VDO raising procedure (B).





Behavioral testing

The nociceptive response testing was performed after the completion of the protocols. Rats were transported in their home cages to the testing room, approximately at 8 a.m. and allowed to acclimate for an hour prior to testing. The nociception assessment was performed in the tail flick and hot plate test under previously described procedure (22). The equipment for both tests was cleaned with water and 70% ethanol for each animal, to remove potential interfering scent.

Tail flick test

The tail flick test is used to assess the nociception. The high-intensity heat stimulus was directed at the rat's tail, according to the method described in our previous paper (22). The animals were placed on a raised grid and covered with an appropriately sized tube to prevent the movement. When the temperature reached 75 °C, a thermal stimulus was placed in the mid portion of the tail and the reaction of each rat was monitored individually. By measuring the duration from the initiation of the painful stimulus to the manifested form of the expected reaction (the tail flick), the results of this test were quantified and expressed in seconds.

Hot plate test

The hot plate test was implemented according to the algorithm previously defined in our lab (23, 24). The appliance consisted of a square metal plate measuring 43 x 43 cm, and glass walls 30 cm high. Each animal was placed in the central part of the plate, keeping the temperature constant at 51.5±0.5°C. It is considered that the test was finished when some of the specific reactions to the thermal stimulus occur, such as paw licking, stomping, shaking the hind paw or bouncing off the ground with all four limbs at the same time. The test time was limited to 180 seconds to prevent the development of burns. The parameter monitored in this test is the reaction time expressed in seconds.

All research procedures were carried out in accordance with the European Directive for the welfare of laboratory animals No 86/609/EEC and the principles of Good Laboratory Practice, and in accordance with the ARRIVE guidelines. All experiments were approved by the Ethical Committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia.

Statistical analysis

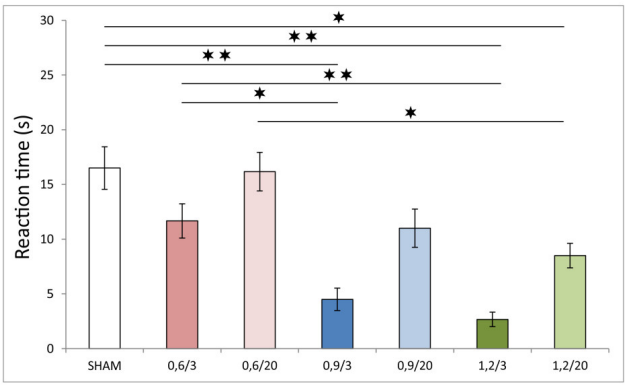
The results were expressed as the means±SEM. Parameters obtained in HP test and TF test and oxidative stress markers were initially submitted to Levene's test for homogeneity of variance and to Shapiro-Wilk test of normality. Comparisons between groups were performed using One-way ANOVA, followed by Bonferroni test. The significance was determined at $p<0.05$ for all tests.

RESULTS

As shown in Figure 2, the applied protocols significantly altered the reaction time in the tail flick test ($dF=6$, $F=13.120$). The significant reduction in reaction time, as an early response to VDO raise, was observed with the higher increase (0.9 and 1.2 mm) in VDO, when compared to the control group ($p<0.01$). Interestingly, that hyperalgesic effect observed also in 0.9/3 and 1.2/3 groups was even significant

when compared to the lowest VDO increase (0.6 mm) in the early phase ($p<0.05$ and $p<0.01$, respectively). The accommodation to VDO raise, evaluated on day 20, showed that although not significant when compared to early response (day 3), was sufficient to attenuate the hyperalgesic effect in the tail flick test in 0.9/20 group, but the reaction time in 1.2/20 group remained significantly below both the control and 0.6/20 groups ($p<0.05$).

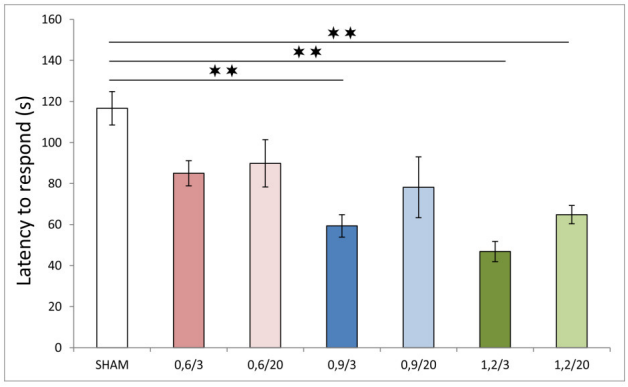
Figure 2. Tail flick test.



The values are mean ± standard error of the mean (SEM), *denotes a significant difference $p<0.05$, **denotes a significant difference $p<0.01$.

The protocols with the stepwise increase in VDO also significantly affected latency to respond in the hot plate test (Figure 3, $F=6.974$). While the lowest VDO increase (0.6 mm) had no significant impact on response to thermal stimuli in the hot plate test in both estimated time-lapses, the gradually increasing hyperalgesic effect, when compared to control, was observed in 0.9 group only in an early phase (day 3, $p<0.01$). At the same time, the highest raise in VDO (1.2 mm) resulted in significant decline in the latency to respond in the hot plate test that was observed on day 3 ($p<0.01$) that persisted even after 20 days neither on day 20 ($p<0.01$) when compared to the control.

Figure 3. Hot plate test.



The values are mean ± standard error of the mean (SEM), **denotes a significant difference $p<0.01$.

DISCUSSION

As previously described, literature data offer the confirmation that change in VDO (by means of an increased dimension) may result in characteristic stress-response behavioral features (25). Since the alterations in VDO represent the specific trauma that causes chronic stress (7), it has been observed that this prosthetic intervention leads to increased sensitivity to pain, manifested by the decline in pain threshold. The key findings obtained by the tail flick test in this study suggest that the raise in VDO in the early phase (evaluated three days following the intervention) resulted in increased sensitivity to pain, while it seems that this hyperalgesic effect was diminished in a time-dependent manner. Protocols with a stepwise increase in VDO also confirmed that the augmentation of VDO simultaneously produced the decline in the pain tolerance by means of estimation in dominantly reflex reaction to thermal stimuli (mainly controlled at the spinal level), as estimated in the tail flick test.

Interestingly, the quantification of response to thermal stimuli in hot plate test showed less pronounced impact of VDO alterations when compared to reflex mechanisms. However, again the stepwise increase in VDO resulted in the altered latency to respond varying from non-significant hyperalgesic effect observed with the lowest VDO increase (0.6 mm) to significant decline in the pain tolerance achieved with the higher VDO increase (0.9 and 1.2 mm). Furthermore, the impact of the incremental VDO raise was strongly confirmed by the observed hyperalgesic effect of VDO increase that persisted even after twenty days in the group in the highest VDO.

Unfortunately, due to lack of data for the interconnection between VDO raise and the pain tolerance in animal experimental models, we are not able to compare our results with the literature. Therefore, we can only try to make an indirect explanation based on the fact that VDO increase has been reported to result in chronic stress (7), while the chronic exposure to stressors resulted in the nociceptive changes, producing hyperalgesia (16). Hormozi and co-workers reported that after three weeks of electric foot-shock stress, hyperalgesia was observed due to decreased expression of spinal cord μ-opioid receptors. These authors observed a significant decrease in tail flick latency on day 22, which coincides with the results of our study 20 days after increased VDO (26). Since Pinto-Ribeiro and co-workers (27) showed that chronic unpredictable stress inhibited pain-like behavior by increasing the nociceptive threshold (stress-induced analgesia), which is not in accordance with the results obtained in this study, this agreement should be addressed to different experimental design and methodological approach.

At the end, it seems very important to comment the observed attenuation of nociceptive alterations induced by VDO increase. Our results are in accordance with the results of the clinical study that confirmed the relationship between the increase in VDO level and neuroplastic changes that reflect the adaptation of the CNS (28). Also, Abduo and

associates noted that subjective symptoms, such as muscle tension and pain, were significantly reduced two weeks after VDO elevation (6). The results obtained on animal experimental models also showed that prolonged and chronic stress stimulated neurohumoral reactions to plastic alterations, leading to behavioral changes (29, 30). Although the sensitivity of TMJ mechanoreceptors is not regulated by the CNS, Naito and associates reported that increased VDO can temporarily change the properties of mechanoreceptors of the TMJ, while the adaptation of peripheral sensory receptors occurred within 6-7 weeks (31).

Finally, summarizing the results of two tests specifically used for the estimation of nociceptive alterations induced by thermal stimuli in rodent experimental model, it seems necessary to comment the observed differences. Namely, although both tests showed the same type of reaction (hyperalgesic) to VDO increase, the evident differences could be addressed to the different levels of pain control affected in the two employed tests. According to results of this study, it seems that central analgesic mechanisms are sufficient to diminish the alterations in reflex response to VDO increase. However, this observation should be additionally confirmed by future investigations that will involve the estimation of specific pain control mechanisms in the CNS.

CONCLUSION

In summary, the results of our study may be considered as an experimental confirmation that VDO raising, may produce alterations in the pain tolerance in the rodent model such as performed in this study. However, the observed stepwise hyperalgesic effect may be attenuated in time-dependent manner confirming the existence of powerful adaptation mechanisms in CNS.

CONFLICTS OF INTEREST

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SYSTEMIC DISEASES WITH ORAL MANIFESTATIONS AND THEIR IMPACT ON HEALTH-RELATED QUALITY OF LIFE

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ABSTRACT

Health-related quality of life is a multidimensional concept established to evaluate the physical, psychological, and social impacts of health conditions on individuals' well-being. Various tools for measuring health-related quality of life can be categorized into two subsets: generic and disease-specific instruments. The oral cavity can be stricken by a broad range of local and systemic diseases and their systemic treatment modalities. The most common systemic illnesses associated with oral lesions are hematologic disorders, endocrinopathies, neurological disorders, gastrointestinal conditions, mucocutaneous and rheumatic diseases, and neoplastic processes. Their manifestations in the oral cavity are, in most cases, rather nonspecific but should not be overlooked. Oral health is one of the most important parts of overall health, thus it has been proposed that poor oral health may affect health-related quality of life. The presence of oral manifestations of systemic diseases has a negative impact on the daily functioning of patients, decreasing their overall well-being. This article will review the most common systemic diseases with oral manifestations and their impact on the health-related quality of life. Oral health researchers should put a stronger emphasis on the patient-reported quality of life as a primary outcome in future clinical trials. The significance of this area has still not been widely understood in the current dental literature even though it could help improve patients' health-related quality of life.

Keywords: *Oral manifestations, systemic diseases, health-related quality of life.*

ABBREVIATIONS

COMDQ - Chronic Oral Mucosal Disease Questionnaire
EuroQol-5D - European Quality of Life 5D
HRQoL - Health-Related Quality of Life
IBD - Inflammatory Bowel Disease
OHRQoL - Oral Health-Related Quality of Life
OLP - Oral lichen planus
SF-36 - 36 - Item Short Form Survey Instrument
SLE - Systemic lupus erythematosus
SS - Sjögren syndrome
SSc - Systemic sclerosis
SScQoL - The Systemic Sclerosis Quality of Life Questionnaire
QoL - Quality of Life
MS - Multiple sclerosis
AD - Alzheimer's disease
PD - Parkinson's disease

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INTRODUCTION

Health-related quality of life (HRQoL) is a multidimensional concept established to evaluate the physical, psychological, and social impacts of health conditions on individuals' well-being (1). It cannot be measured using only one domain, so key aspects of HRQoL include physical status and functioning, mental health, and social interactions (2). Oral health is one of the most important parts of overall health, thus it has been proposed that poor oral health may impact health-related quality of life (3). The oral cavity can be stricken by a broad range of both local and systemic diseases and their systemic treatment modalities (4). Oral changes in systemic disorders may help in their diagnosis, as many of them first present in the oral cavity and ensure guidance for their adequate treatment (5, 6). This article will review the most common systemic diseases with oral manifestations and their impact on the health-related quality of life.

HEALTH-RELATED QUALITY OF LIFE

The World Health Organization defines the quality of life as "individuals' position in life in the context of the culture and value systems in which they live and in relation to their goals, expectations, standards, and concerns" (7). Quantification of quality of life, used in health studies to assess physical, psychological, and social functioning, refers to the term health-related quality of life (8). Various tools for measuring HRQoL can be categorized into two subsets: generic and disease-specific instruments. Generic tools are used in the general population, their validity has been confirmed in different chronic conditions and disorders, but they are not specific for a certain disease or a group of patients (9, 10). SF - 36 (36-Item Short Form Survey Instrument) and EuroQol-5D (European Quality of Life 5D) questionnaires are generic instruments and, according to published studies, the most broadly used tools for measuring HRQoL (11, 12). Their validity and reliability have been confirmed in a great number of clinical studies, and they are available in multiple world languages. Disease-specific HRQoL questionnaires are focused on a particular illness and are characterized by greater sensitivity and specificity than the generic ones (13). Their main limitation is that they are unable to compare health conditions of different natures, which is not the case with generic instruments (14). In recent years, many questionnaires for HRQoL evaluation have been developed because this concept has been recognized as one of the most important outcomes in clinical and health services research. It is a valuable predictor of treatment success, and it has a prognostic significance (15). Therefore, its routine use in clinical trials is highly recommended (16). Expressing HRQoL as a single value, called health state utility value, scored on a scale from 0 (death) to 1 (perfect health), is significant for health economic evaluations. It allows for comparison between different therapeutic areas since outcomes are given in the standard units (17). They can be derived through direct (standard gamble and time trade-off) and indirect (generic HRQoL questionnaires) methods (18). Health state utility values are used for assessing disease burden and calculating quality-adjusted life years (QALYs) that comprise both quantity and quality of life.

ORAL HEALTH-RELATED QUALITY OF LIFE

Even though most oral health problems are not considered life-threatening, they still pose a serious public health burden due its high prevalence and major socioeconomic and psychological consequences, and thus effects on the quality of life (19). Oral health-related quality of life (OHRQoL) is a construct that represents the impact of oral conditions on daily functioning, and it is associated with functional factors, psychological factors, social factors, and experience of pain or discomfort (20, 21). An impairment of overall quality of life can also potentially affect OHRQoL because OHRQoL is an essential part of the general health-related quality of life and contributes to it at biological, social, and psychological levels (3, 22). Its important role has been recognized in different domains of dentistry, such as clinical dentistry, dental research, and dental education (23). OHRQoL can be assessed using various tools like socio-dental indicators, global self-ratings of oral health, and multiple item questionnaires. Social indicators measure OHRQoL at a community level, global self-ratings estimate an individual's oral health at one point in time, and multiple item questionnaires, generic or specific, are usually used for OHRQoL evaluation (24). OHRQoL instruments have been developed and tested for adults and children, respectively. Some of the most widely used tools include General Oral Health Assessment Index, Subjective Oral Health Status Indicators, Dental Impact on Daily Living, Oral Impact on Daily Performances, and Oral Health Impact Profile-14. The number of questionnaires is increased daily, but their utilization has to be verified in the clinical trials (25). OHRQol contains three domains - physical, social, and psychological and therefore, used along with the clinical indicators, provide a more comprehensive picture of one's health status than a simple clinical evaluation. Assessment of OHRQoL is crucial for insight into individuals' feelings related to their health, a better understanding of the role that oral health plays in patients' daily lives, and developing new protocols for evidence-based clinical practice (26, 27).

ORAL MANIFESTATIONS OF SYSTEMIC DISEASES

There are a lot of conditions and diseases that primarily affect the mouth, but a wide range of systemic illnesses and the effects of their systemic treatment could also manifest in the oral cavity (6). The most common systemic diseases associated with oral lesions are hematologic disorders, endocrinopathies, neurological disorders, gastrointestinal conditions, mucocutaneous and rheumatic diseases, and neoplastic processes (4). Oral manifestations of systemic diseases are, in most cases, rather nonspecific and may be found in a plethora of different conditions. Both soft and hard tissues can be affected during the clinical course of the disease. Gingival

bleeding and enlargement, oral ulcers, bacterial, viral, and fungal infections, dry mouth, and mucosal inflammation are some of the most common soft tissues' findings. When it comes to hard tissues, dental caries, periodontitis, enamel hypoplasia, and loss of the teeth are often diagnosed among patients with systemic illnesses (28). Oral lesions should not be neglected, especially because they may be the initial clinical manifestation of several grave health conditions, and some of the systemic diseases could also be potentially identified based on the oral cavity findings during dental examination (5). Systemic diseases can have a direct effect on the status of the oral tissues but inversely, oral conditions, such as periodontal disease, may negatively impact the general health of the patients with systemic disorders (4).

RHEUMATIC DISEASES

Sjögren syndrome

Sjögren syndrome (SS) is an autoimmune disease characterized by lymphocytic infiltration of exocrine glands, especially lacrimal and salivary glands, resulting in their dysfunction and destruction (29). SS appears in 0.5-3% of the general population, predominantly in women. Some of the patients only experience symptoms related to the eyes and mouth (primary SS) and about 50% of them also have another autoimmune condition, such as systemic lupus erythematosus or rheumatoid arthritis (secondary SS) (30). The main clinical signs of SS are manifested in the oral cavity. Due to the reduction of saliva secretion and consequential hyposalivation, patients are prone to tooth caries, gingival inflammation, and opportunistic infections (31, 32). Chronic xerostomia may impair all of the orofacial functions, including chewing, swallowing, and talking, alter the taste and cause sleeping problems (33). Patients also often complain of dry lips, angular cheilitis, and halitosis (34, 35). Still, some other oral lesions, such as ulcers, are not related to a decrease of salivary flow but are probably directly associated with SS (34).

Patients with Sjögren syndrome have significantly decreased HRQoL, which is correlated not only with the main symptoms of the disease but also with psychological, ocular, oral, and sleep disorders. Pain and fatigue are two factors that mainly affect HRQoL, and saliva flow significantly impacts OHRQoL (36). Researchers of the European League Against Rheumatism (EULAR) have developed a set of outcome measures for clinical trials and practice that include both systemic activity and patients' symptoms. The EULAR SS Disease Activity Index (ESSDAI) is a clinical index that clinicians use for the evaluation of disease activity in patients with systemic complications of primary SS. Today it is recommended as a gold standard and primary outcome measure in randomized clinical studies (37). The EULAR SS Patient Reported Index (ESSPRI) has been designated as an instrument for the assessment of patients' symptoms. It consists of three domains that are recognized as the main symptoms that have an impact on HRQoL - overall dryness, pain, and fatigue (38). These tools should be used together for a better understanding of all of the disease's aspects, especially disease activity after therapeutic intervention (39). The first disease-specific instrument for evaluation of HRQoL in patients with SS has been developed in 2018, and it has shown good psychometric properties (40). There is an evident lack of research regarding health state utility values for SS, with only one study reporting impaired utility values compared to the general population (41). Having that in mind, new endpoints that are focused on HRQoL domains and validated exclusively for SS are needed for future clinical trials.

Systemic lupus erythematosus

The systemic lupus erythematosus (SLE) is a chronic, autoimmune disease with a variable clinical course (4, 29). SLE predominantly affects the skin, joints, muscles, lungs, kidneys, blood cells, and nervous system (42). Oral lesions appear in 8-45 % of patients, and they represent one of the most significant clinical aspects of SLE (4). They can manifest as white plaque or erosive lesions in the center, with lines in the periphery, most commonly localized on the labial, buccal, and gingival mucosa (43). Mucosal macules, plaque, and lesions that resemble oral lichen planus or leukoplakia can also be noted in patients with SLE (4). Petechial bleeding and erosive mucosal lesions may be a sign of serious thrombocytopenia (29). Recurrent aphthous stomatitis and ulcers not only accompany SLE but are also linked to the disease activity. Sometimes they might be the initial manifestations of SLE. Chronic ulcers in SLE have a higher risk of malignant transformation into squamous cell carcinoma compared to other ulcerative lesions (4). Some of the less frequent oral symptoms include xerostomia, dysphagia, and fungal infections (44, 45).

SLE has a detrimental effect on the patients' HRQoL because of the pain, physical appearance, and neurological impairment. Several specific patient-reported outcome measures have been developed so far for assessment of their HRQoL. Lupus Quality of Life questionnaire consists of 34 items, with a 5-point response format and a score ranging from 0 (worst HRQoL) to 100 (best HRQoL). SLE-specific Quality of Life questionnaire covers six domains with a 7-point response scale, and scores range from 40 to 280, with higher values corresponding to worse HRQoL. SLE Quality of Life Questionnaire includes 25 items and a "true/not true" response format with a score ranging from 0 to 25 (higher scores representing worse HRQoL) (46). Mean health state utility values for patients with SLE may vary, depending on a measure used, from 0,67 to 0,80, as results from a recent study showed (47).

Systemic sclerosis (Scleroderma)

Systemic sclerosis (SSc) is a rare connective tissue disorder characterized by progressive fibrosis of the skin, organs, and systems (48). The orofacial region is among the most affected by SSc (49). Sclerosis of the skin around the mouth area leads to the limited mouth opening called microstomia, resulting in problems with eating and potential malnutrition, speaking, oral hygiene, and dental treatments (50, 51). Submucosal fibrosis causes atrophy of the oral mucosa, which

becomes pale, with telangiectasias located at the tongue (52). Patients frequently complain of burning mouth syndrome, dysesthesia, and xerostomia (50). SSc might be associated with trigeminal neuralgia, temporomandibular joint dysfunction, and idiopathic tooth resorption (53, 54).

Results of previous studies suggest that patients with SSc have poorer HRQoL than patients with other rheumatic diseases, such as rheumatoid arthritis and systemic lupus erythematosus (55). The extent of skin involvement is a significant factor associated with reduced physical and mental domains of HRQoL in SSc patients. Several instruments are validated for use, but only a few of them are specific for SSc, such as The Systemic Sclerosis Quality of Life Questionnaire (SScQoL) (56). SScQoL consists of five domains - physical functioning, emotional functioning, social functioning, sleep, and pain. Higher scores indicate the more prominent effect of the disease on HRQoL. Results of a recent study showed that mean health state utility values for patients with shorter disease duration (less than two years) ranged from 0,63 to 0,80, whereas for longer disease span, they varied from 0,67 to 0,87, depending on the technique used (57).

MUCOCUTANEOUS DISEASES

Lichen planus

Lichen planus is a chronic inflammatory condition of unknown etiology that can affect the skin and mucous membranes, including the oral mucosa (58). The oral lichen planus (OLP) is a relatively common form, with a prevalence of 0,5 to 2% in the general adult population (59). Oral lesions often appear before skin lesions, and most of the time remain the sole manifestation of the disease (60). All parts of the oral cavity can be affected, but the lesions are predominantly located on the buccal mucosa, with bilateral and symmetrical distribution (61, 62). There are six forms of OLP: reticular, papular, plaque-like, atrophic, erosive-ulcerous, and bullous-erosive (60, 63, 64). The reticular OLP is presented as white keratotic lesions, resembling lace, called Wickham striae (65). The papular OLP is characterized by papules on the buccal mucosa predominantly. The plaque-like type is hard to differentiate from leukoplakia because they share the same localization - the dorsal surface of the tongue (64). These forms of OLP are generally asymptomatic (60). Atrophic, erosive, and bullous types are followed by a different range of symptoms, from mild to intense pain. Clinically, atrophic and erosive forms present like atrophic lesions with white radiating lines at the periphery, and the bullous one can resemble erosive type as the fragile bullae easily get ruptured (64, 66). Oral lichen planus is characterized by periods of remissions and exacerbations and can be triggered by different factors (58, 67).

In the last decade, several disease-specific HRQoL instruments for OLP have been designed to assess all the aspects of disease important to patients, including the Chronic Oral Mucosal Disease Questionnaire (COMDQ) (68). COMDQ is a Likert - type scale that covers four areas - physical discomfort, medication and treatment, social and emotional, and patient support, with 60 points being the highest score. It is considered the most appropriate instrument for measuring HRQoL in OLP patients, and it is the only one designed with input from them (68). General tools have also been used for assessing HRQoL, but their psychometric properties or interpretability have not been tested in the OLP population (69). Therefore, it is necessary to develop a core set of outcome measures, especially patient-related, to improve the quality of the future clinical studies and clinical meaningfulness of their results. Pain, disease activity, and depression are factors associated with impairment of the patients' HRQoL (70).

Pemphigus

Pemphigus is a group of chronic, antibody-mediated autoimmune vesiculobullous diseases that manifest on the skin and mucous membranes (71). Pemphigus vulgaris and pemphigus foliaceus are the most widespread subtypes (72). The majority of the patients (up to 70%) with pemphigus vulgaris have mucosal lesions that initially appear in the oral cavity, while the skin is not always affected. On the opposite, patients with pemphigus foliaceus do not experience mucosal changes (73). Oral lesions in pemphigus vulgaris are often the first and only sign of the disease. They appear as vesicles and bullae, filled with fluid, that burst very easily and leave irregular erosions and ulcers (74). Most lesions do not tend to leave a scar but can be very painful for the patients. The commonest parts of the mouth that are affected include gingival, buccal, and palatal tissues (75). In severe cases, desquamative gingivitis can also be found (4).

Even though treatment options for pemphigus have improved in recent years, there is still a great need for new therapy modalities. To enhance the quality of clinical trials that are testing innovative pharmacological agents, disease severity and HRQoL should be appropriately evaluated. HRQoL is usually measured with generic tools, but a disease-specific instrument has also been developed - the Autoimmune Bullous Disease Quality of Life (76). It represents a valid and reliable patient-based measure that correlates with disease severity indices and can accurately quantify how the disease impacts a patient's HRQoL. This instrument could also be used as an endpoint in clinical trials as it may successfully measure disease burden, activity, and therapy response (76). A recent study reported a mean utility value of 0,76 for pemphigus patients, which is relatively low compared with other chronic dermatological conditions, such as psoriasis and atopic dermatitis (77).

Pemphigoid

Pemphigoid diseases are autoimmune diseases represented by autoantibodies directed against various structural components of the dermal-epidermal junction, resulting in the formation of subepithelial blisters (4). Of all the entities in this group, bullous pemphigoid and mucous membrane pemphigoid are the most commonly presented in the oral cavity. Their clinical manifestations are fairly similar - vesicles and bullae, sometimes blood-filled, located

predominantly on epithelialized mucosa (especially gingiva), bleeding, and pain. Irregular erosions and ulcers can be noted after blisters burst, and the Nikolsky sign might be positive (78). Oral lesions are prone to scarring, forming multiple mucosal contractures (4).

Bullous pemphigoid has a significant effect on the psychological status of the patients due to the appearance of the lesions on the skin and oral mucosa, functional problems, and disease chronicity (79). Their HRQoL can be measured using both generic and disease-specific instruments, such as the Autoimmune Bullous Disease Quality of Life (77). It is a 17-item scale that includes questions about pain, pruritus, healing, anxiety, and depression. The maximum score is 51, and the higher results indicate more severe impairment of HRQoL (80). Bullous pemphigoid affects all aspects of patients' daily lives, so it is of importance to incorporate HRQoL as the primary outcome in future clinical trials to accurately measure treatment efficacy.

GASTROINTESTINAL DISEASES

Ulcerative colitis and Crohn's disease

Ulcerative colitis and Crohn's disease are systemic diseases classified as chronic inflammatory bowel disease (IBD) (81). Periodontitis, aphthous stomatitis, and in severe cases, pyostomatitis vegetans are particularly pronounced in patients with Crohn's disease (81, 82). Patients in the acute phase of the disease show a significantly higher degree of oral lesions (83). The most commonly affected regions are the lips, gingiva, and less often the vestibule and tongue, with predominant clinical symptoms in the form of ulcers, papules, and edema. Mucogingivitis, labial swelling, and linear ulcerations located in the buccal sulci often occur (83). A specific indicator of ulcerative colitis is pyostomatitis vegetans, while other manifestations often appear but are not specific, such as aphthae, halitosis, dry mouth, and various forms of gingivitis (84).

Ulcerative colitis and Crohn's disease are associated with the significant psychological and social burden. The use of HRQoL as an endpoint in clinical trials involving IBD has increased dramatically over the past decades and has become an essential outcome measure. Inflammatory Bowel Disease Questionnaire is a 32-item-instrument, divided into four domains: bowel symptoms, systemic symptoms, social and emotional functioning (85). The total score ranges from 32 (poor HRQoL) to 224 (good HRQoL). A score higher than 170 is usually found in patients in clinical remission (86). Health state utility values, reported from a recent analysis, for patients with the active form of the disease were 0.69 (for ulcerative colitis) and 0.75 (for Crohn's disease). Patients in remission recorded significantly higher values of 0.87 and 0.84 (87).

Celiac disease

Celiac disease is an autoimmune disease that implies a permanent sensitivity to gluten, and it develops in people with a genetic predisposition under the influence of environmental factors. Gluten sensitivity leads to various intestinal symptoms with accompanying extraintestinal manifestations, and it often happens that extraintestinal symptoms are pronounced more significantly than intestinal ones (88). Oral lesions appear in the form of hypoplasia of the dental enamel, which has a higher prevalence in children than in adults. Aphthous lesions that regress after a gluten-free diet can be noticed (89). Glossodynia and atrophic glossitis, more frequent incidence of caries and bleeding tendency are also clinical indicators of Celiac disease (88).

Intestinal and extraintestinal symptoms, medical comorbidities, and major dietary restrictions are associated with impairment of HRQoL in patients with celiac disease (90). Celiac Disease Quality of Life is a specific tool that consists of 20 questions, distributed into four domains: dysphoria, limitations, health concerns, and inadequate treatment (91). The overall Celiac Disease Quality of Life score is expressed in a 100-point scale (0 - worst HRQoL, 100 - best HRQoL). HRQoL should become a key outcome in the evaluation of novel treatments for celiac disease, so new valid and well-designed patient-related measures are necessary.

HEMATOLOGIC DISEASES

Anemia

Anemia is a condition characterized by the decline in the content of hemoglobin and oxygen due to the reduced number or function of red blood cells (92). Anemia can present as an iron deficiency or pernicious anemia. Both mentioned could manifest in the oral cavity. Iron deficiency anemia is associated with atrophic glossitis and pale mucosa. The condition known as the magenta tongue is characteristic of pernicious anemia, which manifests as tongue erythema with atrophy (5). An extremely reddened tongue with atrophic borders is usually the preliminary sign of anemia (92). Angular cheilitis, burning sensations of the tongue, lips, and buccal mucosa are very common findings for anemia (5).

Thrombocytopenia

Thrombocytopenia has been identified as a disorder associated with a substantial decrease in platelets. A reduction in the number of platelets leads to various oral manifestations, most commonly petechiae and purpura. Such oral signs include the occurrence of bleeding from minor cuts, inefficiency in the formation of clots, and gingival hyperplasia, and depending on the platelet counts, bleeding on trauma or spontaneous bleeding (5, 74).

The intensity and recurrence of the oral manifestations of hematological diseases can cause significant discomfort and have a great impact on a patient's HRQoL. Swollen lips, dry mouth, cuts, pain, and bleeding in the mouth often compromise mastication. Patients also complain about feeling embarrassed during mealtimes because of the judgment from

others. All that can cause a patient to feel different and excluded from the environment. Regarding speech problems, patients mainly complain of the lack of saliva. They often state that their social interaction, being with others, and recreational activities are impaired and jeopardized by oral alterations as a result of their primary disease (93). HRQoL of patients with hematologic diseases has mostly been measured using generic instruments so far, but an effort should be made to include more patient-reported outcome measures as endpoints in future clinical trials.

ENDOCRINOPATHIES

Diabetes mellitus

Diabetes mellitus is a disease that changes the resistance of tissues to local irritations due to the deposition of acidic mucopolysaccharides and glycoproteins on the walls of blood vessels. Because of the mentioned disorders, proliferative changes, microthrombosis, and microhemorrhage on the endothelium are likely to occur (94). Microcirculatory pathways, nutritional imbalance, and oxygenation disorders affect periodontal tissues (95). Oral changes appear in the form of inflammation of the gingiva, which is swollen, hyperemic, the periodontium is hypersensitive, and its destruction is accelerated, while the periodontal pockets are very deep with abundant suppuration present. Alveolar bone resorption is extremely pronounced and rapid (96). In addition to the above, the main oral manifestation of diabetes mellitus is the appearance of multiple periodontal abscesses (95). Xerostomia, candidiasis, and burning in the mouth are present, as well as slowed healing of wounds and oral ulcers. Diabetes does not cause periodontitis, but it can lead to its deterioration, while oral manifestations of diabetes may help in its diagnosis (97). Severe periodontal disease is associated with worsening glycemic control (74). The relationship is explained by the fact that chronic infection with Gram-negative bacteria in dental plaque in diabetics leads to increased insulin resistance in tissues and increased hyperglycemia (74, 98, 99). Periodontal therapy affects the metabolic control of diabetes, so if this disease is suspected, appropriate diagnostic tests should be performed immediately (99).

Many disease-specific instruments for diabetes mellitus exist in the literature, and they cover all the relevant aspects of HRQoL concept. The use of appropriate, up-to-date measures is very important for the evaluation of patient's experiences with their disease and treatment (100). Diabetes mellitus affects various parts of the organism and therefore has physical, psychological, and social implications. The impaired HRQoL in patients with diabetes mellitus is mostly a result of the local and systemic complications of the disease, including oral alterations (101). Diabetes-related complications are associated with lower health utility scores, and the impact of various complications on the HRqOL differs significantly (102). Painful neuropathy and amputations have the greatest decline in the health utility scores (up to 0,1), while heart disease and peripheral vascular disease show a relatively small decrease (less than 0,03) (102). Utility scores, generated from the HRQoL instruments, could be used for multiple purposes: measuring quality-adjusted life-years and evaluating improvement of diabetes management (103).

Addison's disease

Addison's disease develops as the result of the destruction of the adrenal cortex. Characteristic clinical signs of adrenal disease begin with general patient weakness, malaise, and fatigue accompanied by weight loss due to loss of appetite (104). Oral manifestations of this disease are characterized by the pigmentation of the mucous membranes, lips, and skin. Hyperpigmentation of the oral mucosa occurs due to elevated adrenocorticotrophic hormone and is one of the first signs of Addison's disease (105). Bluish-black spots are seen in the mouth in up to 82% of patients (6). During treatment, skin pigments most often disappear while oral pigments stay longer (106).

AddiQoL is Addison's disease-specific instrument that consists of 30 items, with a total score ranging from 30 to 120. Higher scores indicate better HRQoL. Patients with Addison disease have reduced HRQoL mainly due to fear of potentially life-threatening adrenal crisis (107).

NEUROLOGICAL DISORDERS

Multiple sclerosis

Multiple sclerosis (MS) is a chronic, demyelinating, neurodegenerative disease of the central nervous system (108). It is the most common neurologic disorder of the young population, with more than 2,8 million people affected worldwide, predominantly women (109). MS is characterized by fatigue, pain, muscle weakness, vision and speech impairment, and cognitive dysfunction (110). Various oral health problems may also be associated with MS. They are mostly caused by patients' inability to maintain adequate oral hygiene due to severe fatigue and motoric disability. Studies suggest a higher prevalence of dry mouth, dental caries, periodontal disease, temporomandibular joint dysfunction, and other neurological disorders such as trigeminal neuralgia and dysgeusia in MS patients (111, 112). Side effects of the medications used in MS treatment might affect oral mucosa, resulting in deterioration of the overall oral health (110).

Both generic and disease-specific tools are widely used in the HRQoL evaluation of MS patients. Several disease-specific instruments are available, including the Multiple Sclerosis Quality of Life-54 questionnaire, the Multiple Sclerosis Quality of Life Inventory, Functional Assessment of Multiple Sclerosis, Multiple Sclerosis Impact Scale-29, and Multiple Sclerosis International Quality of Life questionnaire (113). Cognitive and physical impairment are the most important predictors of HRQoL in MS patients (114). Future clinical studies are needed for a more profound understanding of oral manifestations' impact on HRQoL aspects of patients suffering from MS.

Alzheimer's disease

Alzheimer's disease (AD) represents a progressive neurodegenerative disorder associated with a decline in cognitive functions. The number of cases is constantly growing, and it is estimated that 5-7% of all people over the age of 60 are affected worldwide (115). AD is characterized by the deposition of amyloid plaques in the brain, leading to a decrease in cognitive and physical abilities (116). With the progression of the disease, patients' oral health status is worsening, mainly due to their reduced capacity to maintain adequate oral hygiene. That can result in a higher prevalence of dental caries, periodontal disease, and mucosal lesions, which might affect certain HRQoL domains (117). Patients also report problems with wearing dentures and subsequent insufficient mastication.

The presence of less than five natural teeth, carious teeth, and poor periodontal status are predictive factors of OHRQoL impairment in AD patients (117). Various measures have been developed for measuring their HRQoL, such as Alzheimer Disease Related Quality of Life and Quality of Life in Alzheimer's Disease (118). Recent research showed that non-cognitive symptoms, like depression and loss of functional ability, influenced HRQoL in AD more than cognitive ones (119). Prevention and timely treatment of oral disease are necessary for proper nutrition and oral health of patients with AD, which will positively impact their overall HRQoL.

Parkinson's disease

Parkinson's disease (PD) is a chronic neurodegenerative disease characterized by tremor, rigidity, and hypokinesia. It affects 2-3% of all people over the age of 65, mostly men (120). Motor inability often leads to improper oral hygiene, which results in a higher prevalence of caries, periodontal disease, and loss of teeth (121). Drooling, xerostomia, and dysphagia are also common symptoms in the later stages of PD. They have a significant detrimental influence on OHRQoL in PD patients (121). Decreased saliva production due to medication or autonomic dysfunction results in the onset of caries and periodontal disease. Adequate oral hygiene is therefore essential for PD patients and improvement of their OHRQoL.

Evaluation of HRQoL in PD patients poses a great challenge. Research suggests that fatigue, walking problems, and non-motor symptoms, like nocturia and forgetfulness, remarkably contribute to their worse HRQoL (120). HRQoL in PD might be assessed using different generic and disease-specific instruments, such as 39-item Parkinson's Disease Questionnaire. Frequent evaluation of HRQoL of PD patients is fundamental for the identification of factors associated with its decline and their proper management.

ONCOLOGICAL DISEASES

Leukemia

Leukemia is considered one of the most life-threatening cancers and represents a major therapeutic challenge. Early diagnosis of the disease is of great importance, where the doctor of dental medicine can also play an essential role because the illness causes oral changes (106). Clinical manifestations of leukemia include anemia, pallor, bleeding, splenomegaly, hepatomegaly, and lymphadenopathy (74). Oral manifestations exist both in the case of chronic and acute leukemia. They can be primary (a direct consequence of leukemic cell infiltration) or secondary (a result of neutropenia, thrombocytopenia, reduced granulocyte function) (122). Common oral manifestations of leukemia are mucosal pallor and significant gingival bleeding, which may be the disease's first symptom (5, 74, 106, 122). Hemorrhages on the hard and soft palate may occur when the platelet count falls below 20,000 / μL (20×10^9 / L) (5). An enlargement of the gingiva can also be noticed, which is explained by the cellular infiltrate and inadequate oral hygiene (5, 74, 106, 122). Ulcerations that can be found are usually very deep, with a necrotic base (5). Opportunistic infections often accompany leukemia, and usually, Candida albicans or Herpesviruses cause them (5, 122).

The Functional Assessment of Cancer Therapy-Leukemia and Medical Research Council/EORTC Quality of Life Questionnaire Leukemia Module are used for evaluation of HRQoL in patients with leukemia. Health utility values cannot be derived from these tools since they are not preference-based, so generic instruments are implemented for that purpose (e.g. EuroQoL-5D) (123). The mean utility score of patients with leukemia is lower when compared to the general population, which highlights the negative effect of leukemia on HRQoL. Infections, anemia, and bleeding are factors mainly associated with worse HRQoL, while social support seems to be the predictor of better HRQoL (124).

Multiple myeloma

Oral manifestations may also develop in the later stages of multiple myeloma. The most common changes occur in the mandible, and asymmetry can even be very noticeable. Patients frequently complain of pain, swelling, paresthesias, and tooth mobility (5, 125). Imaging exams often show osteolytic lesions, which can also lead to pathological fractures (125). Macroglossia can sometimes be seen as a consequence of amyloid deposition (5).

Patients with multiple myeloma have reduced HRQoL due to pain, fatigue, psychological and social factors (126). Multiple Myeloma-Specific Core Questionnaire 20 is a validated instrument that consists of 20 questions and includes four myeloma-specific aspects of HRQoL: body image, disease symptoms, treatment side effects, and future perspective (127). Domain scores are transformed to a linear score

ranging from 0 to 100. Higher scores for disease symptoms and treatment side effects indicate more severe symptomatology, whereas high scores for body image and future perspective are associated with better outcomes (128). Health utility values derived from EuroQol-5D may range from -0,025 to 0,9 (for asymptomatic patients), according to a systematic review that analyzed 26 publications and included 11 112 patients (129).

Oral squamous cell carcinoma

The most common cancer in the oral cavity is squamous cell carcinoma. The symptoms that accompany its appearance are discomfort, dysphagia, odynophagia, limited movement, neck mass, and weight loss. It is clinically manifested as long-term ulceration, which does not go away with the removal of potential irritating factors. It may have an irregular, granular, rough, or crusty structure and may be present as a red or white lesion (92, 130).

Patients with oral squamous cell carcinoma report a great number of symptoms that reduce their HRQoL, including pain and problems with chewing, swallowing, and talking (131). Psychological and social domains are also affected. The Basal and Squamous Cell Carcinoma Quality of Life is a recently developed questionnaire that encompasses five subscales, scored from 0 to 3, with higher scores representing worse HRQoL (132). It can be used in future clinical trials for comparison of different cancer treatment modalities.

Head and neck cancer therapy

Cancer therapy of the head and neck region can have various side effects and impact significantly OHRQoL. Anesthesia, paraesthesia, hyperesthesia, dysgeusia, tongue mobility changes, and trismus may occur as surgical therapy complications (92). The most common accompanying complications of radiotherapy are oral mucositis, opportunistic infections, and osteoradionecrosis. Periodontal tissue damage can also happen (92, 133).

Impairment of HRQoL of cancer patients that received therapy might persist over a long time after treatment in the form of various physical and psychological problems. Different health utility values for head and neck cancer survivors are reported in the literature, depending on the technique used, and range from 0,45 (distant metastasis at initial treatment) to 0,79 (patients initially treated at a locally advanced stage) (134).

The main oral manifestations and disease-specific instruments for measuring HRQoL of the aforementioned systemic diseases are summarized in Table 1.

Table 1. Various systemic diseases - main oral manifestations and HRQoL disease-specific tools

Disease	Main oral manifestations	Disease-specific tools	Published	Authors	References
Sjögren syndrome	Xerostomia	pSS - QoL	2018.	Lackner A. et al.	(33, 40)
Systemic lupus erythematosus	Aphthous stomatitis, ulcers	The SLE QoL Questionnaire	2009.	Galen Research	(43, 135)
Systemic sclerosis	Microstomia, dysphagia	The Systemic Sclerosis QoL Questionnaire	2008.	Reay N.	(50, 56)
Lichen planus	Symmetrical white lesions	COMDQ	2011.	Riordain N. et al.	(65, 136)
Pemphigus	Vesicles and bullae, ulcers	Autoimmune Bullous Disease QoL	2012.	Murrell D. et al.	(67, 77)
Pemphigoid	Vesicles and bullae, ulcers	Autoimmune Bullous Disease QoL	2012.	Murrell D. et al.	(77, 78)
Ulcerative colitis	Pyostomatitis vegetans	Inflammatory Bowel Disease Questionnaire	1989.	Guyatt G. et al.	(84, 137)
Crohn's disease	Aphthous stomatitis	Crohn's Life Impact Questionnaire	2015.	Galen Research	(81, 138)
Celiac disease	Aphthous lesions, atrophic glossitis	Celiac Disease QoL	2009.	Drossman D, Dorn S	(88, 139)
Diabetes mellitus	Periodontitis, candidiasis, ulcers	Diabetes QoL Questionnaire	2010.	Speight J. et al.	(97, 140)

Disease	Main oral manifestations	Disease-specific tools	Published	Authors	References
Addison's disease	Mucosal hyperpigmentation	AD-specific QoL Questionnaire	2010.	Euradrenal	(105, 141)
Multiple sclerosis	Xerostomia, periodontal disease, trigeminal neuralgia	Multiple Sclerosis QoL-54	1995.	Ellison GW. et al.	(111, 113)
Alzheimer's disease	Dental caries, mucosal lesions	Alzheimer Disease Related QoL	1999.	Black Bs. et al.	(117, 118)
Parkinson's disease	Drooling, xerostomia, dysphagia	Parkinson's Disease QoL Questionnaire	1996.	De Boer A.	(120, 121)
Leukemia	Mucosal pallor, gingival infiltration	The FACT-Leukemia	2012.	Cella D. et al.	(74, 123)
Multiple myeloma	Swelling, paresthesias	QoL - Multiple Myeloma	1999.	EORTC	(125, 127)
Squamous cell carcinoma	Persistent ulceration	The Basal and Squamous Cell Carcinoma QoL	2018.	Waalboer-Spuij R. et al.	(92, 132)

CONCLUSION

Various systemic diseases, such as rheumatic and mucocutaneous diseases, gastrointestinal conditions, hematologic disorders, endocrinopathies, neurological disorders, and neoplastic processes manifest in the oral cavity and cause a wide range of oral symptoms and signs. Their presence affects both oral and general health-related quality of life and negatively impacts the daily functioning of patients, decreasing their overall well-being. Oral health researchers should put a stronger emphasis on the patient-reported quality of life as a primary outcome in future clinical trials. The significance of this area has still not been widely understood in the current dental literature, even though it could help improve patients' health-related quality of life.

CONFLICT OF INTEREST

All authors of the manuscript declare there is no conflict of interest regarding this paper.

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APERT SYNDROME WITH AGENESIS OF THE CORPUS COLLOSUM - CASE REPORT

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ABSTRACT

Apert syndrome (Acrocephalosyndactylia type 1) belongs to the group of extremely rare congenital dysmorphic syndromes. It is characterized by craniostenosis with very early fusion of the skull and / or cranial base sutures, facial hypoplasia, symmetrical syndactyly of the fingers and toes and other systemic malformations. Multiple CNS abnormalities are common, but simultaneous occurrence of Apert syndrome and agenesis of corpus callosum is estimated about 10%.

We present a male patient born after first, normal and controlled pregnancy and term, naturally birth, in which the Apert syndrome was diagnosed, based on the clinical presentation of the skull and face and syndactyly offingers and toes. Neurological examination established generalized hypotonia and aggravated provocation of the primitive reflexes. Neurosonography showed complete agenesis of the corpus callosum, which makes this case a rare form of this syndrome. The head X-rays showed turricephalic skull shape and suture's synostoses, which is demonstrated in more detail at computed tomography finding. The hands and feet X-rays showed bone synostosis of fingers and toes. The video-electroencephalogram recorded intermittent depression of electrocortical activity. There was conducted the multidisciplinary examination in order to examine the possibility of reconstructive and plastic surgical correction of the anomalies.

The neurodevelopmental disorders in patients with Apert syndrome can be manifested by a variety of congenital malformations, but considering its rare occurrence, the significance of these abnormalities remains unknown.

Keywords: *Acrocephalosyndactylia type 1, syndactylia, agenesis of corpus callosum.*

INTRODUCTION

Apert syndrome (AS), called Acrocephalosyndactylia type 1, is a congenital complex dysmorphic syndromes with the cranio-mandibular-facial dysostosis and syndactyly of fingers and/or toes [1, 2]. AS is inherited as an autosomal dominant disorder, but most often manifested sporadically, indicating fresh mutations and/or environmental effects on the genome [3]. The syndrome is characterized by premature synostosis of the coronal suture in the first place, and then all the sutures with a protuberance of the skull towards the frontal fontanelle, where the skull becomes high and peaked [1, 2]. A typical facial appearance is tall and dominant forehead, flat back of the skull, shallow orbits, broadly spaced eyes, low-set ears, high-arched palate, irregularity in the dentition and mandibular prognathism. The syndactyly of fingers and/or toes is visible at the extremities (skin or skin-bone fusions) [4, 5]. The syndrome is accompanied with difficult or mild developmental delay [6-8].

A cerebral dysgenesis of various degrees is usually present. Main cerebral abnormalities are prominent convolutional markings, ventriculomegaly, crowded foramen magnum and more rarely deficient septum pellucidum and corpus callosum agenesis (ACC) [6, 8]. AS with complete ACC is extremely rare, and only a dozen of cases have been described so far [9-12].

Aetiology of the ACC is unknown. The agenesis can be partial, complete or atypical, isolated, syndromic and non-syndromic, it is significantly more common in male children, and often associated with abnormalities of the septum pellucidum. It is also described by other congenital malformations and found in people with mental health disorders, epilepsy, neurologic manifestations, visual impairment, although it may be asymptomatic or accompanied by minimal functional disorders [9-13].

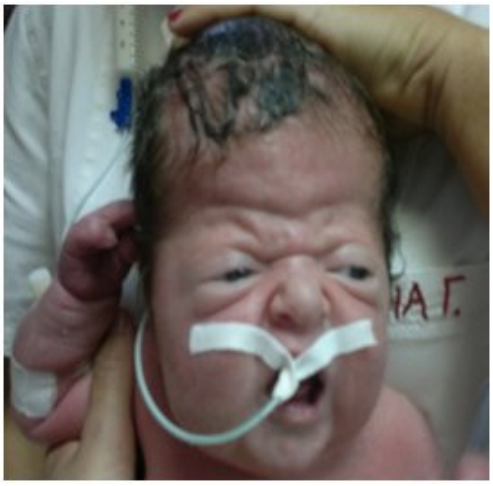
The AS with or without ACC could be diagnosed prenatally using the fetal ultrasonography, nuclear magnetic resonance and amniocentesis in the early neonatal period. After birth the diagnosis is based on the typical clinical appearance documented by additional X-ray and CT diagnostics, while molecular genetic tests, unavailable and expensive in our country, confirm the diagnosis [14, 15]. Apert Syndrome has no specific treatment. Numerous non-surgical methods are used, surgical correction of abnormal connections between the bones of the skull, face and extremities, antibiotic therapy for frequent infections of the inner ear and sinus etc [16, 17].

CASE REPORT

Male child, born after the first, normal, controlled and term pregnancy, with no history of the trauma, infection or drug use during the term, delivered naturally, with birth weight of 4310 g, birth body length of 60 cm and head circumference of 37 cm. The family does not have history of the hereditary diseases. Immediately after birth, the child transferred to the Center of Neonatology, Clinical Centre of Kragujevac due to suspicion of craniosynostosis.

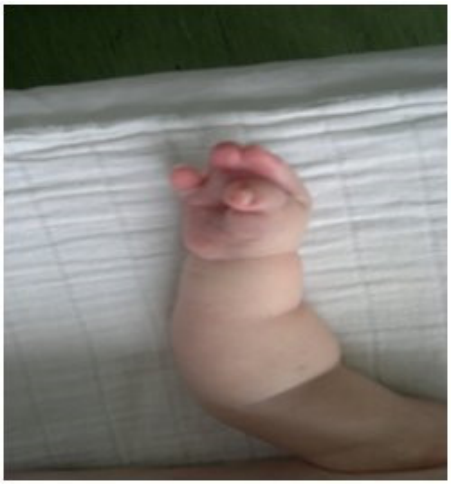
The newborn had normal vital functions and weak cry. Upon inspection, turricephalic head shape was determined. The palpation was used to determine that the frontal fontanelle was shifted forward, widely open and connected via expanded sagittal suture with posterior fontanelle, making improper formation of diameter 15x4 cm, above the level of the bony framework. The examination showed numerous malformations: hypertelorism, exophthalmos, high-arched palate, low-set ears, and short neck (Figure 1).

Figure 1.



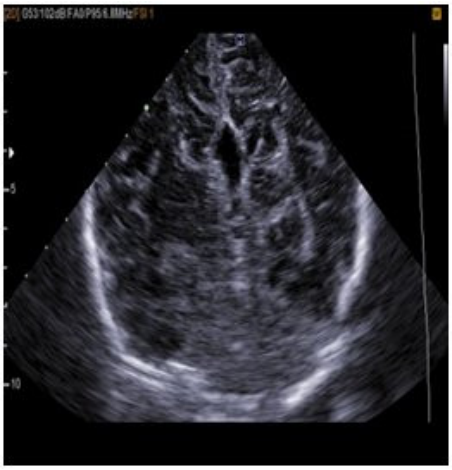
The bilateral syndactyly on hands and feet was noticed (Figure 2). The neurological examination revealed a moderate generalized hypotonia, while primitive reflexes were harder to provoke. We suspected on the Apert syndrome because of the typical anomalies that were noticed, and detailed diagnostics had begun.

Figure 2.



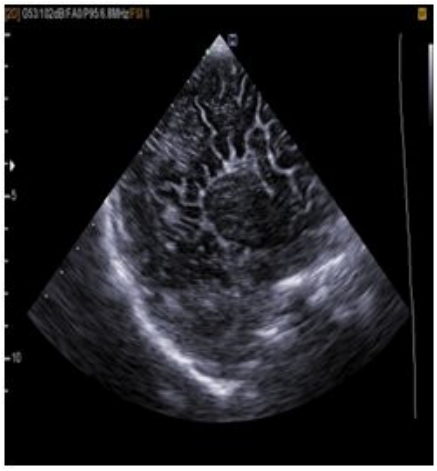
The transfontanelle neurosonography revealed the complete ACC. The coronal sections showed that the interhemispheric fissure was enlarged and it continued onto the third ventricle. The frontal horns of lateral ventricles were widely separated, because there was no corpus callosum above them, while the back parts of the lateral ventricles were grossly dilated (trigone, occipital and temporal horns) (Figure 3).

Figure 3.



The parasagittal scans showed a lack of the corpus callosum and pericallosal artery and radial (not parallel) propagation of sulci on the inner side of the hemispheres (Probst bundles) (Figure 4).

Figure 4.



The head X-rays showed the turricephalic shape of the skull and the synostoses of the sutures (Figure 5). The hands and feet X-rays showed the multiple bone synostosis of fingers and toes (Figure 6).

Figure 5.

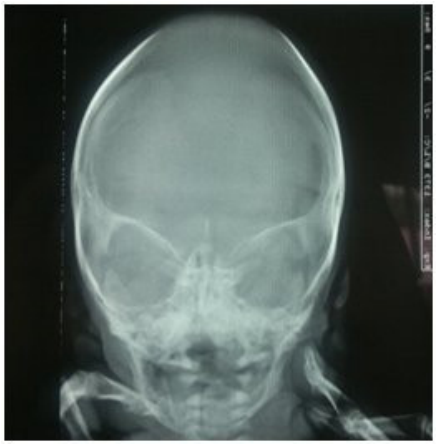
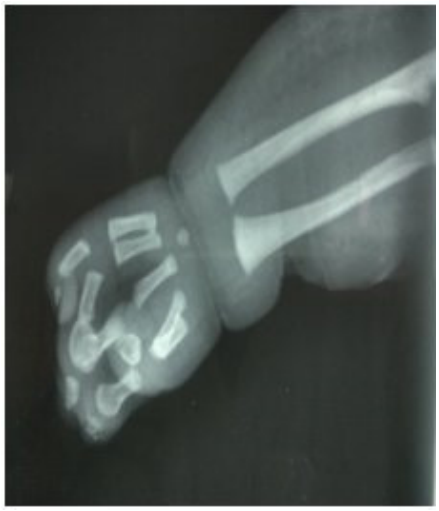
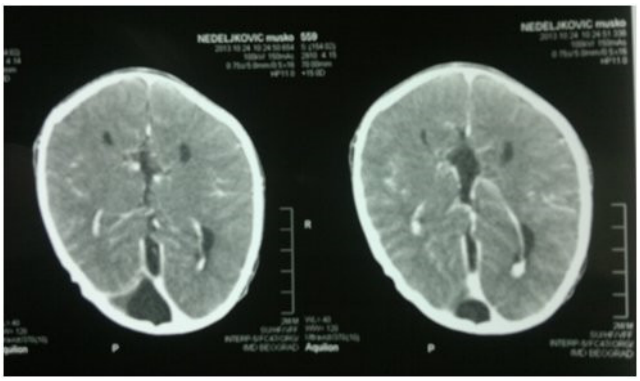


Figure 6.



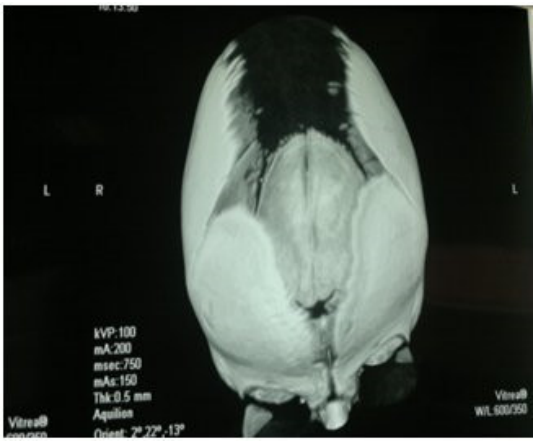
The CT examination of the head (native, followed by IV administration of contrast media) showed parallel lateral ventricles, with emphasized dilatation of the occipital and temporal horns. The frontal horns are oriented upwards and give an image characteristic for the ACC. The third ventricle is elevated and dilated to 12 mm, normal appearance of the fourth ventricle. It was also observed mega cisterna magna (Figure 7).

Figure 7.



The multi slice CT (MSCT) with three dimensional reconstruction of the scull showed the asymmetrical turricephalic shaped skull, with a wide sagittal suture and open metopic suture as a result of coronal craniosynostosis and the bilateral exophthalmos (Figure 8).

Figure 8.

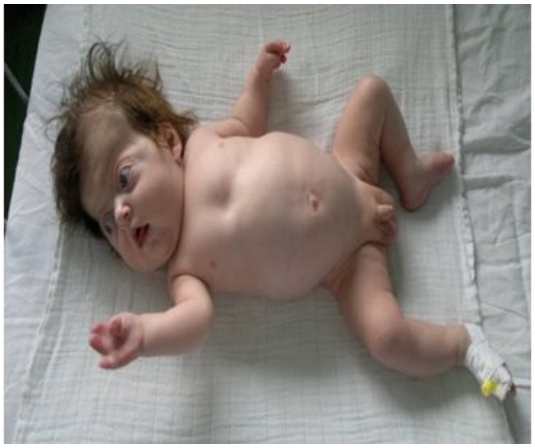


The video-electroencephalogram recorded intermittent depression of electrocortical activity. The karyotype was normal male, 46XY. The AS with ACC was diagnosed based on the clinical findings and examinations.

The additional tests were conducted for eventual reconstructive surgical corrections and plastic surgeries. The fiberoptic nasolaryngoscopy showed bilateral narrow nasal passages and choanae, with deformation of the septum. During the oropharyngoscopy it was determined laryngomalacia and elongated epiglottis. The genetic testing was not carried out due to poor cooperation from the parents (material costs).

At the age of 3 months, the child had normal vital functions and the basic laboratory analyses were within reference ranges. Due to anomalies, the child required regular aspiration of secretions from the upper respiratory tract, not dependent on oxygen therapy, without the apnea crises. He was transferred to the competent reception center at the age of 6 months (Figure 9).

Figure 9.



DISCUSSION

The first clinical description of Apert syndrome dates from 1906, the work of the French physician Eugene Apert, who described 9 patients with similar abnormalities [4, 5]. Considering that the syndrome occurs sporadically, new mutations and/or the influences of environmental factors on genome blamed for its occurrence [3]. Prevalence is estimated at 1:64.500, which makes 3-4% of all craniosynostoses or 10-20% of syndromic craniosynostoses [2]. There is no difference in the occurrence of the syndrome in relation to sex [1]. The abortive cases show an autosomal dominant form, and the results in about 98% cases are point mutations of the fibroblast growth factor receptor 2 (FGFR2) gene, which is located on the long arm of chromosome 10, most commonly derived from the father, which explains the higher incidence in older fathers [1, 2]. The suture progenitor cells with mutated FGFR2 (different expression of mutations of the three isoforms) can not transduce signals from extracellular FGF, as a result they do not produce the fibrous material required for the normal calvarias sutures. There are mutation of the gene for FGFR2-isoforms for keratinocyte growth factor receptor, active in metaphysis and interphalangeal joints, FGFR1 in bone diaphysis, and FGFR2-Bek isoform in metaphysis, diaphysis and in interdigital joint syndactin. When gene mutations for FGFR2 are activating, FGFR2 loses its specificity, and so is activated by other FGF, causing increased osteoblast proliferation and differentiation and early fusion of multiple skull sutures, and other facial bone abnormalities. FGF also suppresses mesenchymal cell apoptosis, the interdigital mesenchyme is not resorbed, and that causing syndactyly [1, 2].

The karyotype is usually normal, or there are separate findings of deletions - translocation (from chromosome 2 to chromosome 11 or 12) [3].

The coronal suture is the most commonly first affected and after that all the sutures with a protuberance of the skull towards the frontal fontanelle, where the skull becomes high (acrocephaly), tower (turricephaly) or conical (oxycephaly) [1, 2]. In our patient the head shape was the closest form of towers. In addition, he had all the characteristic facial malformations and syndactyly of fingers and toes, so that diagnosis or at least suspicion of the diagnosis was not so difficult to set up quickly after birth. The diagnostic dilemma has been Crouzon syndrome, but according to the literature, Apert and Crouzon syndrome seem to be variant of the same syndrome, with the exception of the syndactyly of hands and feet in AS. Cleft or pseudo cleft palate is a frequent finding in Apert syndrome (our patient did not have them), whereas these traits are extremely rare in Crouzon syndrome [1].

On the other hand, the lack of corpus callosum leads to the clinical findings of hindered transfer of information from one to another side of cerebral cortex. The corpus callosum is the largest commissure of the brain and because it's specific structure and topographical organization, the lesions of some parts result in the predictable deficit in

interhemispheric transfer of information. This defect is described in the context of mental disorders, epileptic seizures, neurologic manifestations, optical damages, and may be asymptomatic or accompanied by minimal functional disorders, in which case it can be diagnosed accidentally [11, 13].

Etiology of the ACC remains unknown. It is linked to chromosome aberrations, infection, errors of metabolism and the influence of various harmful toxins (e.g. fetal alcohol, cigarettes and drugs exposure) from 12-22 week of gestation. The agenesis may be associated with other congenital malformations or syndromes (Down, Aicardi, Andermann, macrocephaly, arhinencephaly, lissencephaly etc), where a more severe psychomotor retardation and epileptic seizures are expected. Our patient has not expressed the loss of consciousness, but due to the possible occurrence of it, the electrophysiological monitoring must be regularly done. There are indications in the literature that epilepsy may occur in early childhood until the age of 3 years or later [11, 13].

Thanks to the early use of neurosonography, which is done as a screening in a maternity ward of Clinical Centre of Kragujevac, we diagnosed brain malformations in all neonates in the 2nd day of life, even when they were not symptomatic as well as the described defect in our patient. The ACC in combination with the AS is an extremely rare anomaly. Breik et al. found the prominent convolutional markings (67%) as most frequent CNS abnormality associated with Apert syndrome, after that ventriculomegaly (48%), crowded foramen magnum (36%), deficient septum pellucidum (13%), and ACC in only 11% [8]. There is described a familial occurrence of ACC, but it is not confirmed in our patient [11]. The course of pregnancy was completely normal, in family does not have heredodegenerative diseases, also father is in late twenty years (some authors indicated on paternal age effect [16]), so that we believe that our patient's anomalies are result of new mutations of the genome.

The recent neurobiological evidence have demonstrated that L1 cell adhesion molecule (L1CAM) gene plays a major role in the development of the white matter and its mutation in humans causes similar defects of the corpus callosum, septum pellucidum and cortico-spinal tracts. L1CAM need interactions with FGFR. It seems logical to assumes that the FGFR defects generate both the skull abnormalities and, by lack of interaction with L1CAM, the primary defect of the white matter. The mental deficiency that is common in these patients therefore is likely to be part of the disease, rather than a consequence of the skull size or of the associated hydrocephalus [3, 8].

The children with AS without visible cerebral malformations, if they survive early childhood, can have a normal life expectancy. That was achieved by a considerable improvement in quality of care for these children, and multidisciplinary approach of experts from different medical specialties (primarily reconstructive and plastic surgery). The main treatment is the surgical correction of abnormal connections between the bones of the skull, face and extremities, which is usually performed in 3 phases. The craniotomy is often performed during 6 months of age to treat the craniosynostosis. In the second phase the corrective surgery for syndactyly is done in first year of life and completed by 3 to 4 years of age. The cosmetic corrections for midface deficiency are at 4 to 6 years age. The treatment goals focused on the prevention of avoidable developmental delays (from raised intracranial pressure and sleep apnea) and reducing operative interventions may potentially improve developmental outcomes. Non-surgical methods are symptomatic measures that include: 1. Eye drops that prevent the mucous membrane of the conjunctiva from drying out, with incomplete closure of the eyelids. 2. Continuous positive airway pressure for present obstructive sleep apnea, possibly tracheostomy and intubation 3. Antibiotic therapy for frequent infections of the inner ear and sinus, with possible implantation of aeration tubes, etc [17].

The contribution of the family in order to achieve a better psychomotor development and quality of life is also significant. Some studies showed that grew up in a family environment increased the possibility of normal psychic development (normal IQ), as opposed to children who lived in residential institutions. Therefore, there is a wide variability in IQ of these children [7]. For now, it is not clear why mutation in the same gene can lead to such large variations [3, 8, 16]. Unfortunately, our patient has associated anomalies and psychomotor retardation, which will undoubtedly go to the progression, so the prognosis is very uncertain.

CONCLUSION

We present the experience during the diagnosis and further treatment plan of a male infant with extremely rare Apert syndrome, especially associated with corpus callosum agenesis, which can be qualitative contribution to a broader knowledge of the issue. For now, there is no successful treatment of this disease, besides multidisciplinary approach. Severe psychomotor retardation is always present. It should be noted the important role of the fetal ultrasonography, magnetic resonance imaging and amniocentesis early in the prenatal period, and after birth, ultrasound, CT and MSCT, in order to establish an early diagnosis of the AS with or without associated anomalies. Certainly, the question remains of molecular genetic confirmation of the diagnosis, which, as in many other cases in our country, cannot be made, both because of its unavailability and the high cost of testing. The long term care of children with Apert syndrome as well as other dysmorphic syndromes that carry less or greater morbidity and require a great deal of commitment from both the family and the wider community remains an open question.

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SINGLE-STAGE REDUCTION OF NEGLECTED PERILUNATE DISLOCATION: A CASE REPORT

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ABSTRACT

Neglected perilunate dislocations are rare and challenging injuries. We report a 59 days old perilunate dislocation in a 55 year old right-handed male. X-ray and CT imaging revealed a dorsal perilunate dislocation with no additional fractures and no increased density or microarchitectural collapse of the lunate or scaphoid. A single-stage open reduction and internal K-wire fixation procedure through a dorsal and volar approach was done. Eighteen months after the procedure, the patient was pain free during movement and had a ROM of 70%. His grip strength was 80.8% the opposite side and the Mayo wrist score was calculated 75. Full range of finger movement was achieved and he carried out his routine without any other complication.

Keywords: *Perilunate dislocation, wrist, chronic injury, lunate bone.*

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INTRODUCTION

Perilunate dislocation is a rare high-energy injury (1). It is characterized by a disruption of most capsular and ligamentous connections of the lunate and the adjacent carpal bones. The bone stock needs to be strong enough to resist the amount of torque involved in lunate dislocations without causing fractures (2). Reasonably, most patients are therefore young males (3). The ligament disruption may also be associated with different carpal fractures (4) and thus encompasses a spectrum of injuries. Data documenting the global epidemiology of these injuries is limited. Duckworth et al. (3) demonstrated an incidence of $0.5/10^5$ individuals per year. In neglected cases a soft tissue contracture develops, with reduces the space to seat the bones. Additionally, there is a increased chance of avascular necrosis. This injury is therefore very difficult to treat (1,5). The natural history of untreated or poorly treated perilunate injuries is progressive wrist arthritis, median nerve dysfunction, chronic regional pain syndrome and weak grip (6). Up to our knowledge, there is a lack of information in the literature regarding the management of neglected fracture dislocation of the carpal bones presenting 6 weeks after injury (7). Most authors agree that open reduction and internal fixation is necessary and may be used up to 2 months following injury (6-8) mainly using a two-stage procedure (5,7). We present a successful single-stage reconstruction in a neglected perilunate dislocation. Our procedure achieved an excellent postoperative result despite the observed capitolunate synostosis.

CASE REPORT

An 55 year old, right-handed male school teacher sustained a fall from height on a dorsal flexed hand 59 days ago. The patient did not seek immediate medical attention. He presented as regularly scheduled patient with a painful and stiff wrist without any neurovascular deficit. The anteroposterior and lateral X-ray of the right wrist revealed a dorsal perilunate dislocation (Mayfield III) with no additional fractures (Figure 1).

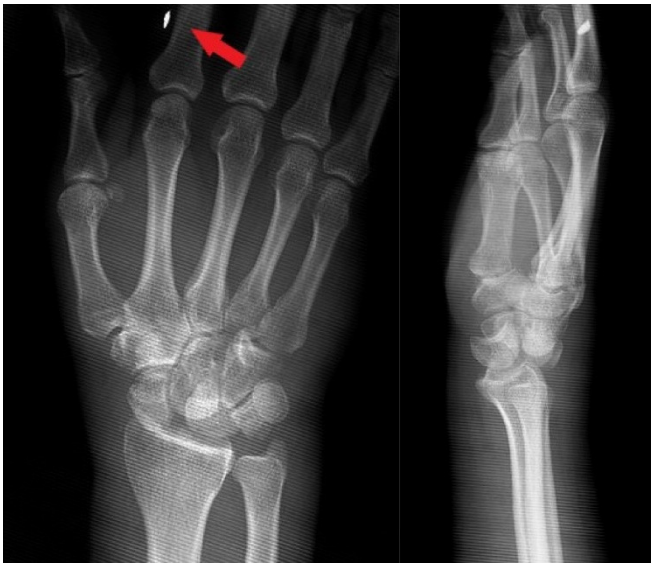

Figure 1. Preoperative antero-posterior and lateral radiography of the right wrist. Near the index proximal phalanx a metal foreign body (arrow).

Computed tomography (CT) of the wrist showed no carpal bone fractures and no increased density or microarchitectural collapse of the lunate or scaphoid (Figure 2).

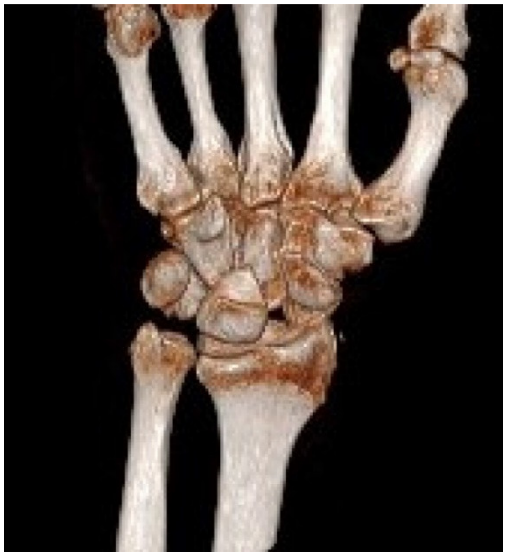

Figure 2. Preoperative CT image of the right wrist with perilunate luxation.

A single stage treatment under tourniquet control was planned. Open reduction and internal fixation was done by a combined volar and dorsal approach to the wrist joint. Initially, through a volar approach, the carpal tunnel was released. Realignment of the bones was assisted by a second longitudinal dorsal incision. No damage to the articular surface of the capitate or lunate was noted (Figure 3).

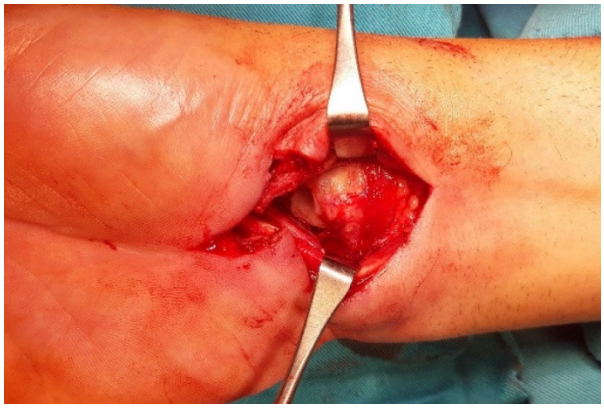

Figure 3. Periperative photograph showing the lunate denuded of soft tissue. No damage to the articular surface is seen.

The lunate, which was denuded of soft tissue attachments on the radial and ulnar side (Scapholunate and Lunotriquetral ligament tear), was stabilized with tree Kirschner (K) wires (Narcissus, Ada, Serbia) to the capitate, triquetrum and scaphoid. A fourth K wire was applied securing the scaphoid, capitate and hamate bone (Figure 4). The K-wire position was checked under fluoroscopy and the wires were cut above the skin. The volar lunate ligaments were secured with two 1,5 mm bone anchors. After the reduction and fixation of the bones to their normal position, an below elbow spica slab was applied in functional position of hand and forearm.

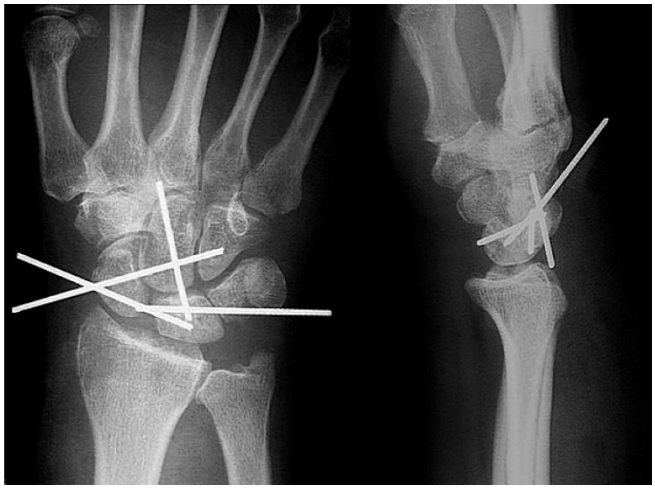

Figure 4. Postoperative anteroposterior and lateral radiography showing lunate bone stabilization with four K-wires.

Suture removal was done at 2 weeks and a below elbow spica slab was continued for 4 weeks. The K-wires were removed after 9 weeks. Even before the last K-wire was removed, the patient was mobilized, for active/assisted hand and wrist mobilization. Strengthening exercises were started after complete K-wire removal.

Eighteen months after the procedure, the patient was pain free during movement and had 30° wrist flexion, 40° wrist extension (range of motion (ROM) 70°) (Figure 5). He had 25° ulnar and 20° radial deviation. His grip strength was 25.8kg (80.8% the opposite side) and the Mayo wrist score was calculated 75 with is referred as „good“. Full range of finger movement was achieved and he carried out his routine without any other complication. Due to a metal foreign body in his index finger a follow up MRI scan was not done. The radiography and CT scan revealed abnormal bone positions, no evidence of radiocarpal arthritis and a capitolunate synostosis. His carpal indices were: Scapholunate angle 70°, lunocapitate angle -20°, and scapholunate distance 3 mm (Figure 6).

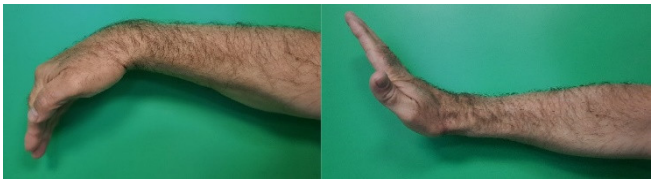

Figure 5. Clinical photographs showing range of movement of the right wrist.

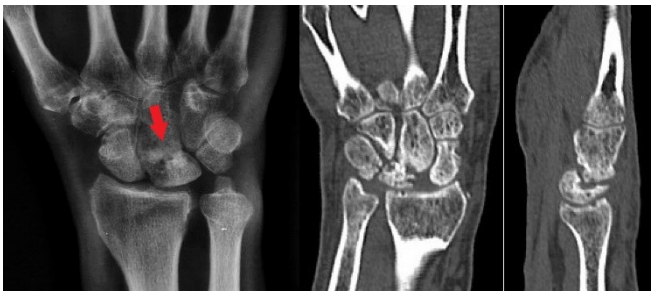

Figure 6. Radiography and CT scan 18 months after the procedure reveals a capitolunate synostosis (arrow).

DISCUSSION

Direct comparison of the operative results for chronic perilunate injuries is difficult because of the variability of cases and low incidence of these injuries. In our patient, there was no evidence of lunate osteonecrosis or instability, despite the duration of displacement and the absence of any soft tissue attachments ascertained during the operation. Perilunate dislocation and fracture-dislocation extremely alter the intercarpal relationships, still they are frequently misinterpreted during initial evaluations. Late reduction of perilunate dislocations is difficult and salvage procedures such as proximal row carpectomy, excision of the lunate or wrist arthrodesis

have been recommended (9). One of the first articles dealing with neglected perilunate dislocations was published in 1988 by *Siegert* et al. (10). In their study of sixteen neglected perilunate dislocations the best outcome was achieved with open reduction and internal fixation as in our procedure. *Inoue* et al. (8) treated twenty-eight patients with neglected perilunate dislocation. They concluded that proximal row carpectomy should be considered in chronic perilunate dislocations who are seen 2 months after injury. Their findings are in accordance with our position. *Kailu* et al. (11) also reported good follow up results in their six chronic perilunate dislocations concluding that open reduction and internal fixation can be applied in the treatment of delayed and some chronic cases. *Garg* et al. reported sixteen patients treated more than 3 months after injury in a two stage reconstruction procedure (4).

We performed an open reduction single stage fixation with K-wire through a dorsal and volar approach. There is a number of reasons for our single stage procedure. Firstly, we did not have access to a gradual distraction external fixator. Secondly, we did not discover any fractues, meaning that an open reduction was more difficult to achieve, but finally the lunatum was more stable. Lastly, the length of treatment is significantly shorter.

Massoud and *Naam* (6) conducted a study treating 19 chronic perilunate dislocations and gained a postoperative ROM averaged 72° (42° flexion, 30° extension), which is similar to our study. They used a single volar approach for 12 patient and a combined approach for the remaining 7 patients. *Israel* et al. showed their operative results of 16 perilunate dislocations and had substantial better ROM (127°± 26°) (5). However their patients were treated mostly hours after admission and the study participants had 33 years on average. It's worth mentioning that a wide range of results (ROM 66°-144°) are achieved in similar studies (5). At the final follow-up of our patient, the lunate was relatively dorsally flexed on the lateral view but the lunocapitate angle was -20°, this might be the reason for decreased dorsiflexion.

Komurcu et al. (9) showed that their delayed treated group had a grip strength of 26.33 ± 13.48 kg with is similar to our patient. Compared with the contralateral side *Massoud* and *Naam* (6) accomplished an averaged postoperative grip strength of 87%. Their study group, of chronic perilunate injuries, consisted of younger individuals averaged 27 years. As with the previous measure, a wide range of results (59% - 87% of opposite side) is achieved in similar studies (5).

Kremer et al. (12) reported a decreased Mayo wrist score in patients treated with a combined approach compared with those treated with a single dorsal approach (64.3 vs 79.4). Our patient had a better outcome score although he was treated with a combined approach. *Forli* et al. (13) reported the long-term outcomes of 18 patients with perilunate luxation and at least 10 years of follow-up. They found a average Mayo wrist score of 76. Their result is similar to ours although we did not have a such long follow up. *Massoud* and *Naam* (6) reported in their study of chronic perilunate injuries a average Mayo wrist score of 56. Similarly, *Israeli* et al. (5) reported a mean Mayo wrist score of 68±12 in their 16 perilunate dislocations. These scores are lower than the score of our patient. The difference may be attributed to the fact that our patient is not a manual worker, and he has not experienced pain during his daily routine.

The authors are aware that a larger number of single-stage reconstructions is necessary in order to have a better insight into the success of this procedure.

CONCLUSION

Neglected perilunate dislocations are rare and challenging injuries. We believe that better results can be achieved with open reduction and internal fixation rather then lunate excision. Although radiologic result does not always correlate with the clinical outcome, acceptable result in our case indicates that open reduction and internal fixation can be preferred as a treatment in delayed cases.

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INSTITUTIONAL REVIEW BOARD STATEMENT

Approval no. 00-4; January 16, 2020

INFORMED CONSENT STATEMENT

Patient informed consent was aquired before writing of this report.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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INFORMATION FOR AUTHORS

AIMS AND SCOPE

Experimental and Applied Biomedical Research (*EABR)* former *Serbian Journal of Experimental and Clinical Research* is a peer-reviewed, open access journal which publishes original research articles, reviews, case reports and letters to the editor in all areas of the biomedical sciences that have not been published previously. The journal comprises both basic and clinical research in the field of biomedicine. Current acceptance rate is 60%. *EABR* was founded in 2000 under the name *Medicus* and over more than two decades has grown into one of the leading national journals in the field of biomedical sciences. *Experimental and Applied Biomedical Research* is owned and published by Faculty of Medical Sciences University of Kragujevac. The journal adheres to the policies of the International Committee of Medical Journal Editors (ICMJE) and publishing ethics guidelines provided by the Committee on Publication Ethics (COPE).

TYPES OF MANUSCRIPTS

- *Original research articles*: *EABR* considers all original research manuscripts which present the results of an original research study (experimental or clinical). These manuscripts must contain sufficient information on all relevant research methods, as well as a detailed analysis of the results obtained.

- *Reviews*: *EABR* considers literature reviews, systematic reviews and meta analyses addressed to a particular subject area, with special reference to new knowledge and facts. Manuscripts in this category must not be shorter than 6000 words, the text must cite more than 70 references of which 50% have been published in the previous 5 years. Systematic reviews should follow the PRISMA guidelines.

- *Case reports*: *EABR* considers case reports presenting detailed information on the symptoms, signs, diagnosis, treatment (including all types of interventions), and outcomes of an individual patient. Case reports should usually describe new or uncommon conditions that serve to enhance medical care or highlight diagnostic approaches. Case reports should follow the CARE guidelines.

- *Letters to the editor: EABR* considers letters to the editor related to different clinico-laboratory observations. They should be titled, not exceed 500 words, and have a maximum of 5 references. Up to 1 table or figure may be submitted, but will be published at the discretion of the Editor. No more than 3 authors should appear.

MANUSCRIPT SUBMISSION

Manuscripts submitted to *Experimental and Applied Biomedical Research* must neither be published previously nor be under consideration for publication in another journal. Manuscripts are acompanied with a suitable *cover letter* stating that: the manuscript is not

submitted for publication elsewhere; all authors have agreed to submission; the study is carried out in accordance with relevant ethical international guidelines.

EABR considers only manuscripts written in English using *Microsoft Office Word* format and uploaded online at https://www.editorialmanager.com/sjecr/.

Plagiarism, data fabrication and image manipulation are not tolerated. Plagiarism includes copying text, ideas, images, or data from another source, even from authors own publications, without providing any reference to the original source. If a study's design or the manuscript's structure or language has been inspired by previous works, these papers must be explicitly cited. All manuscripts submitted to *Experimental and Applied Biomedical Research* are checked for plagiarism using the academic standard software prior to the first step of the editorial process.

MANUSCRIPT PREPARATION AND ORGANISATION

Title Page

The Title Page should contain the following informations:

- Manuscript title
- Full author(s) names
- The affiliation(s) of the author(s)
- A clear indication and an active e-mail address of the corresponding author

Manuscript title should be concise and informative.

It is necessary to state the full names and surnames (middle letter or name is optional) of all authors and the exact affiliations of all authors - institution, (department), city, (state), country. *Experimental and Applied Biomedical Research* remains neutral with regard to jurisdictional claims in institutional affiliations. Responsibility for affiliations ultimately rests with the author.

Abstract

Provide an abstract of 150 to 250 words. Abstract should be structured (Background, Methods, Results, Conclusion), citation-free, without abbreviations if possible.

Keywords

Three to five relevant keywords need to be added after the abstract. Keywords should be specific to the manuscript, yet reasonably common within the subject discipline.

Text Formatting

Manuscripts should be submitted in *Microsoft Office Word*. The authors should use normal, plain *Times New Roman* font (12pt) for text. Pages should be numbered automatically. Italics may be used for emphasis. Abbreviations should be defined at the first mentioning in the text and used consistently thereafter (do not use a separate subtitle for abbreviations only). Please use no more than three levels of displayed headings. International System (SI) of Units should be used (imperial, US customary and other units should be converted to SI units).

Original research articles should contain following sections: Introduction, Materials and Methods, Results, Discussion, Conclusions, Acknowledgments, Conflict of Interest, and References. *Reviews* may require different formats, while *Case reports* manuscripts should follow the CARE guidelines.

Introduction. This section should contain context or background for the study, rationale, clear aim of research or tested hypothesis.

Materials and Methods. This section should provide sufficient detail for replication of the study. If more than one method is used in the research, use subsections with appropriate subheadings. The *Materials and Methods* section should also contain following statements:

a) Informed Consent Statement. In cases where the identification of personal information is necessary for scientific reasons, authors should obtain informed consent from all individuals included in the study
b) Human Right Statement. Manuscripts containing information related to human should clearly state that the research has complied with all relevant international and national regulations and institutional policies and has been approved by the authors' institutional Ethics committee.
c) Animal Right Statement. Manuscripts containing information related to animals should clearly state that the research has complied with all relevant international and national regulations and institutional policies and has been approved by the authors' institutional Ethics committee.

For details and examples of statements please see part ‘Research and publication ethics’.

Results. The results should be presented in logical sequence in the manuscript. Do not repeat all the data in the tables or figures in the text.

Conclusions. Within the *Conclusions* section the authors should clearly explain the main conclusions of the article, highlighting its importance and relevance.

Acknowledgments. Acknowledgments of people, grants, funds, etc. should be placed in a separate section after the Conclusions section. The names of funding organizations should be written in full. This may include administrative and technical support, or donations in kind (e.g., materials used for experiments).

Conflict of Interest. Authors must declare all relevant interests that could be perceived as conflicting. If there is no conflicts exist, the authors should state this. Submitting authors are responsible for coauthors declaring their interests.

References. *References* must be numbered in order of appearance in the text (including table captions and figure legends) and listed individually at the end of the manuscript. In the text, reference numbers should be placed in round brackets ( ), and placed before the punctuation – e.g. (1), (1–3) or (1, 3). The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text.

The reference list should include contain surnames and the first letter of the author's name, full title, abbreviated title of the journal, year of publication, volume, number and pagination (Vancouver style guide). In case where the list of authors are more than six, please use et al. after the sixth author.

The examples of correct referencing:

For journal papers:
Shoji F, Haro A, Yoshida T, Ito K, Morodomi Y, Yano T, et al. Prognostic significance of intratumoral blood vessel in- vasion in pathologic stage IA non-small cell lung cancer. Ann Thorac Surg. 2010;89(3):864-9.

For journal papers by DOI:
Ewy MW, Patel A, Abdelmagid MG, Mohamed Elfadil O, Bonnes SL, Salonen BR, et al. Plant-Based Diet: Is It as Good as an Animal-Based Diet When It Comes to Protein? Curr Nutr Rep. 2022. doi: 10.1007/s13668-022-00401-8.

For books:
Kleiner FS, Mamiya CJ, Tansey RG. 2001. Gardner's art through the ages (11th ed.). Fort Worth, USA: Harcourt College Publishers.

For chapter in an edited book:
Mettam GR, Adams LB. How to prepare an electronic version of your article. In: Jones BS, Smith RZ, editors. Introduction to the electronic age, New York: E-Publishing Inc; 2009, p. 281–304.

Tables, figures and images

Tables

Tables should always be cited in text in consecutive numerical order. For each table, please supply a table caption (title) explaining the components of the table. Identify any previously published material by giving the original source in the form of a reference at the end of the table caption. Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

Figures

Please submit each figure as an individual file separate from the manuscript text. All figures are to be numbered using Arabic numerals. Figures should always be cited in text in consecutive numerical order. Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file.

For vector graphics, the preferred format is EPS, for halftones, please use TIFF format. *Microsoft Office* files are also acceptable. Vector graphics containing fonts must have the fonts embedded in the files.

Line art:

- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
- All lines should be at least 0.1 mm (0.3 pt) wide.
- Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.
- Vector graphics containing fonts must have the fonts embedded in the files.

Halftone art:

- Definition: Photographs, drawings, or paintings with fine shading, etc.
- If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.
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Images

Supply vector-based files such as those produced by *CorelDraw*, *Adobe Illustrator* or similar software. Vector files give us maximum flexibility for sizing your figures properly. Do not rasterize line art or text. Photographic images should have a minimum resolution of 300 dpi at final print size. Embedded images within a vector file should also have a minimum resolution of 300 dpi. Up sampling artwork (artificially increasing file size or resolution) will not improve quality and causes production problems. At final print size, line weights can be no thinner than 0.28 pt.

PEER REVIEW PROCESS

All submitted manuscripts received by the Editorial Office will be evaluated by a professional *Editorial board* to determine whether they posses sufficient quality, are they properly prepared and follow the ethical policies of *Experimental and Applied Biomedical Research*. Manuscripts that do not fit with the quality and ethical standards of *EABR* will be rejected before peer-review. Manuscripts that are not properly prepared according to the Instruction for authors will be returned to the authors for revision and resubmission.

Once a manuscript passes the initial evaluation, it will be assigned to at least two independent experts for single-blind peer-review process. If the outcomes of the performed reviews are opposite, the third review is required. The peer-review outcomes are one of following:

- *Accept (without any changes)* - the journal will publish the paper in its original form. This type of decision outcome is rare.
- *Minor revision* - the manuscript have high chance to be accepted after fulfillment of minor corrections. Authors will be asked to resubmit the revised manuscript within a suitable time frame, and the revised version will be returned to the reviewer for further comments.
- *Reconsider after Major Revision* - the acceptance of the manuscript would depend on the revisions. The authors are required to perform extensive and significant

improvements in their manuscript. Authors will be asked to resubmit the revised manuscript within a suitable time frame, and the revised version will be returned to the reviewer for further comments.

- *Reject* - the manuscript is rejected for two reasons: 1. it has serious flaws, and/or makes no original significant contribution; 2. corrections and improvements during the (major) revision were not sufficient and satisfactory. No offer of resubmission to the journal is provided.

All reviewer comments should be responded point-by-point in a separate document entitled 'Answers to reviewers comments'. Corrections should be marked within the text in a red colour or as a track changes. During the submission process, author should suggest two potential reviewers with the appropriate expertise to review the manuscript. Proposed reviewers should be from different institutions than the authors.

Upon editor's approval, after received positive manuscript reviews, the manuscript is accepted in the system, and the corresponding author receives information about the manuscript accepted for publication to the email address. Once accepted, the manuscript will undergo professional copy-editing, English editing, proofreading by the authors, pagination and publication. The Editorial board reserves the right to correct the English language after proofreading by the authors.

DOI number is assigned to the paper and, after proofreading and text break according to the Journal instructions, the paper is published as *Ahead of Print* first on *Sciendo* platform (https://sciendo.com/journal/sjecr) and then in one of the next issues of the Journal.

RESEARCH AND PUBLICATION ETHICS

Research Involving Human Subjects

When reporting on research that involves human subjects, human material, human tissues, or human data, authors must declare that the investigation was carried out following the rules of the Declaration of Helsinki of 1975 (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/), revised in 2013. As a minimum, a statement including number of approval and name of the ethics committee must be stated in Section 'Statement of Human Rights' of the article. In addition, the protection of privacy is a legal right that must not be breached without individual informed consent. In cases where the identification of personal information is necessary for scientific reasons, authors should obtain full documentation of informed consent, including written permission from the patient prior to inclusion in the study.

Example of Statement of Human Rights: "The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of *Name of the Institution* (No. *number of approval*)."

Example of Statement of Informed Consent: "All subjects gave their informed consent for inclusion before they participated in the study".

For non-interventional studies (e.g. surveys, questionnaires, social media research), all participants must be fully informed if the anonymity is assured, why the research is being conducted, how their data will be used and if there are any risks associated. As with all

research involving humans, ethical approval from an appropriate ethics committee must be obtained prior to conducting the study.

Research involving Animals

When reporting on research that involves animal subjects, animal material or animal tissues, authors must declare that the investigation was carried out following the rules of the European Directive for the welfare of laboratory animals (No. 2010/63/EU) and national and institutional regulations. As a minimum, a statement including number of approval and name of the ethics committee must be stated in Section 'Statement of Animal Rights' of the article. Statements on animal welfare should confirm that the study complied with all relevant legislation. Also, authors must include details on housing, husbandry and pain management in their manuscript (section Materials and methods).

Example of Statement of Animal Rights: "All research procedures were carried out in strict accordance with the European Union Directive for the welfare of laboratory animals (No. 2010/63/EU) and approved by the Ethics Committee of *Name of the Institution* (No. *number of approval*)."

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