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REVIEW PAPER

# THE USE OF PROTON PUMP INHIBITORS IN INTENSIVE CARE UNITS

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## ABSTRACT

*The bleeding from the upper gastrointestinal tract represent a significant medical but also socio-economic problem.A special group of patients et increased risk consists of critically ill patients in intensive care units. Particularly significant cause of bleeding in intensive care unit patients is bleeding resulting from the stress ulcers caused by damage of themucosa of the stomach and duodenum. The purpose of this review is to present current experience in prevention of upper gastrointestinal tract bleeding using proton pump inhibitors in intensive care units. Combination of endoscopic hemostatic methods and proton pump inhibitors represents golden standard in most cases. Despite some adverse effects treatment with proton pump blockers is essential when upper gastrointestinal tract bleeding appears in critically ill patients in intensive care units. Proton pump inhibitors are more effective in acid suppression, as well as in the prevention of recurrent bleeding after endoscopic hemostasis than histamine 2 receptor blockers. The efficacy of proton pump blockers is higher in the case of a continuous intravenous infusion than in the intermittent mode of administration of the drug. The need for highly elaborate strategy for the prophylaxis of bleeding from the upper parts of gastrointestinal tract in intensive care units is essential, because when it occurs in intensive care units, mortality is high, and therapeutic options become narrow.*

***Keywords***: *Proton pump inhibitors, acid suppression, upper gastrointestinal tract, bleeding, prophylaxis.*

## INTRODUCTION

### The bleeding from the upper gastrointestinal tract

Evidence based medicine is the concept of scientific thinking and processing results and involves rational interpretation and use of the currently best available, scientifically proven and evidence-based approaches in the treatment of patients. The methodology of evidence-based medicine can be successfully demonstrated evaluating studies that have observed the occurrence of bleeding from the upper gastrointestinal tract, acid suppression and application of proton pump inhibitors in critically ill patients in intensive care units (1).

The bleeding from the upper gastrointestinal tract represent a significant medical but also socio-economic problem of our time. It is estimated that each year about 4 million people are diagnosed with peptic ulcer disease worldwide (2). Previously, bleeding from the digestive tract ulcer was a common cause of surgical treatment, but this is a rarity today, thanks to advances in pharmacological therapy. On the other hand, the way of life of modern man certainly contributes to the increase of diseases such as gastric and duodenal ulcers. A special group of patients consists of critically ill patients in intensive care units who are at increased risk for the occurrence of stress ulcer and consecutive bleeding from the upper gastrointestinal tract. Together with the underlying disease and comorbidities bleeding from the digestive tract is often life-threatening when patients are concerned. Many clinical trials have focused on acid suppression treatment and prevention of stress ulcer but it is still a common problem and a major challenge for clinicians who deal with treatment of critically ill patients in intensive care units worldwide (3).

There is a big difference between the group of patients who had signs of acute bleeding from the upper gastrointestinal tract at the time of admission and the group of patients who bleeding from the upper gastrointestinal tract developed after hospitalization. Mortality in the first group is about 9.1% and the mortality rate in the second group is about 50% (4).

### Causes of gastrointestinal bleeding

The causes of bleeding from the upper gastrointestinal tract are differ not only in place and the pathogenesis, as well as by the intensity, dynamics of occurrence and possible consequences. The main cause of gastrointestinal bleeding in critically ill patients are the varices of the distal esophagus and proximal stomach with hyperacidity related causes such as peptic ulcer disease and stress-related damage to the lining of the gastrointestinal tract. Particularly significant cause of bleeding in intensive care units patients is bleeding resulting from the stress ulcers caused by damage of the mucosa of the stomach and duodenum.

The pathophysiological mechanisms by which stress leads to lesions of gastric and duodenal mucosa are numerous, often mutually complementary and the most significant are hypotension with hypo perfusion of mucosa, tachycardia, anemia, hypoproteinemia, etc... Hyperacidity inhibits platelet aggregation and activate pepsin, so that it has a double negative effect. Decreased platelet aggregation leads to increased bleeding tendency due to the difficulty or inability to create the formation of blood clot while by acid activated pepsin can lyse clots that already formed (5). The causes of bleeding from the upper gastrointestinal tract may be relatively rare conditions such as Mallory-Weiss syndrome, coagulopathy, vascular lesions.

### Treatment of patients with acute gastrointestinal bleeding

The initial treatment of patients with signs of acute bleeding from the upper gastrointestinal tract is generally identical in all patients, and reduced to the basic resuscitation and resuscitation. Particular attention should be paid to the protection of the airway during massive hemorrhage, restoration of circulating blood volume, correction of comorbidity. After stabilization of vital functions some of the methods of hemostasis should be carried out as causal treatment (6).

According to several studies performed mechanical ventilation is one of the most provocative factor when for bleeding from the upper gastrointestinal tract in critically ill patients in intensive care units (5).

H. pylori infection is the main cause of chronic gastritis type B and duodenal ulcer. Its presence can be detected in a biopsy sample of intestinal mucosa during gastroduodenoscopy, serology or breath test. Although it has long been known for its role in the development of gastric and duodenal ulcers, its role in the occurrence of acute bleeding from the upper gastrointestinal tract is still unclear. Results from the studies are contradictory. There are studies that indicate that eradication of H. pylori significantly reduces the incidence of ulcer recurrence or occurrence at a recurrent bleeding after already achieved hemostasis in patients with duodenal ulcer (7). There are also studies that have shown that there is no statistically significant correlation between the presence of H. pylori and gastrointestinal bleeding in critically ill patients. On the other hand, in the same studies a causal relationship with the bleeding by applying mechanical ventilation has been confirmed, which is already previously known (8).

The therapeutic modalities in case of bleeding from the upper parts of the gastrointestinal tract are pharmacotherapy, endoscopic intervention methods and operative treatment.

Development of interventional endoscopic procedures has significantly reduced the need for surgical intervention in case of bleeding peptic ulcers. Endoscopic hemostatic methods have numerous advantages as compared to other treatment modalities. First of all, trauma is significantly lower compared to surgery, which is very important for seriously ill patients who usually except the bleeding

**Table 1.** Pre-endoscopy Rockall Score for Risk Assessment after Upper Gastrointestinal Tract Bleeding

| | | |
|---|---|---|
| **Age** | < 60 years | 0 |
| | 60-79 years | +1 |
| | ≥ 80 years | +2 |
| **Shock** | No shock (SBP ≥100 and HR <100 | 0 |
| | Tachycardia (SBP ≥100 and HR ≥100) | +1 |
| | Hypotension (SBP <100) | +2 |
| **Comorbidities** | No major comorbidity | 0 |
| | Any comorbidity except renal failure, liver failure, and/or disseminated malignancy | +2 |
| | Renal failure, liver failure, and/or disseminated malignancy | +3 |

from the digestive tract have life-threatening underlying disease. On the other hand, interventional endoscopic hemostasis is a significantly faster than pharmacological methods. However, the aforementioned methods of treatment are not competing but are complementary. When establishing hemostasis by endoscopy emphasis is placed on prevention of recurrent bleeding, which occurs in about 20% of patients (9). The main endoscopic techniques for establishing hemostasis in case of bleeding from the upper gastrointestinal tract are injection therapy and termocoagulation and these two techniques are complementary. The injection therapy is most commonly used solution with adrenaline which has vasoconstrictor effect (10). The two most commonly used techniques are the method of heating and bipolar coagulation methods (11). One of widely used scoring systems for risk assessment after acute upper gastrointestinal hemorrhage is the Rockall score (Tables 1 and 2) (12-14).

Acid suppressive agents such as histamine-2 receptor antagonists have been for many years in use. The first drug of this type, which is set to use in the US was cimetidine, which went into service in 1977. The fact that drugs in this group had a great effectiveness in the treatment of peptic ulcer disease is a prerequisite that they enter into wider use in the field of prevention of repeated bleeding after already achieved hemostasis. However, histamine 2 receptor blockers have not met the expectations in this regard and did not show a statistically significant effect on reducing the number of required transfusion, duration of bleeding, repeated bleeding or the need for surgical intervention, as proven by numerous studies (15, 16).

## Proton pump inhibitors

Proton pump inhibitors belong to the group of benzimidazole drugs in chemical composition. Omeprazole (Prilosec®, AstraZeneca, Wilmington, DE) was approved by the FDA in 1989 and was the first drug from a group of PPI approved for use in the United States; and then followed lansoprazole, pantoprazole, rabeprazole, esomeprazole, omeprazole bicarbonate, naproxen and esomeprazole-dekslansoprazol. These drugs inhibit H + / K + -ATP-ase and reduce gastric acid secretion. Some of the indications for use of proton pump inhibitors include gastroesophageal reflux disease (GERD), erosive esophagitis, gastric ulcer, Helicobacter pylori eradication, as well as the treatment of gastric ulcers caused by excessive use of non-steroidal anti-inflammatory drugs (17). Application of proton pump inhibitors in critically ill patients with signs of bleeding from the upper gastrointestinal tract has led to significant advances in treatment, which has proven by randomized, double-blind, placebo-controlled studies (18, 19). H+, K+ -ATP-ase is the key enzyme in the process of acid secretion (20). It is mainly localized in idle parietal cells and it is mobilized in a situation when it comes to the activation of the parietal cells by some of stimuli such as histamine, acetylcholine or gastrin (21). Proton pump blockers such as lansoprazole [2 - [[[3-methyl-4- (2, 2, 2-trifluoroethoxy) -2-pyridyl] methyl] sulfinyl] -1H-benzimidazole), omeprazole, rabeprazole and pantoprazole inhibit gastric H +, K + -ATP-ase covalent attachment to the sulfhydryl group, thus inhibiting the secretion of acid (22). Although proton pump inhibitors show a powerful acid suppression activity, still many pharmaceutical laboratories try to improve the pharmacological properties of the drug, as proton pump inhibitors bind only for the activated H +, K + -ATP-ase, so that it takes 4-5 days to achieve maximal therapeutic effect (23). Proton pump inhibitors alter its molecular configuration and therefore acid suppression activity depends on pH conditions, i.e. acidity of the environment, so that their activity is considerably lower at a neutral pH environment.

**Table 2.** Complete Rockall Score for Risk Assessment after Upper Gastrointestinal Tract Bleeding

| | | |
|---|---|---|
| **Age** | < 60 years | 0 |
| | 60-79 years | +1 |
| | ≥ 80 years | +2 |
| **Shock** | No shock (SBP ≥100 AND HR <100 | 0 |
| | Tachycardia (SBP ≥100 AND HR ≥100) | +1 |
| | Hypotension (SBP <100) | +2 |
| **Comorbidities** | No major comorbidity | 0 |
| | Any comorbidity EXCEPT renal failure, liver failure, and/or disseminated malignancy | +2 |
| | Renal failure, liver failure, and/or disseminated malignancy | +3 |
| **Diagnosis** | Mallory-Weiss tear | 0 |
| | No lesion identified and no stigmata of recent hemorrhage | 0 |
| | All other diagnoses | +1 |
| | Malignancy of upper GI tract | +2 |
| **Mallory -Weiss tear** | None | 0 |
| | Dark spot only | 0 |
| | Blood in upper GI tract | +2 |
| | Adherent clot | +2 |
| | Visible or spurting vessel | +2 |

Proton pump inhibitors have a relatively short plasma half-life period, so their activity over night is in question, even in the repeated bolus administration (24). Proton pump inhibitors have adverse effects including development of life threatening Clostridium difficile colitis. Some novel studies declare that proton pump inhibitors do not affect risk for Clostridium difficile infection in the intensive care unit, but it is still unclear (25-27).

In order to improve the pharmacodynamics a new group of drugs has been proposed. In contrast to the proton pump inhibitors, a novel class of medicaments, known as potassium-competitive blockers of acid suppression (P-CABs) or acid pump antagonists inhibit the gastric H +, K + -ATP-ase K + -competition, through reversible mechanism (28). 3- (Cyanomethyl) -2-methyl, 8- (phenylmethoxy) imidazo (1, 2-a) pyridine (SCH28080) is the prototype of the P-CAB classes of drugs. It binds to fosfoenzim monovalent cation sites (E2P) on H +, K + -ATP-ase and it is strictly K + -competitive (Mendlein and Sachs, 1990). This mechanism allows the rapid inhibition of the pump without the need for an acidic medium. Several structural imidazopyridine derivatives, pyrimidine, imidazonaphthyridine and pirolopiridazin have been studied as a P-CABs. These compounds are stable at low pH values. Therefore, P-CABs are highly concentrated in the extremely acidic environment of gastric parietal cells on the luminal surface of the H +, K + -ATP-ase, and their effectiveness is less variable, because, unlike the proton pump inhibitor does not require a gastro protective formulations (29). P-CABs show rapid development of acid inhibition based on the rapid achievement of therapeutic doses in plasma, so that the maximum effect is already achieved during the first day of implementation (30). However, P-CABs group of drugs has its drawbacks, primarily hepatotoxicity (31).

## CONCLUSION

Based on studies on a large number of patients as scientifically valid the following conclusions can be imposed:

Although the stress ulcers of the digestive tract in critically ill patients are common, clinically significant acute bleeding from the upper gastrointestinal tract is not so often (32). During the first 24 hours of admission to the intensive care unit, 75% to 100% of critically ill patients develop endoscopically visible damage of the mucosa of the upper gastrointestinal tract, however, relatively few of them, 1% to 4% develop clinically overt bleeding (33).

Clinically significant acute bleeding from the upper gastrointestinal tract caused by mucosal stress damage is accompanied by signs of hemodynamic instability and the need for transfusion, but need for surgical intervention is relatively rare, even when viewed from only a sample of patients in intensive care units. Also it is in constant decline which is associated with the progress of medicine and therapeutic possibilities as well as a better understanding of the pathophysiology of the problem (34).

The need for highly elaborate strategy for the prophylaxis of bleeding from the upper parts of GIT is essential, because when it occurs in intensive care units, mortality is high, and therapeutic options become narrow (35).

Treatment with proton pump blockers is essential when upper gastrointestinal tract bleeding appears in critically ill patients in intensive care units.

Proton pump inhibitors are more effective in acid suppression, as well as in the prevention of recurrent bleeding after endoscopic hemostasis than histamine 2 receptor blockers (6).

Prophylaxis of stress ulcers has led to a reduction of frequency of clinically significant bleeding, but it has not been proven to increase the survival rate (36).

The cause of death of critically ill patients who developed stress ulcer bleeding is often one or more preexisting comorbidities, while bleeding from the upper parts of GIT represents only contributing, additional factor. Therefore, it is recommended that each institution has a guide which determines what the patient has enough factors of risk and is in need of acid suppression prophylaxis (37).

Acid suppression therapy has its adverse effects. There is evidence to suggest that this therapy is associated with increased risk of hip fracture, but this connection is likely to apply only to patients who already have some of the other risk factors, such as osteoporosis (38).

The efficacy of proton pump blockers is higher in the case of a continuous intravenous infusion than in the intermittent mode of administration of the drug (39).

Although the price of the proton pump inhibitor is greater than the price of histamine 2 receptor blockers, there is a clear benefit from their use not only in medical terms but also in terms of economic profitability because costs are much higher if the treatment with lower efficiency histamine 2 receptor blockers contributes to the formation of stress ulcers and consequent bleeding from the upper gastrointestinal tract (6).

As proton pump inhibitors should be considered as serious risk factor for Clostridium Difficile infection some of prevention methods mustn't be neglected, such as reducing use of broad–spectrum antimicrobials, probiotics administration, isolation of cases and personal protective equipment (40).

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**Conflict of interest**

The authors have declared that no competing interests exist.

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None.

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ORIGINAL SCIENTIFIC ARTICLE

# METFORMIN REVERSES THE EFFECTS OF ANGIOTENSIN 2 IN HUMAN MAMMARY ARTERIES BY MODULATING THE EXPRESSION OF NITRIC OXIDE SYNTHASES

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## ABSTRACT

*Angiotensin 2 impairs vascular function by activation of reactive oxygen species (ROS) production and development of endothelial dysfunction. Metformin, the first-line therapeutic agent for type 2 diabetes mellitus, has vascular protective properties, beyond its glucose lowering effects. The aim of the present study was to investigate the interaction between metformin and angiotensin 2 in human internal mammary arteries harvested from patients with coronary heart disease undergoing revascularization procedure, by evaluation of vascular function, reactive oxygen species (ROS) production and the gene expression of nitric oxide (NO) synthases (endothelial – eNOS, neuronal – nNOS and inducible – iNOS). To this aim, vascular samples were incubated with angiotensin 2 (Ang2, 12 h) with/without metformin (Metf, 10 μM) and used for ROS measurement (FOX assay), vascular reactivity in organ bath (contractility to phenylephrine, relaxation to acetylcholine, contractility to NG-nitro-L-arginine methyl ester/L-NAME) and RT-PCT studies. Acute incubation of the vascular rings with Ang2 impaired vascular reactivity (increase contractility, decrease relaxation), increased ROS production, supressed eNOS/nNOS and increased iNOS mRNA expression. Ex vivo incubation with metformin at a clinically relevant concentration reversed all these effects. These data suggest that Metformin might be useful in alleviating endothelial dysfunction by improving the endothelial-dependent relaxation and mitigating oxidative stress in clinical setting associated with cardiovascular disease regardless the presence of impaired glucose metabolism.*

***Keywords***: *Human mammary arteries, angiotensin 2, metformin, endothelial dysfunction, NO synthases.*

## INTRODUCTION

Increased generation of reactive oxygen species (ROS) contributes to the development of endothelial dysfunction mainly by limiting the bioavailability of nitric oxide (NO) and by inducing the activation of endothelial cells. Activation of both classic and local renin-angiotensin systems are recognized one of the most important contributors to the endothelial dysfunction in cardiovascular pathologies (1). In the vascular system the effects of angiotensin 2 (Ang 2) have been systematically investigated during the past decades and include vasoconstriction and prothrombotic effect, vascular remodeling with the proliferation of vascular smooth muscle cells and fibroblasts, increased expression of adhesion molecules in endothelial cells, and impaired nitric oxide (NO) generation/signaling (2-4).

Metformin, the first-line treatment for type 2 diabetes mellitus, used for more than 60 years, promotes also cardiovascular protection via pleiotropic, partially elucidated mechanisms (5). The beneficial effects in alleviating endothelial dysfunction have been reported in the setting of diabetes (6-8), metabolic syndrome (9), obesity (10, 11) albeit the mechanisms of action remains elusive. The mechanisms responsible for the regulation of endothelial cell functions by the drug in the setting of diabetes have been nicely covered by a recent review (12). However, direct anti-inflammatory effects have been reported irrespective of the diabetic status via a decrease in the secretion of proinflammatory cytokines and the markers of systemic inflammation (13). The anti-inflammatory properties of metformin have been unequivocally demonstrated in preclinical models, yet its use in the setting of diseases associated with chronic inflammation still remains elusive, as recently reviewed in ref. (14).

The present study was aimed to investigate the effects of metformin on vasomotor function, reactive oxygen species (ROS) level together with expression of nitric oxide (NO) synthases (endothelial, neuronal and inducible, eNOS, nNOS and iNOS) after Ang 2 stimulation of human internal mammary arterial samples harvested from patients with coronary heart disease subjected to coronary artery by-pass grafting.

## PATIENTS AND METHODS

### Study group

This study was conducted according to the World Medical Association Declaration of Helsinki and was approved by the Committee for Research Ethics of "Victor Babeș" University of Medicine and Pharmacy, Timișoara, RO (no. 04/28.02.2020 and 04p/17/12/2020). All participants provided a written informed consent.

The characteristics of patients included in the study are summarized in Table 1.

**Table 1.** Characteristics of the study group (n=7).

| Parameter | Value (mean± SEM) |
|---|---|
| Age (years) | 63.71±2.91 |
| Sex (M/F) | 6/1 |
| Body Mass Index (kg/m$^2$) | 27.75±1.48 |
| Systolic Blood Pressure (mmHg) | 136.28±3.36 |
| Diastolic Blood Pressure (mmHg) | 77.42±2.78 |
| Heart Rate (b/min) | 68.85±5.15 |
| Erythrocyte Sedimentation Rate (mm/h) | 31.57±9.79 |
| Erythrocytes (mil/mm$^3$) | 4.79±0.29 |
| Hematocrit (%) | 43.88±2.26 |
| Hemoglobin (g/dL) | 14.55±0.76 |
| Leucocytes (*1000/mm$^3$) | 8.42±0.83 |
| Thrombocytes (*1000/mm$^3$) | 248.71±11.67 |
| Creatinine (mg/dL) | 1.01±0.08 |
| Uric Acid (mg/dL) | 6.84±1.84 |
| Total Cholesterol (mg/dL) | 217.29±25.02 |
| HDL-Cholesterol (mg/dL) | 47.4±7.4 |
| LDL-Cholesterol (mg/dL) | 131.66±43.86 |
| Fasting plasma glucose (mg/dL) | 105.28±5.85 |
| ALAT (U/L) | 42.71±11.16 |
| ASAT (U/L) | 26±4.34 |
| Na (mmol/L) | 139.28±1.58 |
| K (mmol/L) | 4.24±0.13 |
| CK (IU) | 69.16±13.19 |
| CK-MB (IU) | 18.42±3.26 |
| **Main diagnostics** | |
| Stabile angina pectoris, unstable angina pectoris, myocardial infarction, trivascular coronary artery disease, carotid atherosclerotic artery disease, mitral/tricuspid regurgitation, hypertension, heart failure NYHA II, hypercholesterolemia | |
| **Preoperative medication** | |
| Beta-blockers, loop diuretics, thiazide diuretics, ACEi, ARBs, statins, antiplatelet drugs, oral anticoagulants, nitrates, cerebral vasodilators | |

### Study design

Human internal mammary arteries were dissected under sterile conditions, cleaned, and incubated (12 h, 37°C in EBM culture medium with 0.1% BSA) in the presence or absence of Ang2 (100 nM) with/without Metf (10 μM). Subsequently, the vessels were used for vascular reactivity studies using the DMT myograph (contraction to Phenylephrine-Phe, relaxation to Acetylcholine-ACh and contractility in the presence of NG-nitro-L-arginine methyl ester - L-NAME, the classic eNOS inhibitor), ROS measurement (FOX assay) and gene expression of NO synthases (RT-PCR). We assessed the lowest concentration of Metf (10 μM) since the plasma therapeutic window of Metf in humans was reported to be between 10 - 40 μM (15).

### Vascular reactivity studies

The human internal mammary arteries rings were suspended in the myograph chambers containing 5 ml of Krebs solution added with the cyclooxygenase inhibitor, diclofenac (10 μmol/L) aerated with 95% O2-5% CO2 gas mixture (pH 7.4, 37°C). Each vascular ring was fixed to a force transducer for the isometric force recording. Firstly, the vascular rings were stretched to a tension of 2 g and equilibrated for 1 h, and further exposed twice to 80 mM KCl. The concentration of Phe used for constriction was adjusted to obtain 80% of the KCl contraction. Subsequently, the endothelial-dependent relaxation to Ach and contractility to eNOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME, 10μM) were recorded.

### Assessment of oxidative stress

Hydrogen peroxide ($H_2O_2$) level was measured in human internal mammary arteries treated or not with Ang2 (100 nM) and Metf (10 μM) using the Ferrous iron xylenol orange OXidation (FOX) assay (PeroxiDetect Kit, Sigma Aldrich) as previously described (16-18). The production of $H_2O_2$ was calculated using a standard curve and the results were expressed in nmol $H_2O_2$/mg tissue/ h/.

### Assessment of gene expression

Quantitative RT-PCR was performed in human internal mammary arteries rings. Total RNA was isolated (Aurum Total RNA Mini Kit, Biorad) and used for reverse transcription (iScript Advanced cDNA Synthesis Kit, Biorad). In order to determine gene expression, primers against were designed using sequence information from the NCBI database (5'→ 3'): eNOS-fw CTC ACC ATA GCT GTG CTG GCT TAC, eNOS-rv GAT GCA GGG CAA GTT AGG ATC AGG, nNOS-fw CTC CCG CCT CGG GCA AA CAG, nNOS-rv GTG CAC CCC GTT TCC AGC GT, iNOS- fw GCT CGC TTT GCC ACG GAC GA, iNOS-rv AAG GCA GCG GGC ACA TGC AA. The housekeeping gene (EEF2, eukaryotic elongation factor 2) and its primers were as follow (5'→ 3'): EEF2fw: GAC ATC ACC AAG GGT GTG CAG and EEF2rv: GCG GTC AGC ACA CTG GCA TA.

### Statistics

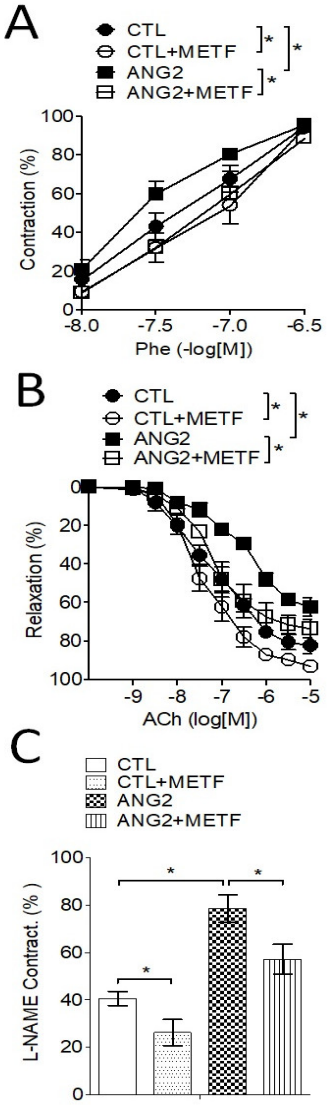
Statistical data processing was performed with the GraphPad Prism software Version 9.3.1 (GraphPad, USA). Data are presented as mean ± SEM and were analyzed using one-way ANOVA or Student t-test when appropriate. Data analysis of the dose–effect response curves was performed using the ANOVA F-test (comparisons of bottom and top values, EC50 and the Hill slope). Values of $p < 0.05$ were considered statistically significant.

## RESULTS

### Metformin improved vascular function in human internal mammary arteries

In this study we investigated the interaction between metformin and Ang2 in human internal mammary arteries. We evaluated the vascular reactivity (contraction to Phe and the endothelium-dependent relaxation to ACh) in vascular rings stimulated or not with Ang2 in the presence *vs* absence of Metf. Ang2 impaired vascular function by increasing contractility and reducing relaxation and Metf was able to attenuate these effects (Fig. 1 A,B). The beneficial vasomotor effects of Metf were observed in all vascular rings (stimulated or not with Ang2), demonstrating the activation of the local renin-angiotensin system in the mammary arteries harvested from patients with coronary heart disease. As depicted in Fig. 1 A, B the magnitude of response appear to be higher in the Ang2-stimulated group, albeit the statistical significance was not evident due to the reduced number of samples. In the same experiments we measured the contractility response to eNOS inhibitor, NG-nitro-L-arginine methyl ester (L-NAME, Fig. 1C), as an indirect approach to assess the nitric oxide (NO) bioavailability.

**Figure 1.** Metformin improves vascular function in human internal mammary arteries stimulated of not with Ang2.



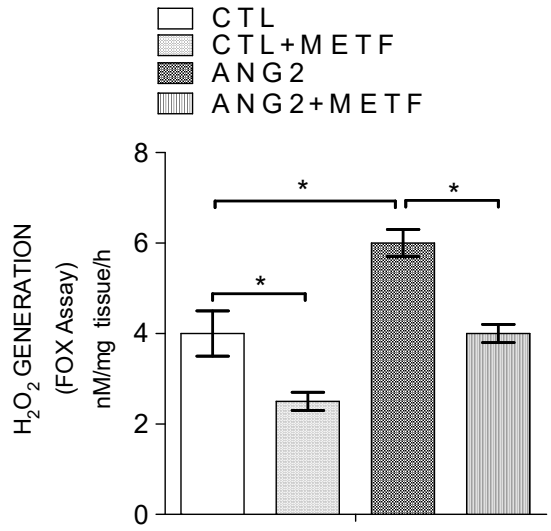

A. Phe-induced contraction; B. ACh endothelial-dependent relaxation; C. L-NAME induced contraction. n=7, *p<0.05

The contractility response was augmented by Ang2, but incubation with Metf was able to attenuate this effect suggesting the interference with the NO generation/signaling cascade as possible mechanism of vascular protection elicited by Metf.

**Metformin reduced oxidative stress in human internal mammary arteries**

To elucidate whether the improvement of vascular function was a consequence of ROS production modulation, in another set of experiments we investigated the effect of Metf on the $H_2O_2$ formation in human internal mammary arteries. As shown in Fig. 2, after *ex vivo* Ang2 stimulation, vascular $H_2O_2$ formation determined by the FOX assay increased. Co-incubation with Metf significantly attenuated this effect (Fig.2). Similar to the vasomotor effect, the drug was able to mitigate ROS prodution in CTL vessels (non-stimulated with Ang2) thus demonstrating that increased oxidative stress was present along with endothelial dysfunction at the level of mammary arteries used for grafting.

**Figure 2.** Metformin attenuated $H_2O_2$ generation in human internal mammary arteries stimulated of not with Ang2.




The $H_2O_2$ levels are expressed as nM/mg tissue/h, n=7, *p<0.05.

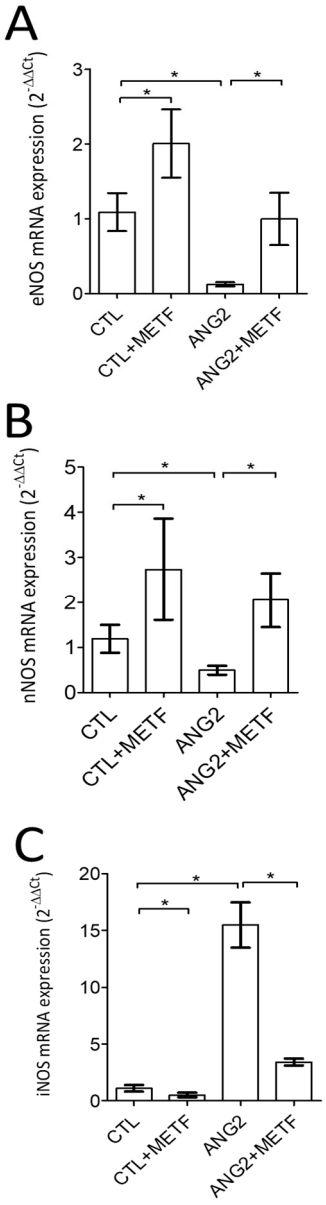
**Metformin modulated the expression of nitric oxide synthases in human internal mammary arteries**

As Metf was able to attenuate ROS generation and to improve vascular function, including reduction of NG-nitro-L-arginine methyl ester (L-NAME)-induced contractility, we further investigated whether Metf interfere with the mRNA gene expression of the enzymes responsible for NO generation in vessels. To address this aspect we performed qRT-PCR studies of the NOS isoforms (eNOS, nNOS, iNOS) in human internal mammary arteries stimulated or not with Ang2 and Metf.

In CTL samples, acute incubation with Metf elicited an increased expression of eNOS and nNOS and had no effect on iNOS.

Incubation of vascular segments with Ang2 in organic culture decreased expression of eNOS and nNOS and increased expression of iNOS, respectively. Metf was able to reverse all these effects (Fig. 3). Collectively, these experiments identify eNOS, nNOS and iNOS as targets of Metf in the human mammary arteries.

**Figure 3.** Metformin modulates NOS isoforms expression in human internal mammary arteries.
qRT-PCR mRNA gene expression.
A. eNOS, B. nNOS, C. iNOS, n=7, *p<0.05



## DISCUSSIONS

This pilot study was purported to assess the effect of Metf on vascular reactivity, oxidative stress and NO synthases expression in human internal mammary arteries harvested during the revascularization procedure (aorto-coronary by-pass grafting) and stimulated or not *in vitro* with Ang2. The main finding of this study is that Metf was able to reverse the deleterious effects of Ang2 on vascular reactivity, ROS generation and gene expression of the enzymatic sources responsible for vascular NO generation.

Metformin has been introduced as diabetic medication in 1957 and considerable efforts have been made ever since to understand its beneficial effects at cellular and molecular level in order to argue for the drug repurposing in the cardiovascular pathologies (19). In the past decades the drug has also been systematically investigated for its protective effects in several other pathologies, such as: cancer, neurodegenerative diseases, inflammatory bowel disease, tuberculosis, polycystic ovarian syndrome, osteoporosis, periodontitis, and more recent, COVID-19 via modulating several signaling pathways such as: NF-κB, PI3K/AKT/mTOR, SIRT1/PGC-1α, NLRP3, ERK, P38 MAPK, Wnt/β-catenin, Nrf2, JNK as recently reviewed in ref. (20).

Currently, activation of AMP-protein kinase (AMPK) is considered the main molecular mechanism behind the protective effects of metformin in cardiovascular disease and cancer; moreover, the moderate increase in lactate, which act as a major energy shuttle in several lactate consumer-organs, has been recently proposed as being beneficial rather than deleterious after Metf administration (21).

The observation that Metf is able to improve the endothelium-dependent relaxation, an effect mediated by NO, has been reported 2 decades ago (22).

The mechanisms underlying the vascular beneficial effects of Metf have been studied in several experimental models. Thus, Davies et al firstly demonstrated that chronic administration of metformin in the wild-type C57BL6 mice enhanced eNOS activation and increased NO bioactivity (assessed as cyclic GMP), effects that were mediated via AMPK (since the effect was ablated in the AMPK-alpha1 knockout mice) (23). Three years later, Lefer's group demonstrated that chronic activation of AMPK with low-dose metformin elicited beneficial effects on cardiac function and survival in a murine model of heart failure; cardioprotection was mediated via increased eNOS phosphorylation in cardiac myocytes and was abolished in mice lacking functional eNOS (24).

Controversial data are available in the literature regarding the effects of Metf on NO release and NOS activity/expression in diabetic animals. Thus, while two studies reported the increase in eNOS expression (25, 26), no effect was observed by another group (27). As regarding the inducible NO synthase, the drug has been reported to negatively regulate the iNOS expression in vascular smooth muscle cells treated with TNFα (28). In a very recent data, Dawood et al demonstrated the activation of renal artery angiotensin 1 receptor, upregulation of endothelin-1 and iNOS, as well as downregulation of eNOS in a rat model of diabetic nephropathy, effects that were reversed by Metf (29).

In mesenteric arteries harvested from aged Otsuka Long-Evans Tokushima fatty (OLETF) rats, a type 2 diabetes model, a 4 week protocol of Metf administration both alleviated endothelial dysfunction and decreased superoxide production (30). We also demonstrated here that Metf is able to mitigate the oxidative stress, in particular the $H_2O_2$ generation. Important, in a previous study we reported that metformin does not have ROS scavenger properties (31), thus the reduction of ROS is the consequence of direct effect on ROS generation and/or on the antioxidant capacity, hypotheses that will be addressed in future studies.

As regarding clinical studies with Metf in humans, one of earliest studies in humans regarding the chronic beneficial vascular effects of administration of Metf (500 mg twice per day, 8 weeks) was performed in 33 non-diabetic women in which *in vivo* assessment of forearm (skin) microvascular function using laser Doppler imaging combined with iontophoresis was performed back to 2006. Endothelium-dependent- (but not independent) responses were improved by Metf *vs* placebo in women with angiographically normal coronary arteries vs placebo (32). In 2008, Meaney et al reported in the MEFISTO study that chronic administration of Metf (850 mg/day for 1 year) elicited a reduction in carotid intima–media thickness levels of carbonyls, dityrosines and AOPP and an increase in NO levels, thus indicating better endothelial function (33).

However, literature regarding the direct effect of Metf in human arterial samples is scarce. The novel observation of the present study is the direct modulation by acute incubation with Metf of all three NOS isoforms in human internal mammary arteries together with the above mentioned antioxidant effect.

## CONCLUSIONS

In summary, Metformin, the first line therapy in diabetes, is able to alleviate endothelial dysfunction induced by Ang2 via the modulation of NOS expression and ROS production in human internal mammary arteries, the nonatherosclerotic repair arteries used for coronary bypass grafting.

Elucidating the molecular mechanisms underlying the Metf-NOS interaction in humans is required for a better management of the chronic vascular complications in cardiometabolic pathologies and/or of the acute vascular reperfusion injury.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was conducted according to the World Medical Association Declaration of Helsinki and was approved by the Committee for Research Ethics of "Victor Babeș" University of Medicine and Pharmacy, Timișoara, RO (no. 04/28.02.2020 and 04p/17/12/2020). All participants provided a written informed consent.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## FUNDING

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ORIGINAL SCIENTIFIC ARTICLE

# SYSTEMIC REDOX STATUS OF RATS TREATED WITH DIFFERENT DOSES OF PERFLUOROCARBON BASED BLOOD SUBSTITUTE- PERFTORAN®

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## ABSTRACT

*The aim of this research was to examine the influence of the intraperitoneal application of PFT in different doses and regimen on systemic oxidative stress and activity of antioxidative enzymes in animals. Depending on whether the animals received only saline or PFT in different doses (8, 12, 16 ml/kg body weight), and time (1, 10, or 20 hours before sacrificing and blood sampling), all animals were divided into control or experimental groups. From plasma samples we measured following biomarkers of oxidative stress: superoxide anion radical ($O_2^-$), hydrogen peroxide ($H_2O_2$), nitrites ($NO_2^-$), index of lipid peroxidation measured as TBARS (thiobarbituric acid reactive substances), and from hemolysate samples activity of the next enzymes: catalase (CAT), superoxide-dismutase (SOD) and reduced glutathione (GSH). All mentioned biochemical parameters of oxidative stress were determined spectrophotometrically (Shimadzu UV-1800UV-VIS spectrophotometer, Japan). Superoxide anion radical was a molecule very affected with the PFT administration. we observed the significantly higher activity of superoxide dismutase in all PFT treated groups in comparison with the CTRL group. The highest activity was observed in group treated with the 8 and 12 ml/kg of PFT nearly to sampling (1 hour). Catalase activity was significantly higher in PFT group in comparison with the CTRL, especially in PFT 16ml/kg group (1 hour). In comparison with the CTRL group, the total content of GSH was significantly lower in the groups treated PFT in dose of 16 ml/kg 1 hour and 10 hours before blood sampling. All these changes in oxidative stress markers seems to be very clear, but we can observe that almost all changes are induced in 1 hour after PFT administration. Probably, PFT solution has short-term protective effects on reducing oxidative stress, but no long term-effects. Maybe the chemical and biological instability of PFT solution could be a reason for that transient antioxidative effects, and developing the nano-formulation of PFT could be potential option for resolving the problem with poor pharmacodynamic of PFT.*

***Keywords***:*Perfluorocarbon, oxidative stress, antioxidative enzymes, blood samples, rat.*

## INTRODUCTION

The development of pefluorocarbon solution (PFT) began during 80' years, when it is suggested that PFT as infusion could be blood substitute and transport oxygen in vivo. As a chemically and biologically inert, pure PFT is not suitable for injection into vascular system, but the nano-emulsion as a best formulation could be used because of the faster clearance, longer retention time in organs and lower risk for side effects (1, 2)

In history, the first experiment with PFT was done by Clark and Gollan who kept Langendorff-heart beating by perfusing it with pure PFT (3). After that, in 1967, Sloviter suggested that component of PFE must be prepared as emulsion, because of the insolubility of glucose and salts, so he was the first scientist who emulsify PFE in water (4, 5). That was a challenge because of the many different components in this solution, such as lipids, glucose, salts, proteins and water.

The second challenge was stability of PFE emulsion. We know that emulsions are metastable because reaches thermodynamic equilibrium only after complete phase separation. Nano emulsion of PFT shows droplets diameter below 1 μm, and it is more stable than micro emulsions.

Almost 30 years ago, PFT emulsion was developed named as Fluosol-DA® which was the first generation of these syntetic solutions. The first use of this commercially-available product was reported in Japan with infusion of 500 ml and 1000 ml given to patients with severe gastrointestinal bleeding and surgery-related blood loss for esophageal cancer, respectively. Fluosol-DA® was only FDA approved PFT for clinical use (6, 7).

After that, in future years were sintethyzed another PFT solutions but not FDA-approved. Most of them were reached only phase II trials. Some of them were Oxygent®, Oxycyte® and they are still in preclinical testing.

In 1996 in Russia, Professor S.I. Vorobyev and her collaborators registered perfluorocarbon emulsion Perftoran® for wide clinical use for the first time in the world (8, 9).

Definitely, PFT solutions are products that in theory seem to be a viable option for clinical use. However, due to the short stability of approximately 8 hours it is difficult to use it in coronary balloon angioplasty, the indication for which the product has gained its approval, and that led to FDA removing Fluosol® from the market in 1994. Developing of the new nano emulsion seems to PFT, especially Peftoran-plus (Ftoremulsion III, Russia) could be approved for clinical use in the future.

On the other hand, examining the association of PFT and level of oxidative stress is still actual, since it is clear that PFT emulsions deliver oxygen to the tissues as a hemoglobin-based carrier.

Oxidative stress as an imbalance of free radicals and antioxidants in organism is also something that could be associated with the effects of PFT. Oxidative stress (OS) has the ability to damage different molecules and cellular structures, altering the correct function of organs and systems (10-13). OS accumulates in the body by endogenous and exogenous mechanisms. Increasing evidence points to the involvement of OS in the physiopathology of various chronic diseases that require prolonged periods of pharmacological treatment (10-13).

In previous studies, PFT was mentioned as potential preventive strategy for ischemia/reperfusion injuries for example in lung graft preservation (14).

Also, it is suggested that PFT could decrease production of inflammatory markers and stress oxidative markers, decrease lung oedema and hydroxyproline level, as well as attenuate histopathological changes and fibrosis (15).

Based on previous literature data, the greatest future of PFT may be not in the treatment of anemia, but in improving the microcirculatory oxygen delivery for treatment of ischemic tissues. For example, PFT solution could be a choice of treatment in stroke, myocardial infarction, ischemic extremities etc.

According to that, the aim of this research was to examine the influence of the intraperitoneal application of PFT in different doses and regimen on systemic oxidative stress and activity of antioxidative enzymes in animals.

## MATERIAL AND METHODS

### Ethics approval

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 Faculty of Medical Sciences, University of Kragujevac, Serbia.

### Study design

In this study we used male Wistar albino rats (10 weeks old, weighed 200 ± 20 g on average) who were housed in a temperature-controlled vivarium (22 ± 2 ° C), with 12-hour alternating light-dark cycles. Standard food and water were available to the animals ad libitum.

This study was aimed to determine the dose of 20% perfluoroemulsion (PFT) on redox parameters in blood samples of rats. Depending on whether the animals received only saline or PFT in different doses (8, 12, 16 ml/kg body weight), and time (1, 10, or 20 hours before sacrificing and blood sampling), all animals were divided into control or experimental groups:

Control groups:

1. Control (CTRL) group - without application of PFT solution (n=6)

Experimental groups:

1. Group PFT 8 ml/kg - 1 hour before sampling (n=6)
2. Group PFT 12 ml/kg - 1 hour before sampling (n=6)
3. Group PFT 16 ml/kg - 1 hour before sampling (n=6)
4. Group PFT 16 ml/kg - 10 hours before sampling (n=6)
5. Group PFT 16 ml/kg - 20 hours before sampling (n=6).

**Perfluorocarbon-based blood substitute (PFT)**

For the purposes of the study, 20% perfluorocarbon emulsion (Perftoran®) was used, which was prepared ex tempore by dissolving equal volumes of 20% PFE for intravenous administration. The chemical composition of the Perfluorocarbon-based blood substitute is: 13,0 g (6,5 ml) of Perfluorodecalin, 6,5 g (3,25 ml) of Perfluoro-N-4- (methylcyclohexyl) - piperidine, 4,0 g of Proxanol-268, 0,6 g of Sodium chloride (NaCl), 0.039 g of Potassium chloride (KCL), 0.019 g of Magnesium chloride ($MgCl_2$), 0.065 g of Sodium bicarbonate ($NaHCO_3$), 0.02 g of Sodium phosphate monobasic ($NaH_2PO_4$), 0,2 g of glucose and 100 ml Distilled Water ($H_2O$). This solution Peftoran® was purchased in the Research Laboratory of Biological and Physico-Chemical Study of perfluorocarbons, Russian Federation.

**Preparation of blood samples**

Rats were anesthetized with a combination of ketamine and xylazine and sacrificed by decapitation using a guillotine for small laboratory animals. Immediately after sacrifice, a whole blood sample was collected in tubes with 3.8% sodium citrate. The blood sample was gently agitated to thoroughly mix the sodium citrate with the blood. Subsequently, the sample was centrifuged three times at 3000 rpm for 10 min at room temperature and dissolved with distilled water for preparing the hemolysate.

**Spectrofotometric determination of biomarkers of oxidative stress**

From plasma samples we measured following biomarkers of oxidative stress: superoxide anion radical ($O_2^-$), hydrogen peroxide ($H_2O_2$), nitrites ($NO_2^-$), index of lipid peroxidation measured as TBARS (thiobarbituric acid reactive substances), and from hemolysate samples activity of the next enzymes: catalase (CAT), superoxide-dismutase (SOD) and reduced glutathione (GSH). All mentioned biochemical parameters of oxidative stress were determined spectrophotometrically (Shimadzu UV-1800UV-VIS spectrophotometer, Japan).

$O_2^-$ concentrations were determined according to Auclair, by using the NTB (Nitro Blue Tetrazolium) reagent in TRIS buffer (assay mixture) with the sample, while the measurement was performed at a wavelength of 530 nm (16).

The determination of $H_2O_2$ was based on the oxidation of phenol red by $H_2O_2$ which is catalysed by horseradish peroxidase. The level of $H_2O_2$ was then measured at 610 nm of wavelength (17).

Nitrites level was measured in order to indirectly asses nitric oxide level. Nitrites were quantified by method according to Green using the Griess-reagent (18). The sample was precipitated with 30% sulfo-salicylic acid, vortexed for 30 min, and centrifuged at 30009g. Equal volumes of the supernatant and Griess's reagent, containing 1% sulfanil-amide in 5% phosphoric acid/0.1% napthalene ethylenediamine-dihydrochloride, were added and incubated for 10 min in the dark and measured at 543 nmol/l.

Index of lipid peroxidation in the blood samples was estimated via measuring of TBARS using 1% thiobarbituric acid (TBA) in 0.05M sodium hydroxide (NaOH) incubated with the sample at 100 C for 15 min and then measured at 530 nm (19).

**Determination of antioxidative enzymes (superoxide dismutase, catalase, reduced glutathione) in the lysate samples from women with PCOS**

SOD activity was evaluated by using epinephrine method according to Beutler (20). Heart tissue homogenate sample was first mixed with carbonate buffer, and afterwards epinephrine was added to the mixture. SOD activity was measured at 470 nm of wave length and was expressed as U/ml/Hb of hemolysate.

Determination of antioxidant enzyme CAT was carried out according to Aebi`s method (21). CAT buffer, prepared lysate sample, and 10 mM $H_2O_2$ were used for CAT determination. The activity of CAT was measured spectrophotometrically at 360 nm of wave length and was expressed in U/ml/Hb of hemolysate.

The level of GSH was determined according to Beutler (22). The method involves the reaction of GSH oxidation with 5.5-dithio-bis-6.2-nitrobenzoic acid. The level of GSH was measured spectrophotometrically at 420 nm of wave length and was expressed in U/ml/Hb of hemolysate.

**Statistical analysis**

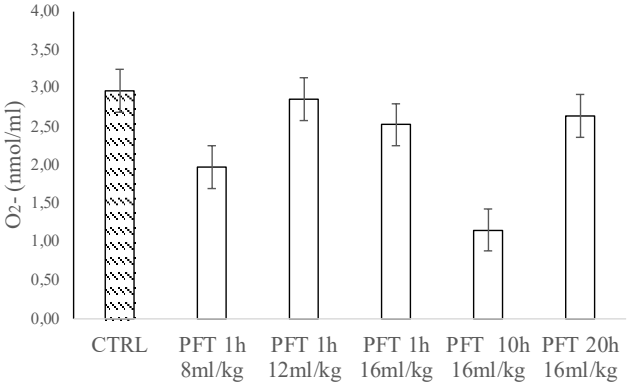
All data were presented in form of tables and figures. Results are presented and analyzed by descriptive analyses (means, standard deviations and standard errors of mean). Analytical statistical analysis is performed using Kruskal-Wallis and Tukey post hoc test for comparison the changes in percent between groups. Statistical significance was set at the level of 0.05. Statistical analysis was done using IBM SPSS statistical software version 26.0.

## RESULTS

### The effects of PFT on superoxide anion radical, hydrogen peroxide, nitric oxide and index of lipid peroxidation

From the figure 1, we can see the mean levels of concentrations of superoxide anion radical. Treatment with PFT in dose of 16ml/kg and 8 ml/kg induced the most significant lower levels of this marker in comparison with untreated rats (CTRL group) (Fig. 1; Table 1).

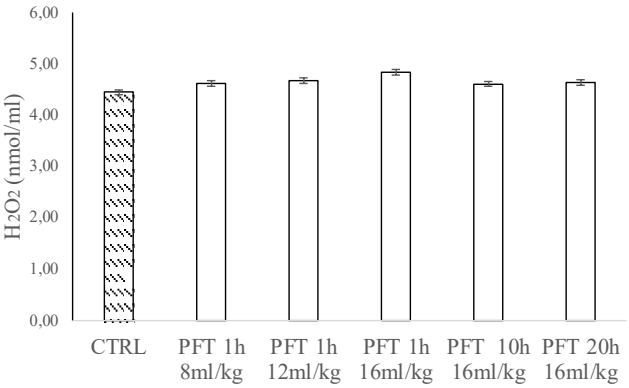
**Figure 1.** The mean values of superoxide anion radical from plasma samples in different groups.




Results are presented as mean ± standard error of mean.

On the other hand, the concentration of plasma hydrogen peroxide was not statistically significant different between groups (Fig. 2; Table 1).

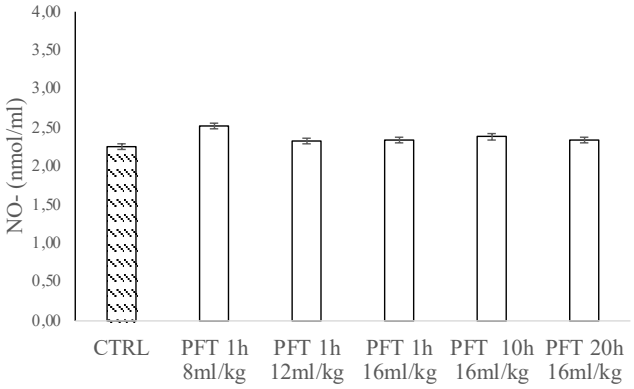
**Figure 2.** The mean values of hydrogen peroxide from plasma samples in different groups.




Results are presented as mean ± standard error of mean.

Bioavailability of nitric oxide was statistically significant higher in group treated with 8ml/kg of PFT 1 hour before sacrificing in comparison with the control group. In other groups, NO was not statistically significantly altered (Fig. 3; Table 1).

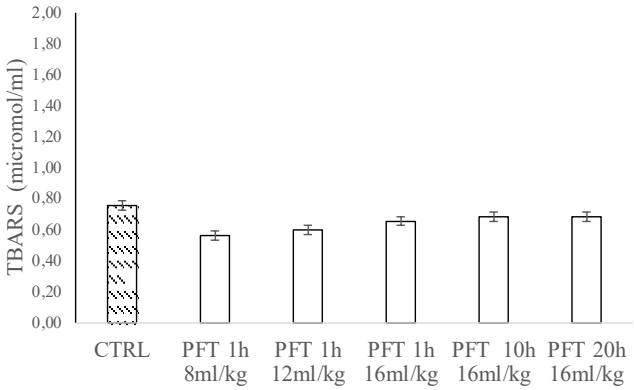
**Figure 3.** The mean values of nitric oxide from plasma samples in different groups.




Results are presented as mean ± standard error of mean.

From the Figures 4 and Table 1, we can see the lower levels of index of lipid peroxidation in group treated with 8 ml/kg and 12 ml/kg of PFT given 1 hour before sampling. Other values were similar with the values in CTRL group (Fig. 4; Table 1).

**Figure 4.** The mean values of index of lipid peroxidation measured as TBARS from plasma samples in different groups.

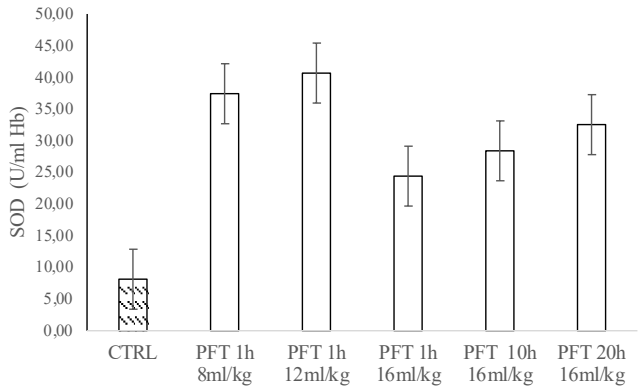



Results are presented as mean ± standard error of mean.

### The effects of PFT on superoxide superoxide dismutase, catalase and reduced glutathione

From the Figure 5 and Table 1, we observed the significantly higher activity of superoxide dismutase in all PFT treated groups in comparison with the CTRL group. The highest activity was observed in group treated with the 8 and 12 ml/kg of PFT nearly to sampling (1 hour) (Fig. 5; Table 1).

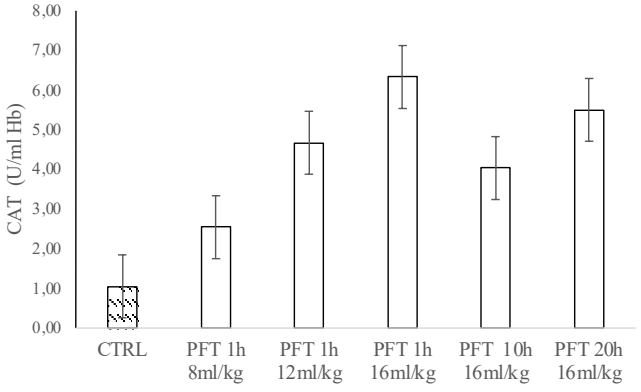
**Figure 5.** Activity of superoxide dismutase from hemolysate samples in different groups.




Results are presented as mean ± standard error of mean.

Also, catalase activity was significantly higher in PFT group in comparison with the CTRL, especially in PFT 16ml/kg group (1 hour) (Fig. 6).

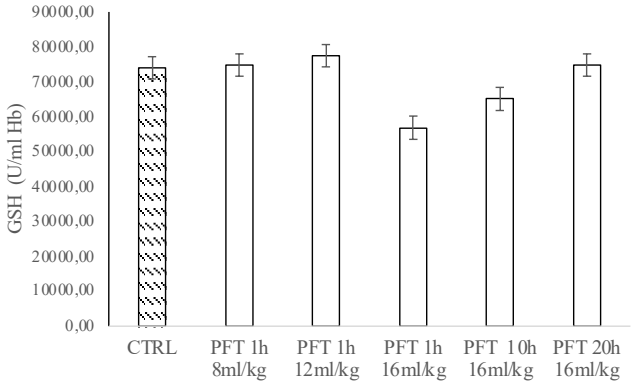
**Figure 6.** Activity of catalase from hemolysate samples in different groups.




Results are presented as mean ± standard error of mean.

In comparison with the CTRL group, the total content of GSH was significantly lower in the groups treated PFT in dose of 16 ml/kg 1 hour and 10 hours before blood sampling (Fig. 7).

**Figure 7.** The mean content of reduced glutathione from hemolysate samples in different groups.




Results are presented as mean ± standard error of mean.

**Table 1.** Comparison of the values of PFT-treated rats with the values in CTRL group.

| Comparison | O2- | H2O2 | NO- | TBARS | SOD | CAT | GSH |
|---|---|---|---|---|---|---|---|
| CTRL vs. PFT 1h 8ml/kg | p=0.02* | p>0.05 | p=0.038* | p=0.041* | p<0.001** | p=0.002** | p>0.05 |
| CTRL vs. PFT 1h 12ml/kg | p>0.05 | p>0.05 | p>0.05 | p=0.046* | p<0.001** | p<0.001** | p>0.05 |
| CTRL vs. PFT 1h 16ml/kg | p=0.03* | p>0.05 | p>0.05 | p>0.05 | p<0.001** | p<0.001** | p=0.021* |
| CTRL vs. PFT 10h 16ml/kg | p=0.02* | p>0.05 | p>0.05 | p>0.05 | p<0.001** | p<0.001** | p=0.028* |
| CTRL vs. PFT 20h 16ml/kg | p>0.05 | p>0.05 | p>0.05 | p>0.05 | p<0.001** | p<0.001** | p>0.05 |

Statistical analysis was done using Kruskal-Wallis Test.
Single asterisk (*) represents statistical significance, while double asterisk (**) represents high statistical significance.

## DISCUSSION

The main aim of this research was to examine the influence of the intraperitoneal application of PFT in different doses and regimen on systemic oxidative stress and activity of antioxidative enzymes in animals. Actually, this study is a second part of our research of the effects of PFT on cardiovascular system, where we reported that PFT administered before ischemia (1 hour) has less positive effects on myocardial function in an isolated rat heart model compared to earlier administration (10 and 20 hours). Also, the effects of 20% PFE are more pronounced if there is a longer period of time from application to ischemia, i.e., immediate application of PFE before ischemia (1 hour) gave the weakest effects on the change of cardiodynamics of isolated rat heart. Therefore, the future of PFE use is in new indications and application methods, and PFE can also be referred to as anti-hypoxic and anti-ischemic blood substitute with mild membranotropic effects (22).

Based on these conclusions, we continued our research and using standard methods for determining the concentrations of biomarkers of oxidative stress in rat blood, we evaluated the effects of PFT administration on their changes. Definitely, we have founded that PFE administration induced decreasing the superoxide anion radical and increasing the activity of antioxidative enzymes, which confirms the antioxidative role of PFT in vivo in comparison with the control conditions (Figs. 1-7; Table 1).

Superoxide anion radical was a molecule very affected with the PFT administration. Production of superoxide anion radical is essential for the life of aerobic organisms who acts as a signaling molecule in many processes such as apoptosis, aging etc (24). All these processes are associated with the many serious diseases when we observe the lower scavenging of $O_2$-. Also, poor antioxidants capacity of organism is reason for the increased levels of free radicals, such as superoxide anion radical. In our study, PFT showed that has ability to decreased the level of this marker of oxidative stress.

Interestingly, nitric oxide was increased after PFT administration 1 hour before and in dose of 8ml/kg. As we know, NO is actually synthesized from arginine by the endothelial isoform of NO-synthase (eNOS) in response to an appropriate stimulus. Endothelial NO is dispersed in vascular smooth muscle cells where cytosolic guanylate cyclase is activated and increases the production of cyclic guanosine monophosphate, leading to smooth muscle cells relaxation. Loss of endothelium-mediated vasodilator capacity is considered one of the earliest manifestations of cardiovascular damage and precedes the formation of atherosclerotic plaques.

Previous study conducted by Bekyarova et al, concluded that the combined application of alpha-tocopherol and FC-43 perfluorocarbon emulsion immediately after thermal skin injury in rats increases plasma antioxidant capacity, decreases free radical mediated damage of erythrocytes and suppresses their aggregation on the third hour after the injury (25).

Also, Zhang et al reported about the antioxidant potential of PFT. Their research showed that PFC reduced A549 cell damage caused by blast injury. Also, suggested the potential mechanism which may be associated with the following signaling pathways:

the signaling pathways of NF-κB and MAPK, which inhibit inflammation and reactive oxygen species (ROS); and 2) the signaling pathways of Bcl-2/Bax and caspase-3, which inhibit apoptosis (26).

Also, in the pathophysiology of neurological disease oxidative stress has an important role. Previous authors confirmed the preventive role of PFT in schizophrenia (27).

Furthermore, in the state of redox imbalance, high oxidative stress could be reduced by antioxidative defense system, which is crucial to reduce risk factors for the development the disease. In our study, we observed the significantly higher activity of superoxide dismutase in all PFT treated groups in comparison with the CTRL group. The highest activity was observed in group treated with the 8 and 12 ml/kg of PFT nearly to sampling (1 hour) (Fig. 5; Table 1). Also, catalase activity was significantly higher in PFT group in comparison with the CTRL, especially in PFT 16ml/kg group (1 hour) (Fig. 6). In comparison with the CTRL group, the total content of GSH was significantly lower in the groups treated PFT in dose of 16 ml/kg 1 hour and 10 hours before blood sampling (Fig. 7).

In addition, both downregulation of components of the antioxidant synthesis and increases of reactive oxygen species (ROS) have been observed in patients with ischemic disease (28). So, administration the PFT in animals could decrease oxidative stress and improve antioxidant capacity, even for the short time (few hours). Other authors also confirmed these results, such as reducing the production of free radicals and the number of pulmonary structural where changes resulting from cold ischemia (28, 29).

PFT solution which we used, is consist of 20% of total perfluorocarbon in 100 ml of solution, and compared with the other available PFTs, it is with lower percent of PFT. Other PFT are mostly higher concentration based PFT, so maybe that is a reason for the transient antioxidative effect in our study (30, 31). Dani et al investigated the effect of tidal liquid ventilation (TLV) compared to conventional mechanical ventilation (CMV) on oxidative lung damage in the setting of acute respiratory distress syndrome (ARDS). They concluded that animals treated with TLV showed lower oxidative lung damage compared to animals treated with CMV (32).

## CONCLUSION

All these changes in oxidative stress markers seems to be very clear, but we can observe that almost all changes are induced in 1 hour after PFT administration. Probably, PFT solution has short-term protective effects on reducing

oxidative stress, but no long term-effects. Maybe the chemical and biological instability of PFT solution could be a reason for that transient antioxidative effects, and developing the nano-formulation of PFT could be potential option for resolving the problem with poor pharmacodynamic of PFT.

## ETHICS APPROVAL

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 Faculty of Medical Sciences, University of Kragujevac, Serbia.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.


## FUNDING

None.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ORIGINAL SCIENTIFIC ARTICLE

# ANALYSIS OF POTENTIALLY INAPPROPRIATE DRUG PRESCRIBING IN HOSPITALIZED ELDERLY PATIENTS

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## ABSTRACT

*The study was conducted at the Clinical Centre Kragujevac, during the period March-May 2016. The study population consisted of patients older than 65 that were treated at different departments of the Clinic for Internal Medicine. Data were collected from hospital medical records of patients and anonymous semi-structured questionnaires. STOPP (screening tool of older people's prescriptions)/START (screening tool to alert to right treatment) criteria from 2014 were used to monitor the outcome of interest. Based on the outcome PIM (potentially inappropriate drugs)/PPO (potential prescribing omissions), respondents were divided into groups of cases - patients to whom at least one potentially inappropriate drug determined by STOPP criteria was prescribed and those with at least one potential prescribing omission determined by START criteria. Control groups were patients without these outcomes. Most commonly, PIM was detected in the treatment of cardiovascular system diseases (27.12%), followed by the use of drugs that predispose falls in elderly (20.34%). The most important risk factors for the occurrence of PIM were female gender (OR=3.27; 95% CI 1.01-10.64), polypharmacy (5-8 drugs used simultaneously) (OR=3.10; 95% CI 1.11-12.04) and with whom the patient lives (OR=11.26; 95% CI 1.46-86.68). The use of STOPP/START criteria is proved to be efficient in the detection of PIM/PPO at the secondary level of health care. Full attention should always be paid to patients who are at the highest risk for inappropriate drug prescription. Doctors should make their decisions conscientiously and in line with clinical evidence, not blindly believing the pharmaceutical representatives.*

***Keywords***: *STOP/START, potential inappropriate prescribing, secondary health care, elderly.*

## INTRODUCTION

The inappropriate prescribing of drugs to patients older than 65 is one of the major problems across the world due to its association with increased morbidity, mortality and health care costs. The elderly, as a growing population, are the largest drug consumers (1). Appropriate therapy is the use of drugs in clear clinical indication, for which there is a sufficient number of clinical evidence and for which an appropriate cost-benefit ratio is estimated. Potentially inappropriate prescribing (PIP) is considered when the choice of the drug or the length of the prescribed therapy could harm the patient more than help him/her, as well as an omission in prescribing of a drug that may significantly contribute to prevention of onset, alleviation or cure the disease (2). Potential inappropriate medication is defined by STOPP criteria, while potential prescription omission is defined by START criteria (3). The occurrence of potentially inappropriate drugs (PIM) or potential prescribing omissions (PPO) does not mean that medical problems will occur for certain, but there is always a possibility. The most commonly prescribed potentially inappropriate drugs in the literature are: concurrent use of long-acting benzodiazepine and tricyclic antidepressants, first-generation antihistamines, psychotropic drugs which have hypotension as a side effect (benzodiazepines, antipsychotics, etc.) in patients with hypotension which are prone to falls, inadequate and duplicate use of NSAIDs and opioids, unjustified concurrent use of two drugs that have the same mechanism of action and the absence of calcium and vitamin D in patients with osteoporosis. Also, the most common prescribing omission is the use of NSAID in patients with severe hypertension and theophylline in patients with HOBP (4-9). It is considered that the application of these criteria can significantly reduce the use of inappropriate drugs and the consequences, as well as the economic burden of the health system (10).

The aim of this study is to evaluate the prevalence of prescribing potentially inadequate drugs according to STOPP and START criteria from 2014, in hospitalized elderly patients (≥65 years) and to detect associated factors.

## MATERIALS AND METHODS

### Study design

The research was designed as an observational, non-therapeutic, prospective cross-sectional study, with a nested case-control study. The study was conducted at the Clinical Centre of Kragujevac, the Clinic for Internal Medicine, during the period March-May 2016. The study population consisted of patients older than 65 that were treated at different departments of the Clinic for Internal Medicine. Data were derived from the hospital medical records of patients, follow-ups and relevant medical documentation and anonymous semi-structured questionnaires. The questionnaire used for interviewing patients detected the necessary socio-demographic and clinical data: age, sex, place of residence, with whom the patient lives, level of education, workplace before retirement, the number of drugs in chronic therapy, life habits (use of alcohol and smoking, physical activity), comorbidity and allergies. The questionnaire used for interviewing doctors detected the data related to the working years, specialization, working hours per day, as well as the number of visits by pharmaceutical company representatives per month and the use of pharmacotherapy guides. Before the interview, each patient was informed about the goal and the significance of the research, that participation is voluntary and can be dropped at any time. The data were collected after obtaining a written consent for participation in the study. The test was carried out using the face-to-face technique. Identity of patients was protected by assigning an identification number that was used in the further work. Protection of rights and the safety of the respondents included in the study were provided, as confirmed by the positive decision of the Ethics Committee of the Clinical Centre of Kragujevac.

Based on the following outcome - PIM/PPO, respondents were divided into groups of cases - patients to whom at least one potentially inappropriate drug determined by STOPP criteria was prescribed or patients with at least one potential prescribing omission determined by START criteria. Control groups were patients without these outcomes. Cases and controls were matched by age (± 1 year). To monitor the outcome of the interest, STOPP/START criteria from 2014, made up of 80 STOPP and 34 START criteria, were used (3).

### Study population

The study population consisted of patients with different socio-demographic and clinical characteristics, within the following inclusion criteria: aged 65 and above, hospitalization at one of the Internal Clinic Departments during the research period, prescription of at least two drugs at the same time and the presence of at least two concomitant chronic diseases. Average age was 73.44 years. Patients excluded from study were those with incomplete documentation, patients under 65, patients with malignant diseases or terminal stage of the disease and those who received health care through home care services or private health institutions.

A total of 18 prescribing physicians were interviewed: five from the Department of Endocrinology and Metabolic Units, seven from the Department of Gastroenterohepatology, two from the Department of Rheumatology and four from the Department of Allergology.

### The following independent and confounding variables were measured:

**Independent variables** (causes): family history of illnesses, life habits of patients (alcohol use, smoking, physical activity), associated chronic diseases, drug/food allergies, number of drugs for chronic diseases, years of work experience of the physician and the number of patients examined

per day, physicians use of referent literature and participations in the programs of continuous medical education from the areas of rational therapy, number of visits by pharmaceutical company representatives. In order to monitor the influence of the previously mentioned factors on the observed outcome, they are formulated as continuous or categorical variables.

**Confounding variables** (sociodemographic characteristics): age, sex, socio-economic status, education level, independent living or in a community (with a partner or family).

### Data analysis

Data were collected using Microsoft Excel 2007 and were processed descriptively at first. Kolmogorov-Smirnov test was used to determine data distribution. Mann Whitney was used to determine the significance of the difference in the values of the continuous variables between cases and controls. Chi-squared test was used to estimate the significance of the difference in frequency in the categorical variables. The influence of independent and confounding variables on the observed dichotomous outcomes (PIM/PPO) was tested using univariate and multivariate binary logistic regression. The strength of association is shown by a crude and adjusted odds ratio (OR) with a corresponding 95% confidence interval (95% CI). Probability value <0.05 was taken as statistically significant. Statistical program SPSS version 19.0 (SPSS Inc., Chicago, IL) was used for all analyses.

## RESULTS

The study included a total of 109 elderly patients hospitalized at the Clinic for Internal Medicine at Clinical Centre Kragujevac, Serbia 60 (55.05%) of which were women and 49 (44.95%) men. Potentially inappropriate medication was observed in 59 cases, while the absence of a clinically indicated drug was detected in 17 cases (Table 1.). Regarding the distribution of outcomes depending on sex, both PIM and PPO occurred more often in women (n= 36 and 9) than in men (n=23 and 8).

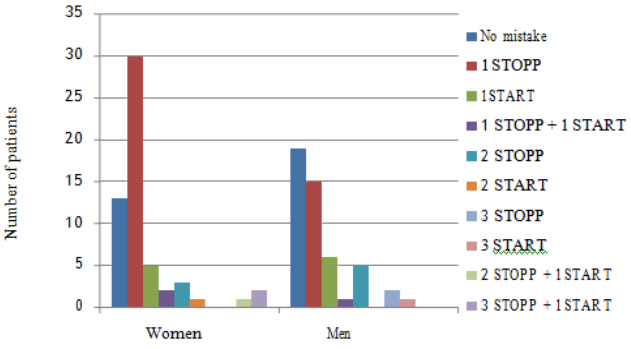
**Table 1**. Observed outcomes of interest depending on the hospital department

| | | Department at the Clinic for Internal Medicine | | | | |
|---|---|---|---|---|---|---|
| | | **Endocrinology** | **Metabolic unit** | **Gastroenterohepatology** | **Rheumathology** | **Allergology** |
| Outcome | No prescribing error | 9 | 4 | 1<br>5 | 3 | 2 |
| | PIM | 14 | 6 | 1<br>8 | 6 | 15 |
| | PPO | 5 | 1 | 8 | 2 | 1 |

### Characteristics of the observed population

Most patients lived in the city or a suburban area (55.5%), with their families (63.3%) and had completed only primary school (59.6%). Most of them were adhering to good living habits – 83.5% of patient were non-smokers and 92.7% of them never used alcohol. Most of the patients also had physical activity on daily basis (72.5%), especially after the deterioration of health status and hospitalization. However, a number of patients had 3 associated chronic diseases (36.7%) for which they were prescribed between 5 and 8 drugs as concomitant daily treatment (62.4%). Patients treated at the Clinic for Internal Medicine mostly had positive history of illnesses in their family (55%). Food or drug allergies were recorded in a small number of patients (24.8%).

**Figure 1.** Distribution of inappropriate prescription depending on gender



A total of 18 prescribing physicians were interviewed from all the departments of the clinic. All physicians stated that they visited some of continuous medical education on rational drug use during the last year and that they were using pharmacotherapy guides or other professional literature in their work, both for the treatment and the diagnostics. Most of them (89%) have been visited monthly by the representatives of pharmaceutical companies.

**Prescribing Potentially Inappropriate Drugs to Older Patients (PIM)**

Potentially inappropriate medication was recorded in 59 cases. In most cases, the prescription of only one PIM was recorded (78.69%), while prescribing 2 or 3 PIMs in the same patient was detected in a smaller percentage (16.39% and 4.92%), respectively.

The largest number of PIM was recorded in the therapy of cardiovascular disorders (27.12%). Prescription of drugs that predispose falls in elderly patients was recorded in 20.34% of cases. In all these cases, benzodiazepines were used. Drug prescribing without a clear indication and use of antihistamine of I generation as the first line of therapy were detected in 13.56%. Duplication of therapy, inappropriate antithrombotic/anticoagulant or endocrinology therapy was detected in 11.86%, 10.17% and 3.39%, respectively. Most often errors made are presented in Table 2.

**Table 2.** Distribution of PIM by pharmacological groups

| PIM | Number of events |
|---|---|
| Cardiovascular system | |
| Application of beta blockers in combination with verapamil (risk of heart failure) | 1 |
| Application of thiazide diuretics in a patient with hypercalcemia | 1 |
| The use of antihypertensive agents with central effect (methyldopa) in the absence of intolerance or inefficiency of other antihypertensive agents | 10 |
| Administration of amiodarone as the first line of therapy in patients with supraventricular arrhythmias (higher risk of adverse effects than beta blockers, digoxin, verapamil or diltiazem) | 4 |
| Endocrine system | |
| Application of long-acting sulfonylurea derivatives (glimepiride) in patients with diabetes mellitus type 2 | 2 |
| Antithrombotic and anticoagulant drugs | |
| NSAID in combination with antithrombotic drugs without present proton pump inhibitor (PPI) therapy as a peptic ulcer prophylaxis | 6 |
| Allergies | |
| Application of first-generation antihistamines (there are safer and less toxic antihistamines) | 8 |
| Duplication therapy | |
| Simultaneous application of two drugs with the same mechanism of action (NSAID) | 7 |
| Use of drugs that predispose falls in the elderly | |
| Benzodiazepines | 12 |
| Indication | |
| Application of the drug without a clear indication | 8 |

Doctors prescribed two non-steroidal anti-inflammatory drugs in some cases, which was unnecessary duplication of therapy, with consequent increase in the risk of adverse effects. Use of NSAIDs in combination with antithrombotic drugs, without simultaneous prophylactic administration of PPI was recognized as a potential prescribing error. Proton pump inhibitor should reduce the risk of peptic ulcer and consequent bleeding in the digestive tract. A trend of frequent prescribing of benzodiazepines to elderly patients was also observed, which according to STOPP criteria were labeled as drugs to be avoided in this population.

**Table 3.** Socio-demographic characteristics of patients in whom PIM is detected

| Characteristics | | Cases n=59 | Controls n=33 | Statistics | | Crude OR with 95% CI |
|---|---|---|---|---|---|---|
| Age (mean value ± standard deviation) | | 73.98 ± 6.72 (66-88) | 72.69 ± 6.65 (66-92) | p=0.266 | - | 1.04 (0.98; 1.11) |
| Days spent in hospital (mean value ± standard deviation) | | 5.61 ±4.09 | 7.70 ±9.53 | p=0.333 | - | 0.95 (0.88; 1.03) |
| Number of drugs for chronic therapy | 1-4 | 3 (5.08%) | 3 (9.09%) | p=0.026 U=1145.0 Z=-0.968 | - | 2.03 (1.03; 3.40) |
| | 5-8 | 35 (59.32%) | 24 (72.72%) | | | |
| | >9 | 24 (40.68%) | 5 (8.47%) | | | |
| History of illness in family | yes | 37 (62.71%) | 11 (33.33%) | p=0.047 U=1043.0 Z= -1.908 | $\chi^2$=0.086 (2.952) | 2.17 (0.98; 4.80) |
| | no | 22 (37.29%) | 22 (66.67%) | | | |
| With whom the patient lives | Alone | 10 (16.95%) | 1 (3.03%) | p=0.468 U=1195.0 Z= -0.725 | $\chi^2$=0.077 (5.132) | 0.72 (0.40; 1.27) |
| | With a spouse | 11 (18.64%) | 11 (33.33%) | | | |
| | With family | 38 (64.41%) | 21 (63.63%) | | | |
| Place of living | Country-side | 27 (45.76%) | 13 (39.39%) | p=0.606 U=1222.0 Z= -0.516 | $\chi^2$=0.750 (0.101) | 0.81 (0.37; 1.78) |
| | City | 32 (54.24%) | 20 (60.61%) | | | |
| Education | Primary school | 37 (62.71%) | 16 (48.48%) | p=0.538 U=1208.0 Z=-0.615 | $\chi^2$=0.608 (0.996) | 0.80 (0.44; 1.43) |
| | High school | 18 (30.51%) | 13 (39.39%) | | | |
| | College | 4 (6.78%) | 4 (12.12%) | | | |
| The department at which the patient is hospitalized* | 1 | 6 (10.17%) | 9 (27.27%) | p=0.097 U=1051.0 Z=-1, 661 | $\chi^2$=0.112 (7,490) | 1,31 (0,97; 1,78) |
| | 2 | 6 (10.17%) | 4 (12.12%) | | | |
| | 3 | 18 (30,51%) | 15 (45,45%) | | | |
| | 4 | 6 (10,17%) | 3 (9,09%) | | | |
| | 5 | 15 (45,45%) | 2 (6,06%) | | | |
| Allergy | yes | 16 (27,12%) | 8 (24,24%) | p=0,860 U=1269.0 Z=-0,177 | $\chi^2$=1,000 (0,000) | 1,10 (0,43; 2,77) |
| | no | 43 (72,88%) | 25 (75,74%) | | | |

*1-Endocrinology, Diabetes and Metabolic Disease, 2-Metabolic Unit, 3- Gastroenterohepatology, 4- Rheumatology, 5-Allergology

Most of the patients (88.13%) in whom PIM was detected reported that they had previously experienced hospitalization at one moment in their life. In the control group, 57.57% of patients have never been hospitalized before. Characteristics of patients in whom PIM was detected are given in Table 4.

**Table 4.** Characteristics: Life habits of patients

| Characteristics | | Cases n=59 | Controls n=33 | Statistics | | Crude OR with 95%CI |
|---|---|---|---|---|---|---|
| Smoker | yes | 10 (16.95%) | 6 (10.17%) | - | $\chi^2$=0.876 (0.024) | 0.79 (0.27; 2.30) |
| | no | 49 (83.05%) | 27 (45.76%) | | | |
| Use of alcohol | Daily | 0 | 0 | p=0.282 U= 1213.5 Z= -1.075 | $\chi^2$=0.355 (3.249) | 1.64 (0.79; 3.42) |
| | Several times per week | 0 | 1 (3.03%) | | | |
| | Once per week | 0 | 0 | | | |
| | Once per month | 2 (3.39%) | 2 (6.06%) | | | |
| | Once per year | 1 (1.69%) | 0 | | | |
| | Never | 56 (94.91%) | 30 (90.91%) | | | |
| Physical activity | Daily | 42 (71.19%) | 21 (63.64%) | p=0.446 U= 1198.0 Z= -0.763 | $\chi^2$=0.496 (1.403) | 1.21 (0.77; 1.90) |
| | >1 in a week | 1 (1.69%) | 2 (6.06%) | | | |
| | Rarely | 16 (27.12%) | 10 (30.30%) | | | |

The largest number of participants from the "case" group had 3 (35.7%) or 5 (19.6%) concurrent chronic diseases. In the control group, most patients had 3 (41.3%) or 4 (21.7%) diseases present at the same time, for which they used chronic therapy. The statistical significance of the relationship between observed outcome and co-morbidity had not been established (p = 0.124).

Using adequate statistical tests as significant factors (p <0.05) for the observed outcome, showed to be factors listed in Table 5. However, when adjusted for other independent and confounding variables, some of factors were lost. By implementing the multivariate model the most important factors were the ones shown in Table 5.

**Table 5**. Results of multivariate binary logistic regression

| Variable | Adjusted OR |
|---|---|
| Sex (women) | 3.27 (1.01; 10.64) |
| Age | 1.09 (1.00; 1.19) |
| Previous hospitalization (yes) | 0.21 (0.05; 0.85) |
| Number of drugs for chronic diseases (5-8) | 3.10 (1.11; 12.04) |
| History of illness in family (yes) | 0.32 (0.11; 0.92) |
| With whom patient lives (with a spouse) | 11.26 (1.46; 86.68) |
| Visits by representatives of pharmaceutical companies | 0.08 (0.003; 2.18) |

## OBSERVED OUTCOME: THE ABSENCE OF PRESCRIBING A CLINICALLY INDICATED DRUG (PPO)

The absence of prescribing a clinically indicated drug was reported in 17 cases, determined by START criteria. One indicated drug that was omitted per patient was detected in most cases (86%), while two or three were left out in a smaller percentage (9% and 5%, respectively).

Potential prescription omission was detected in the treatment of cardiovascular system diseases in 52.94%, followed by musculoskeletal (29.41%) and urogenital tract disease therapy (17.65%). Errors occurred in drug application for central nervous system and eye therapy were detected in 5.88%. Out of 32 START criteria, 8 were detected in this study (Table 6).

**Table 6.** Distribution of PPO depending on the pharmacological group

| Organ system | Number of events |
|---|---|
| Cardiovascular system | |
| Antithrombotic therapy in patients with a history of coronary, cerebral or peripheral vascular events | 3 |
| Application of statins in patients with a history of coronary, cerebral or peripheral vascular events | 4 |
| Beta blocker in patients with heart ischemia | 2 |
| Central nervous system and psychotropic drugs | |
| L-dopa in the treatment of idiopathic Parkinson's diseases | 1 |
| Musculoskeletal system | |
| Application of bisphosphonate, vitamin D and calcium in patients on long-term therapy with systemic corticosteroids | 1 |
| Vitamin D and calcium in patients with osteoporosis and/or previous fractures | 4 |
| Urogenital system | |
| Blocker of alpha-1 receptor in patients with prostatism to whom prostatectomy is not indicated | 3 |
| Eyes | |
| Topical prostaglandin, prostamide or beta blocker in patients with glaucoma open angle | 1 |

**Table 7.** Characteristics of patients in whom PPO was detected - Socio-demographic characteristics

| Characteristics | | Cases n=17 | Control n=33 | Statistics | | Crude OR with 95% of CI |
|---|---|---|---|---|---|---|
| Age (mean value ± standard deviation) | | 69.35 (66-87) | 75.21 (66-92) | p=0.967 U=821.5 Z= 0.041 | - | 0.979 (0.90; 1.06) |
| Days spent in hospital (mean value ± standard deviation) | | 7 | 16,8 | p=0.447 U=811.5 Z= 0.124 | - | 0.96 (0.84; 1.08) |
| Number of drugs for chronic therapy | 1-4 | 4 (23.53%) | 3 (9.09%) | p=0.871 U=809.5 Z=0.162 | $\chi^2$=0.137 (5.144) | 0.85 (0.37; 1.92) |
| | 5-8 | 9 (52.94%) | 24 (72.73%) | | | |
| | >9 | 4 (23.53%) | 6 (18.18%) | | | |
| History of illness in family | yes | 12 (70.59%) | 11 (33.33%) | p=0.159 U=679.0 Z=-1.407 | $\chi^2$=0.246 (1.345) | 2.12 (0.74; 6.08) |
| | no | 5 (29.41%) | 22 (66.67%) | | | |
| With whom the patient lives | Alone | 1 (5.88%) | 1 (3.03%) | p=0.916 U=815.5 Z= -0.105 | $\chi^2$=0.992 (0.016) | 1.04 (0.51; 2.16) |
| | With a spouse | 6 (35.29%) | 11 (33.33%) | | | |
| | With family | 10 (58.82%) | 21 (63.63%) | | | |
| Place of living | Countryside | 9 (52.94%) | 13 (39.39%) | p=0.701 | $\chi^2$=0.896 (0.017) | 0.82 (0.30; 2.23) |

| Characteristics | | Cases n=17 | Control n=33 | Statistics | | Crude OR with 95% of CI |
|---|---|---|---|---|---|---|
| | City | 8 (47.06%) | 20 (60.61%) | U=796.5 Z= -0.384 | | |
| Education | Primary school | 12 (70.59%) | 16 (94.12%) | p=0.547 U=762.5 Z= -0.602 | $\chi^2$=0.192 (3.296) | 0.90 (0.42; 1.94 ) |
| | High school | 3 (17.65%) | 13 (76.47%) | | | |
| | College | 2 (11.76%) | 4 (12.12%) | | | |
| The department at which the patient is hospitalized* | 1 | 5 (29.41%) | 9 (27.27%) | p=0.132 U=651.0 Z= -1.058 | $\chi^2$=0.399 (4.054) | 0.73 (0.49; 1.08) |
| | 2 | 1 (5.88%) | 4 (12.12%) | | | |
| | 3 | 8 (47.06%) | 15 (45.45%) | | | |
| | 4 | 2 (11.76%) | 3 (9.09%) | | | |
| | 5 | 1 (5.88%) | 2 (6.06%) | | | |
| Allergies | yes | 3 (17.64%) | 8 (24.24%) | p=0,433 U=757.5 Z= -0.784 | $\chi^2$=0.628 (0.235) | 0.60 (0.16; 2.22) |
| | no | 14 (82.35%) | 25 (75.76%) | | | |

*1-Endocrinology, Diabetes and Metabolic Disease, 2-Metabolic Unit, 3- Gastroenterohepatology, 4- Rheumatology, 5-Allergology

Most of the patients (88.23%) in whom PPO was detected, reported that they had previously been hospitalized in a certain period of their lives. In the control group, 57.57% of patients have never been hospitalized before. Characteristics of patients in whom PPO is detected are given in Table 8.

**Table 8.** Characteristics of patients in whom PPO was detected: Life habits of patients

| Characteristics | | Cases | Controls | Statistics | | Crude OR 95%CI |
|---|---|---|---|---|---|---|
| Smoker | yes | 2 (11.76%) | 6 (18,.18%) | - | $\chi^2$=0.624 (0.240) | 0.52 (0.11; 2.49) |
| | no | 15 (88.23%) | 27 (81.82%) | | | |
| Use of alcohol | Daily | 0 | 0 | p=0.589 U=796.5 Z= -0.540 | $\chi^2$=0.699 (1.427) | 0.86 (0.41; 1.81) |
| | Several times per week | 0 | 1 (3,03%) | | | |
| | Once per week | 0 | 0 | | | |
| | Once per month | 2 (11.76%) | 2 (6.06%) | | | |
| Use of alcohol | Once per year | 0 | 0 | | | |
| | Never | 15 (88.23%) | 30 (90.91%) | | | |
| Physial activity | Daily | 16 (94.12%) | 21 (63.64%) | p=0.015 U=596.0 Z= -2.241 | $\chi^2$=0.043 (6.080) | 0.33 (0.11; 0.96) |
| | >1 in a week | 0 | 2 (6.06%) | | | |
| | Rarely | 1 (5.88%) | 10 (3.03%) | | | |

Characteristics of patients which were recognized as important factors ($p < 0.05$) for the outcomes of interest - PPOs are listed in Table 9. Based on the value of the implemented binary logistic regression, some of the patients' characteristics were declared statistically insignificant for the observed outcome. However, a new factor was distinguished - the presence of multiple diseases in one patient increases the likelihood of PPO occurrence. The significance and magnitude of impact can be seen in the following table.

**Table 9.** Results of multivariate binary logistic regression

| Risk factor | Adjusted OR (95% CI) |
|---|---|
| Length of hospitalization | 0,071 (0,48; 1,03) |
| Previous hospitalization (yes) | **19,208 (1,13; 326,20)** |
| Number of drugs for chronic therapy (5-8) | 0,404 (0,03; 5,99) |
| Comorbidities | **4,073 (1,71; 9,70)** |
| Allergy | 0,099 (0,01; 0,96) |
| Visits by representatives of pharmaceutical houses (once per month) | **25,05 (1,65; 381,26)** |
| Education (primary school) | 0,061 (0,01; 0,68) |

**Table 10.** Characteristics of prescribing physicians / PIM connection

| Characteristics | Statistics | Crude OR with 95% CI |
|---|---|---|
| Does the doctor attend some forms of continuous medical education regarding rational use of drugs? | U=1288.0 Z=0.000 p=1.00 | 1.22 |
| Does the doctor have visits from pharmaceutical companies? | U=1099.5 Z= 1.517 p=0.129 | 1,35 (0.85; 2.15) |
| Does the doctor use guides and other professional literature when determining therapy? | U=1288.0 Z=0.0 p=1.00 | 2,21 |
| Number of patients examined by a doctor during one working day | U=795.0 Z= - 3.501 p<0.01 | 0,83 (0.74; 0.93) |

**Table 11.** Characteristics of prescribing physicians / PPO connection

| Characteristics | Statistics | Crude OR |
|---|---|---|
| Does the doctor attend some forms of continuous medical education in the field of drug use? | p=1,00 | 0,218 |
| Does the doctor have visits by representatives of pharmaceutical houses? | U=822.5 Z= - 0,039 p=0,049 | 2,14 (0,62; 4,08) |
| Does the doctor use guides and other professional literature when determining therapy? | p=1,00 | 0,218 |
| Number of patients examined by a doctor during one working day | U=766.5 Z= - 0,520 p=0,603 | 1,04 (0,93; 1,18) |

## DISCUSSION

Medication therapy for elderly patients is confronted by major challenges due to the frequent occurrence of comorbidity, polypharmacy, decreased liver and kidney function, greater susceptibility to drug adverse events, and more frequent occurrence of complications (4,5). Compliance with the recommendations given in the pharmacotherapy guides can help to counter the effects of inadequate drug prescribing, reducing the risk of adverse reactions or potential drug interactions, shortening the time of treatment and improving therapeutic outcomes. In order to provide the best therapy and achieve the best possible outcome for a patient, STOPP/START criteria have been formulated (3,4,6,7). The knowledge about factors associated with the wrong choice of therapy in the elderly population allows planning and introducing appropriate corrective measures to improve the quality of drug prescription, which may lead to the significant improvement in the health and economic outcome of the treatment (5,8). Based on the above mentioned, we conclude that there are a number of factors that are directly or indirectly related to inadequate use of drugs. In order to prevent the occurrence of potential prescribing errors, which would significantly contribute to the improvement of the quality of life of elderly patients, there is a need for their control. Researchers from Serbia have been observing the problem of prescribing potentially inappropriate drugs to elderly patients in primary and secondary health care (9-12). Our study showed that mistakes are significantly lower at the secondary health care level than at the primary health care level. In addition to the occurrence in a small percentage, mistakes have also been characterized by low variability - smaller distribution by organ systems in hospital-treated patients, which was expected. Although patients with multiple associated diseases were in worse health condition and using larger number of drugs in their chronic therapy, doctors took more care of the drugs they administered. Prescription of inappropriate drug (PIM) was recorded three times more often than the absence of a clinically indicated drug (PPO).

Evaluating PIM as an outcome of interest, it has been found that its occurrence is more common in women than in men. It was estimated that the chance for its occurrence in the female population is 3 times higher than in males. Simultaneous use of more drugs, polypharmacy, was recognized as the most important risk factor associated with PIM (9-13). Many studies indicate the importance and need for reducing polypharmacy in order to avoid unwanted effects and interactions of drugs (9-13). Considering the overall health of elderly patients and the number of comorbidities requiring treatment, the simultaneous use of a large number of drugs becomes inevitable. As it has been proven in this study, the use of a large number of drugs in chronic therapy is strongly associated with the occurrence of PIM in the observed population. By using the aforementioned adequacy indicators, the risk of such outcome could be reduced in many ways. In addition to using different mechanism of action drugs in the therapy, patients often resort to self-drug administration without informing their physician, which results in drug interactions and/or adverse effect occurrence. By increasing the prescription control and using good clinical practice guides, these unwanted consequences could be minimized. The likelihood of missing the necessary drug in therapy, due to doctor's attempts to avoid polypharmacy in such patients, is even 4 times higher than in patients with smaller comorbidity. Elderly very often come out of doctors' clinics without fully understood therapeutic guidelines, forgetting to take drugs or duplicate them. They are self-initiated by taking new or discontinuing the use of drugs according to the current subjective feeling. Persons with this kind of tendency should be supervised by family or professional persons (14). Polypharmacy was detected as a predisposing factor for PIM in this study, which was defined as the use of 5-8 drugs in a daily therapy. These patients have a triple chance of getting a drug that is not appropriate for their health - PIM. The same findings were obtained by researchers from Kragujevac and Dublin during the examination of the problem of prescribing potentially inappropriate drugs in the elderly population, at the level of primary health care level (12,15). In the study conducted in Kragujevac, it was found that the simultaneous application of more than 9 drugs is one of the predisposing factors. Contrary to the results of this study, they found that PIM in elderly patients is associated with poor life habits of patients and frequent visits by representatives of pharmaceutical companies (12). Our data are in accordance with the results of the study conducted in England, which claims that physicians who more often encounter pharmaceutical representatives often unnecessarily prescribe drugs without clear clinical indication. The reason for this is that the doctors have been influenced by pharmaceutical representatives, blindly accepting their claims (16). Contrary to that, our study showed that visits of pharmaceutical representatives have been the risk factor for the drug absence in prescribing a clinically indicated drug (PPO). There was a slight difference in risk factors between the levels of health care. B. Hill Taylor et al. analyzed the results of 13 relevant clinical studies that examined the application of the STOPP/START criteria and their outcomes, and found that the results of this study largely agree with the findings obtained in other studies (17). They also found that female gender is one of the predictors of prescribing potentially inappropriate drugs in the older population. Most important factors that increase the risk of PIM and PPO as outcomes, in most studies, as in this study are: age (> 75 and 85 years), sex (females), polymedication and comorbidities (17). In a study conducted in nursing homes in New Zealand, it has been demonstrated that the likelihood of PIM occurring is higher in patients with higher and middle risk of falls than those with low risk (18). What has not been done in this study and which is important for discovering the source of the observed problem is the detection of protective factors, which researchers in Kragujevac pointed out in their study (12). At the level of secondary health care, it was noted that errors were distributed in the area of a few organ systems and the same drugs were generally wrongly prescribed. The results of this study generally agree with the results of other studies. In most cases, benzodiazepines should be avoided as

they predispose falls in older patients and the patients should avoid their use for more than a month (8-15,17-20). The use of aspirin often occurs in literature without a clear indication, without the presence of cerebrovascular, cardiovascular or peripheral vascular complications (8,9,13). NSAIDs are found as the most common drugs that were prescribed without a clear indication to the patients of this study, which are very common detected as unduly duplicated therapy. Researchers from Ireland came to the same results (13,15,19). The use of non-steroidal anti-inflammatory drugs in patients with severe essential hypertension is one of the STOPP criteria; however, a trend of their frequent prescription among primary care physicians was observed, followed by theophylline treatment as monotherapy in the treatment of HOBP and proton pump inhibitors in maximal doses longer than 8 weeks, while at the level of secondary health care - in this study, no case was reported (9,12,13,15).

Observing PPOs as outcomes of interest, it has been found that their occurrence is slightly more common in women than in men. In patients who have been hospitalized at least once, the probability of missing clinically indicated drug (PPO) is surprisingly high. Factors that increase the risk of the aforementioned outcome are found to be frequent visits by representatives of pharmaceutical companies (minimum once a month) and comorbidities. Researchers in a study conducted in Serbia in 2014, found that the likelihood of PPO occurring in older patients was increased by their age and comorbidities (9). However, the difference is that they have defined and isolated diseases that are directly related to the observed outcome (DM, osteoporosis, myocardial infarction, HOBP, stroke or angina pectoris), while in this study this has not been performed. Trend of falling under the influence of promotional material and blind belief in the propaganda of pharmaceutical houses was noticed among doctors. They are prone to prescribe drug for personal benefit or pure curiosity about the functioning of new drugs on the market (14).

Most often omitted drugs in literature were antimicrobial drugs and statins in patients with a positive history of cerebral, coronary and peripheral vascular events, as well as vitamin D and calcium supplements in osteoporosis and/or previous fracture and/or T bone density index > -2.5; measured in several places. Most of the studies highlight the same criteria for PPO (12,13,15). In addition, in 15.5% of the study participants in Kragujevac, the use of an ant aggregation drug (aspirin, clopidogrel) in the presence of cerebral, coronary and peripheral vascular events in patients with normal heart rhythm has been omitted (12). Another START criteria that was not detected in this study and is identified in primary health care is the absence of warfarin therapy in patients with chronic atrial fibrillation (13). Researchers found that the probability of missing the necessary drug in therapy is lower in patients who are treated in smaller health institutions and who visit specialist physicians more often. They also concluded that this risk is lower in those patients who have heavier illnesses (18). At the level of secondary health care, it was found that in most cases the errors were 1 PIM and 1 PPO, while at the same time the presence of 2 or more omitted drugs was considerably lower. Results of PPOs found in a study conducted in 2012 do not differ from those found in our study (benzodiazepines, aspirin, NSAIDs, etc.) However, researchers have determined diseases that might be risk factors for the occurrence of PIP: diabetes mellitus type 2, atrial fibrillation, osteoporosis, COPD and ischemic heart disease (20).

This study has several limitations. Primarily, it was conducted in one hospital, mirroring the characteristics of the functioning of the health system of the only one environment, so its results cannot be absolutely generalized. Also, in order to get a more realistic picture of the correctness and rationality of prescribing drugs in hospital conditions, it is necessary to conduct the research on a larger sample of respondents. For the same purpose, a quantitative assessment should also be carried out by a qualitative (e.g. method-based theory) in order to discover the reasons for such prescribing and the factors contributing to it. In this way, we could work on the plan of solving and minimizing inadequate prescription. Although measures have been taken to reduce bias to a minimum, there is still the possibility of information bias. Given that the questionnaires were compiled on the basis of relevant medical literature sources, that the questions for all participants were the same, that they were anonymous, with each respondent having their own identification code, their attendance is expected to be minimal. All data were obtained from the hospital medical records and other relevant sources, so any mistake in them could have influenced the conclusions made in this study.

## CONCLUSION

Detection of mistakes in drug prescribing and determination of their prevalence and/or sources in a particular environment represents an important step in the drug use rationalization. This would reduce the occurrence of drug adverse effects and their interactions, thus preserving the fragile health of geriatric patients. In order to achieve the desired goal, it is necessary to improve recognition of the drugs to be avoided in the elderly population, based on their adverse effects and drug interactions. The use of STOPP/START criteria appeared to be effective in the detection of PIM/PPO at the secondary health care level. Attention should be directed to patients at the highest risk of PIM or PPO. They are persons prone to falls, vascular diseases and osteoporosis, have a number of associated chronic diseases and consequently they take more drugs daily. Physicians should pay more attention to each patient individually. They should be encouraged to make the conscientious decisions that are in line with clinical evidence, avoiding to believe blindly to the pharmaceutical representatives. It is important to reduce unnecessary polypharmacy. Overall, functioning of the health care system should be improved, so that doctors can make decisions in the optimal working conditions. In order to achieve abovementioned, it is necessary to work on education of both patients and doctors.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the ethicalstandards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. Voluntary written and informed consent was obtained from each participant prior toenrollment in the study

## CONFLICT OF INTEREST

There are no conflicts of interest.


## FUNDING

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ORIGINAL SCIENTIFIC ARTICLE

# SELF-MEDICATION WITH ANTIBIOTICS AMONG NURSING STUDENTS IN SERBIA: PILOT STUDY

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## ABSTRACT

*Healthcare professionals should serve as promoters of rational antibiotic use in attempt to decrease antibiotics misuse within the process of self-medication. Current pilot study was undertaken with the aim to identify potential predictors of self-medication with antibiotics (SMA) and to describe SMA practice among nursing students in Serbia. Data have been collected during the period March-May, 2016 in Medical school of bachelor degree, in Belgrade. Self-reported questionnaire served as a data source. Descriptive statistic was used to analyse study sample characteristics. Chi-square test was used to test differences between groups. Study sample have included 138 participants. Almost half of them, 43.8%, practice SMA. Life style (smoking, alcohol consumption, sleeping habits and physical activity) and socio-demographic characteristics (excepted school grade) were not shown as SMA determinants. Time & money savings were stated as the most frequent reason for SMA, while common cold, sore throat and cough were the most common conditions cured through SMA. Pharmacists' recommendations and previous positive experience were specified as the most important in the process of antibiotics selection, indicated by 50.0% and 37.5% participants, respectively. Amoxicillin was the most frequently used antibiotic in SMA, used by 50% of participants who practice SMA. High proportion of SMA and observed practice among nursing students in Serbia call for efforts with regards to relevant education about rational antibiotic use, actual clinical guidelines and potential consequences of misuse.*

***Keywords***: *Self-medication, antibiotics, predictors, nursing students.*

## INTRODUCTION

The World Health Organization (WHO) has defined drug misuse as the use of a substance for the purpose that is not consistent with legal or medical guidelines and has suggested prescription drug use in self-medication as a typical example of drug misuse (1). Such misuse brings over use and non-rational use of antimicrobials which are among the main reasons for development of antimicrobial resistance recognized as one of the highest threats to individual and public health. The WHO reported alarming levels of resistance to antimicrobials presented in humans, animals, food and environment (2). Moreover, the resistance is of high spreading potential and, accordingly, became a global concern. Therefore, coordinated actions have been purposed to minimize the emergence and spreading, while isolated interventions have limited impact, but still are very important (2, 3).

The prevalence of self-medication with antibiotics (SMA) is very different over the world, being the most studied in WHO Southeast Asian Region and Africa. Previously published systematic review studies and meta-analysis have reported the range of SMA from 1% to 100% with overall prevalence between 38.8% and 42.6% (4-6). Studies in Europe have reported the highest prevalence rates for actual SMA in eastern, followed by southern Europe, while the lowest rates were observed in northern and Western Europe (7, 8). Young population was recognized as particularly endangered since overall 50% of adolescents and 96% of students population use to take drugs without consulting a physician, where antibiotics were among the most frequently used drugs (9, 10). Studies among healthcare students' population have shown overall trend of SMA between 39% in Sri Lanka (11), and 66.9% in India (12). There are also reports on differences in SMA practice between medical and non-medical students, but the results are not consistent. While significantly higher prevalence of SMA among medical vs. non-medical students was found in Jordan, 55% vs. 33%, respectively (10), it was not the case in Libya, 43% and 46%, respectively (13). In line with high prevalence of SMA among healthcare students, and their upcoming professional roles and responsibilities related to rational antibiotics use, it is of particular importance to increase the awareness of antibiotics' misuse consequences and, accordingly, decrease the SMA practice.

Among individual determinants of SMA, level of education, age, gender, past successful use, severity of illness and income were reported (5). Additionally, storing antibiotics at home, poor access to healthcare, and SMA intention were also recorded as predictors of SMA (14). Dispensing antibiotics in whole packages and lack of relevant medicine regulations were revealed as healthcare system related determinants (14). Interestingly, healthcare professionals were recognized as contributors to SMA practice when serving for demanding and socially vulnerable patients (14). Revealing of prevalence and determinants of SMA is very useful in creation of well-targeted interventions and prevention of such addictive practice with serious consequences for individual and public health.

Current pilot study was done with the aim to identify practice and predictors of SMA among nursing students in Serbia.

## PATIENTS AND METHODS

This was cross-sectional study. Data have been collected during the period March-May, 2016. Study participants were nursing students of bachelor degree, enrolled at of Medical school of bachelor studies in Belgrade, Serbia. The study was carried out after the study protocol was approved by the school management team. All participants have received detailed information related to study aims and study protocol. Participation in the study was anonymous and voluntary.

Self-reported questionnaire specially designed in line with the aims of the study was used as a data source. Socio-demographic (gender, school grade, weekly allowance, living conditions) and life style characteristics (smoking, alcohol consumption, sleeping habits, physical activity) have been collected along with data related to SMA practice (reasons for SMA, conditions threated by SMA, antibiotic selection and dose related items, side effects, type of antibiotics used within SMA, attitudes related to SMA). SMA was presented to the study participants as use of antibiotics without doctors' prescription, i.e. buying antibiotics in pharmacy without doctors' prescription, use antibiotics from home pharmacy, use antibiotics of somebody else, etc.

### Statistical analysis

Descriptive statistic was used to analyse study sample characteristics. Chi-square test analysis was used to test potential differences between two groups, those who did and who did not practice SMA, with regards to socio-demographic and life style characteristics.

Statistical significance in all analyses was deemed likely if the computed probability value was $<0.05$. Data analysis was performed by using Statistical Package for Social Sciences (SPSS) software (SPSS 18.0 for Windows, SPSS Inc., Chicago, IL, USA).

## RESULTS

There were 138 nursing students who participated in the study. Although 84.5% of participants have indicated that SMA is not acceptable practice, almost half of them, 43.8%, stated that practice SMA. There were no statistical differences between those who did and did not practice SMA with regards to socio-demographic characteristics, excepted school grade (Table 1).

**Table 1**. Students' socio-demographic characteristics with regards to self-medication practice (N=138)

| Variable | Total | SMA users, % | SMA nonusers, % | p-value |
|---|---|---|---|---|
| **Gender** | | | | |
| Male | 8.7 | 33.3 | 66.7 | >0.05 |
| Female | 91.3 | 44.8 | 55.2 | |
| **School grade** | | | | |
| The first, the second | 60.1 | 34.1 | 65.9 | <0.05 |
| The third, the forth | 39.9 | 58.2 | 41.8 | |
| **Weekly allowance** | | | | |
| ≤ 2500 RSD | 70.1 | 43.0 | 57.0 | >0.05 |
| > 2500 RSD | 29.9 | 42.5 | 57.5 | |
| **Live with …** | | | | |
| Parents | 32.6 | 36.4 | 63.6 | >0.05 |
| Friends, cousins, in marriage | 50.0 | 46.4 | 53.6 | |
| Alone | 17.4 | 50.0 | 50.0 | |

Abbreviation: SMA-self-medication with antibiotics

Life style characteristics (smoking, alcohol consumption, sleeping habits and physical activity) were not shown as determinants of SMA (Table 2).

**Table 2.** Students' life style characteristics with regards to self-medication practice (N=138)

| Variable | Total | SMA users, % | SMA nonusers, % | p-value |
|---|---|---|---|---|
| **Smoking** | | | | |
| Yes | 34.8 | 42.6 | 57.4 | >0.05 |
| No | 65.2 | 44.4 | 55.6 | |
| **Number of cigarettes a day** | | | | |
| ≤ 10 | 66.7 | 51.7 | 48.3 | >0.05 |
| > 10 | 33.3 | 33.3 | 66.7 | |
| **Alcohol consumption** | | | | |
| No | 58.4 | 50.0 | 50.0 | >0.05 |
| Yes | 41.6 | 35.7 | 64.3 | |
| **Sleeping per night** | | | | |
| < 8 h | 52.9 | 51.4 | 48.6 | >0.05 |
| ≥ 8 h | 47.1 | 35.4 | 64.6 | |
| **Frequency of physical activity** | | | | |
| ≥ 2 times a week | 50.7 | 46.4 | 53.6 | >0.05 |
| < 1 times a week | 49.3 | 39.4 | 60.6 | |

Abbreviation: SMA-self-medication with antibiotics

Participants have stated time and money savings as the most frequent reason for SMA. Common cold, sore throat and cough were indicated as the most common conditions treated by SMA. Pharmacists' recommendation and previous positive experience were specified as the most important in the process of antibiotics selection, indicated by 50.0% and 37.5% participants, respectively. Additionally, majority of participants, 76%, stated that they consider antibiotic indication in the process of antibiotic selection. Patient information leaflet (PIL), pharmacists and physicians were mostly used sources of information related to relevant antibiotic dose and dosing regimen. However, even one fifth of the SMA users, 21.6%, stated that only partially understand PIL information (Table 3).

**Table 3.** Practice of self-medication with antibiotics among nursing students (N=60)

| Variable | Total |
|---|---|
| **Reason for SMA** | |
| Time & money savings | 91.4 |
| Other | 8.6 |
| **Conditions threated by SMA** (multiple options available) | |
| Common cold | 52.1 |
| Sore throat | 52.1 |
| Cough | 37.0 |
| High temperature/Fever | 26.0 |
| Nasal congestion | 23.3 |
| Headache | 19.2 |
| Toothache | 15.1 |
| Vomiting / diarrhoea | 11.0 |
| Stomach pain | 9.6 |
| Skeen Injuries/wounds | 4.1 |
| Eye infection | 2.7 |
| Other | 2.2 |
| **Antibiotics for self-medication are most often purchased according to ...** (multiple options available) | |
| Pharmacist' recommendation | 50.0 |
| Previous positive experience | 37.5 |
| Family member recommendation | 25.0 |
| Previous physician proscription | 28.2 |
| Friends' recommendation | 2.8 |
| Positive experiences announces at forums/internet | 0.0 |
| **What do you consider when purchasing antibiotic?** (multiple options available) | |
| Antibiotic indication | 76.7 |
| Type of antibiotic | 45.6 |
| Antibiotic price | 17.8 |
| Profile of adverse reactions | 17.8 |
| Antibiotic producer | 6.7 |
| Other | 28.2 |
| **Antibiotic dose and dosage regime selection** (multiple options available) | |
| By the patient information leaflet | 44.9 |
| Consultation with pharmacist | 43.6 |
| Consultation with physician | 35.9 |
| Previous experience | 16.7 |
| Consultation with family/friends | 7.7 |
| Journals/books | 2.6 |
| Alone | 2.6 |
| Internet | 0.0 |
| **Experience of side effects during SMA** | |
| Yes | 5.4 |
| No | 94.6 |
| **The main source of antibiotics** | |
| State/city owned pharmacy pharmacy | 44.6 |
| Private owned pharmacy | 33.9 |
| Home pharmacy (the rest from the previous use) | 7.4 |
| From friends | 0.8 |
| **Do you read antibiotic patient information leaflet?** | |
| Yes, always | 41.8 |
| Yes, occasionally | 52.2 |
| No | 6.0 |
| **How much do you understand patient information leaflet?** | |

| Variable | Total |
|---|---|
| I understand completely | 77.6 |
| I understand partially | 21.6 |
| I don't understand | 0.8 |

Abbreviation: SMA-self-medication with antibiotics

Among antibiotics which were used in SMA, amoxicillin dominated. Even 50% of participants who practice SME reported usage of amoxicillin (Table 4). The list of antibiotics used in SMA also contains antibiotics which are recommended as alternative therapy in treatment of some conditions, like amoxicillin/clavulanic acid and azithromycin.

**Table 4.** The list of antibiotics used in self-medication (N=60)

| ATC code | INN | Percentage, % |
|---|---|---|
| J01AA02 | Doxycycline | 3.3 |
| J01CA01 | Ampicillin | 3.3 |
| J01CA04 | Amoxicillin | 50.0 |
| J01CR02 | Amoxicillin/clavulanic acid | 8.3 |
| J01DB01 | Cephalexin | 13.3 |
| J01EE01 | Trimethoprim/Sulfamethoxazole | 1.7 |
| J01FA10 | Azithromycin | 8.3 |
| J01FA01 | Erythromycin | 5.0 |
| J01XD01 | Metronidazole | 1.7 |
| J01XX01 | Fosfomycin | 1.7 |
| S01AA01 | Chloramphenicol | 1.7 |

Abbreviations: ATC- anatomical-therapeutic-chemical; INN- International Nonproprietary Name

## DISCUSSION

This is one of the first studies which investigated SMA among students population in Serbia. The results are in line with previously published study among Serbian population where SMA has been studied through obtaining an in house inventory of drugs. Amoxicillin was confirmed as the most commonly used antibiotic for self-medication in both studies, while similar indications (common cold, cough) were reported as most commonly treated with SMA (15). However, although antibiotics were encountered in 49.1% of households, only a quarter of packages were used for self-medication, purchased at pharmacy without prescription, 20.65%, or obtained through friends or family member, 6.52%. This indicated lower prevalence of SMA among general population than among medical students in Serbia, 43.8% (15).

Amoxicillin was revealed as most commonly used antibiotic in SMA not only in Serbia, but also wider (16, 17). Such practice could be described as expected since the amoxicillin is recommended as the first line therapy for many infective conditions (18). However, high usage of antibiotics as amoxicillin in combination with clavulanic acid and azithromycin in self-medication is of particular concern. Amoxicillin in combination with clavulanic acid is recommended in some cases (i.e. Sinusitis Acuta and Pharyngitis Acuta in children) as alternative therapy after the first line therapy does not improve health outcomes. Concurrently, it is recommended caution in usage of azithromycin in Serbia, because of recorded increased resistance to Streptococcus Pneumoniae (18). Accordingly, along with high prevalence of SMA practice among nursing students, types of antibiotics recorded in self-medication practice are of additional worry. Accurate education about rational antibiotic use, compliance with guidelines and recommendations related to the first and alternative antibiotics selection have to be the imperative in attempts against the antibiotic resistance.

SMA practice among nursing students was recorded as more prevalent in males 49 (62%), while satisfaction with previous antibiotic use, saving time and money and advises received in drug stores were recognized in the literature as factors with the most influence to starting SMA (19-21). However, more data are necessary on SMA contributing factor in order to create well targeted public health actions to decrease such undesirable practice.

Previous studies reported lack of awareness of the negative implications of self-medication among nursing students (22). Moreover, it was revealed that 50% of nursing students were not familiar with the term „antibiotic resistance“ (17). Soroush at al. suggested that having a relative awareness

about various diseases and medications, which is sometimes associated with taking a few educational courses with an internship, creates a false confidence in nursing student for self-medication and suggesting drugs to others (23). Accordingly, it was suggested as beneficial if the education system and associated tutors could inform the students about the possible consequences of this issue (22).

Pharmacies have been confirmed as the most used sources of antibiotics within the process of SMA, while pharmacists' recommendations were observed among the most important for antibiotic selection. Accordingly, continuing education of pharmacy personal with regards to accurate recommendation about rational antibiotic use and actual data about antibiotic resistance and its' consequences is of very high importance. Additionally, inclusion of well-educated pharmacists in public health actions aimed to decrease of SMA practice could have very good impact at such actions success outcomes.

This is the pilot study which reported results on SMA practice within relatively small study sample that could be considered as limitation. Study with representative sample of nursing students for the whole county is recommended for more relevant and accurate results. This study has been conducted in March-May, 2016, at the beginning of the national campaign directed to increase of rational antibiotic use in Serbia. It will be of particular importance to conduct similar study in the up-coming period, after the National guideline of good clinical practice for the rational antibiotic use is issued, as well as after the national program for bacterial resistance control is adopted by the Serbian government (24).

## CONCLUSION

Accurate education of nursing students about rational antibiotic use in line with clinical guidelines recommendations and misuse consequences have to be the imperative in attempts to decrease SMA. However, more data are necessary on SMA contributing factor in order to create well targeted public health actions to decrease such undesirable practice among nursing students. Additionally, continuing education of pharmacy personal with regards to accurate recommendation, antibiotic resistance and its' consequences is of very high importance. Inclusion of well-educated pharmacists in public health actions aimed to decrease SMA practice could have very desirable impact at such actions outcomes.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

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

## CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.


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ORIGINAL SCIENTIFIC ARTICLE

# TREATMENT OPTIONS IN PATIENTS SUFFERING FROM HEMOLYTIC-UREMIC SYNDROME: THE SERBIAN MILITARY MEDICAL ACADEMY EXPERIENCE

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## ABSTRACT

*Hemolytic-Uremic Syndrome (HUS) is a clinical syndrome with a triad of non-immune Microangiopathic Hemolytic Anemia (MAHA), thrombocytopenia and renal failure. Together with the Thrombotic Thrombocytopenic Purpura (TTP), it belongs to a group of diseases characterized as the Thrombotic Microangiopathy (TMA), which represents a microvascular occlusive disorder with the formation of a predominantly thrombocytic thrombus in the renal and/or systemic circulation. In the period starting from 2001 to 2017, 14 patients with a HUS were diagnosed at the Clinic for Nephrology (unfortunately ADAMTS 13 could not have been done due to technical reasons). In a retrospective clinical laboratory analysis and monitoring, we obtained the following results. Out of 14 patients, 10 were female (or 71.43%) and 4 were male (28.57%), the youngest patient was aged 17 and the oldest one 78, the average age of our patients was 55.33 years, the annual number of patients with the diagnosis of HUS was 0.93 patients per year, or 0.00116 in relation to the total number of patients treated. After monitoring the patients individually for the period ranging from 1 to 14 years, a stable remission was achieved in 5 patients, while a chronic renal insufficiency occurred in 3 patients. In two of our patients, a percutaneous kidney biopsy was performed with pathohistological findings described in references. Having done this retrospective analysis, we can conclude that the survival and complications of this rare, but serious disease correspond to the available world data.*

***Keywords:*** *Hemolytic-uremic syndrome, atypical hemolytic-uremic syndrome, C3, corticosteroids, plasmapheresi.*

## INTRODUCTION

Hemolytic-Uremic Syndrome (HUS) is a clinical syndrome with a triad of non-immune Microangiopathic Hemolytic Anemia (MAHA), thrombocytopenia and renal failure. Together with the Thrombotic Thrombocytopenic Purpura (TTP), it belongs to a group of diseases characterized as the Thrombotic Microangiopathy (TMA), which represents a microvascular occlusive disorder with the formation of a predominantly thrombocytic thrombus in the renal and/or systemic circulation. According to the International Society on Thrombosis and Hemostasis (ISTH) Guidance published in 2005, this term is used for all diseases or conditions with the hemolytic anemia (with schistocyte formation) and consumptive thrombocytopenia (1). The TMA was first mentioned in references named as the Moschcowitz syndrome after Dr Eli Moschcowitz, who first described such a case at a hospital in New York in 1925. The term HUS dates back to 1955, when Dr Conrad von Gasser et al. published a case describing five children with the hemolytic anemia, thrombocytopenia and dominant acute renal insufficiency (2). Karmali first established the relationship between the HUS and E. coli infection in 1985 (3, 4). Nowadays, many years later, the HUS is still defined as a clinical triad, but with significant advances in clarifying the etiology and pathophysiology of this relatively rare disease. D+ HUS, a typical type of HUS or Stx-HUS etiopathogenesis occurs about a week after the episode of bloody diarrhea caused by the enterohemorrhagic E. coli infection, most commonly by serotype 0157: H7 (70%). Other E. coli serotypes (O111: H8, O103: H2, O121, O145, O26 and O113), Shygella dysenteriae serotype 1 and rarely other enteral bacteria can also be the cause of infection (5-8) D−HUS, an atypical type of HUS or non-Stx-HUS etiopathogenesis accounts for only 5 to 10% of all cases. In 1975, Kaplan et al. observed for the very first time that there were two types of the atypical HUS, sporadic and familial, which were characterized by a reduced level of serum C3 protein, the central component of the human complement system. Genetic studies have shown that mutations in the genes encoding the complement regulatory proteins predispose the development of this type of disease. The following factors are important for the development of the disease: factor H, factor I and Membrane Cofactor Protein (MCP). So far, more than 50 mutations have been described for the Complement Factor H (CFH), a plasma protein that inhibits the activation of the alternative complement pathway by the inactivation of C3 convertase. The familial type of HUS makes up for less than 3% of all HUS cases, and occurs as a consequence of a hereditary defect or a complement factor H (CFH) defect. Thompson and Winterborn were the first to describe this disease in 1981 in patients with very low levels of C3 protein in plasma and a decreased CFH level. The disease can be inherited autosomically recessively and autosomically dominantly. The recessive inheritance is combined with a low level of CFH, which can range from 10 to 50% of normal concentration, a reduced C3 complement component, and the development of HUS in early childhood. The prognosis is poor with a mortality rate ranging from 60 to 70%. The autosomal dominant type of the disease is associated with a functionally abnormal CFH level, a normal C3 level, followed by a development of HUS in special conditions such as, for example, an infection or pregnancy. Moreover, there is one more type of HUS, the post-transplantation HUS (pHUS), which represents a known and serious complication that can arise after the transplantation of solid organs (kidney, heart, lung, liver, pancreas, small intestine) and hematopoietic stem cells. It is clinically manifested most commonly with the sudden onset of hemolytic anemia, thrombocytopenia and the development of renal insufficiency (15, 16).

## PATIENTS AND METHODS

In the period from 2001 to 2017, 14 patients with aHUS were diagnosed at the Clinic for Nephrology (unfortunately ADAMTS 13 could not have been done due to technical reasons). The research was carried out by the means of retrospective analysis and monitoring.

## RESULTS

In a retrospective clinical laboratory analysis and monitoring, we obtained the following results. Out of 14 patients, 10 were female (or 71.43%) and 4 were male (28.57%); the youngest patient was aged 17 and the oldest one 78, the average age of our patients was 55.33 years. The total number of patients treated for all diagnoses at the MMA Clinic for Nephrology from 2001 to 2016 was 12,025, with the average of 858.92 patients per year. The annual number of patients with the diagnosis of HUS was 0.93 patients per year, or 0.00116 in relation to the total number of patients treated. Out of 14 patients, 9 patients were O Rh(D) positive, and the rest were A Rh(D) positive blood group. At the time of diagnosis of HUS, the mean serum creatinine levels in these patients were 321.78 umol/l, the mean hemoglobin was 81.83 g/l, while the mean platelet count was 53.49. Hemoglobin levels were reduced in all monitored patients (100%), out of whom 28.57% had hemoglobin levels below 80 g/l at admission. Serum albumin was slightly decreased in 71.43% of the monitored patients. Only in 5 patients, the value ranged from 21-30 mmol/l, (35.17% of cases), while only one of the monitored 14 patients had indirect bilirubin levels in reference values (0.07%) (Table 1).

**Table 1.** The laboratory parameters in our patients

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **SE** | >100 | 7 | >100 | >100 | 60 | 85 | 71 | 88 | >100 | >100 | >100 | >100 | >100 | >100 |
| **CRP** | 1.2 | 1.7 | 1.15 | 24.6 | 1.8 | 48.7 | 188 | 24.8 | 1.5 | 45.8 | 1.52 | 1.8 | 5.86 | 128 |
| **Leu** | 9.8 | 9.11 | 10.2 | 5.07 | 8.9 | 22 | 7.5 | 18.4 | 9.9 | 16 | 11 | 11.1 | 9.9 | 12.8 |
| **Eri** | 3.32 | 2.98 | 3.6 | 2.72 | 3.12 | 2.24 | 2.52 | 2.59 | 2.9 | 2.29 | 2.99 | 2.68 | 2.88 | 2.7 |
| **Hgb** | 98 | 92 | 96 | 92.5 | 87 | 61 | 81.9 | 81 | 88 | 56.4 | 82.4 | 82.4 | 76 | 71 |
| **Tro** | 8 | 69 | 58 | 62.8 | 67 | 17 | 87 | 88 | 67 | 9.9 | 28 | 89 | 91.1 | 7 |
| **sCre** | 286 | 159 | 229 | 190 | 547 | 248 | 884 | 128 | 289 | 150 | 821 | 115 | 458 | 286 |
| **Alb** | 33 | 34 | 34 | 31 | 28 | 28 | 29 | 34 | 31 | 25 | 31 | 35 | 25 | 21 |
| **Bil/ dir** | 128/12 | 108/12 | 89/17 | 91/17 | 11 | 20/6 | 28/4 | 48/17 | 29/11 | 25/7 | 25/8 | 48/12 | 27/8 | 49/9 |
| **Coom.t** | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| **LDH** | 8462 | 8244 | 2548 | 6041 | 884 | 1151 | 2086 | 1154 | 2281 | 1868 | 461 | 984 | 1568 | 1218 |
| **AST** | 57 | 96 | 49 | 59 | 24 | 42 | 111 | 19 | 88 | 56 | 11 | 188 | 78 | 88 |
| **ALT** | 26 | 80 | 81 | 26 | 27 | 24 | 56 | 27 | 48 | 21 | 82 | 78 | 69 | 68 |
| **HPT** | 0.4 | 0.01 | 0.8 | 0.02 | 0.8 | 0.06 | 0.08 | 0.07 | 0.4 | 0.06 | 0.01 | 0.9 | 0.09 | 0.08 |
| **Imun** | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| **Tu** | - | - | - | - | - | - | - | - | - | + | +/- | - | - | +/- |
| **Frag** | rare | rare | 14% | 1% | rare | rare | 12-14% | much | 17% | rare | rare | 1-5% | 5% | 5% |
| **24h Pr urine** | 2.26 gr | b.o. | 1.3 gr | 550 mg | 1.8 gr | 7.53 gr | 330 mg | 547 mg | 2.8 gr | 6.8 gr | 13.2 gr | 342 mg | 7.9 gr | 8.2 gr |

**Note: pink colored fields indicate a pathological finding*

Serum haptoglobin levels were significantly decreased in all patients. Moreover, in 64.28% of patients the levels were barely measurable (0.00 ...). Lactate dehydrogenase was significantly increased in all patients, i.e. in 11 patients the value was higher than 1000 (78.57%), and in 3 patients levels were increased, but lower than 1000. The Coombs test was negative in all patients. C3 and C4 (17) levels were not measured in all patients (due to the lack of reagents, although they were initially required) and were not taken into account in monitoring our patients. In one patient (7.14% of total patients monitored) we observed significantly elevated tumor markers (CEA, CA 19-9, NSE), in two patients (14.28% of total patients monitored) we observed an elevated non-specific tumor marker (CEA) while in one patient, (7.14% of total patients monitored) we observed increased levels of immunological markers. Only in one patient (7.14%) the 24h proteinuria (Biuret method) was in reference values, in 5 patients (35.17%) the 24h proteinuria was nephrotic (or greater than 3.0gr/24h), while in 4 patients (28.57%) the 24h proteinuria was less than 1.0gr/24h (at admission the possibility of prior administration of SSP or sAlb was excluded, with unknown data concerning the 24h proteinuria, i.e. the presence of previously diagnosed glomerulopathy was excluded).

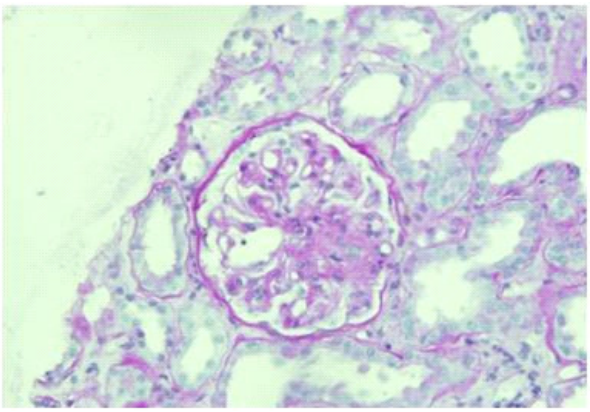
13 patients (86.6%) were treated by the SSP and therapeutic plasma exchange – plasmapheresis and 11 patients (78.57%) were treated by the therapeutic plasma exchange (during plasma treatment, the number of blood platelets, creatinine concentration and serum lactate dehydrogenase is determined daily, 26.) and corticosteroids, while in 7 patients (50%) the treatment was performed together with intermittent hemodialysis (HD) procedures as a supportive therapy. The average number of hemodialysis procedures performed in these patients was 8 (ranging from 3 to 18 HD procedures/patient, Table 2).

**Table 2.**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **TPI** | 20 | 13 | 11 | 4 | 0 | 6 | 2 | 88 | 23 | 2 | 31 | 18 | 5 | 4 |

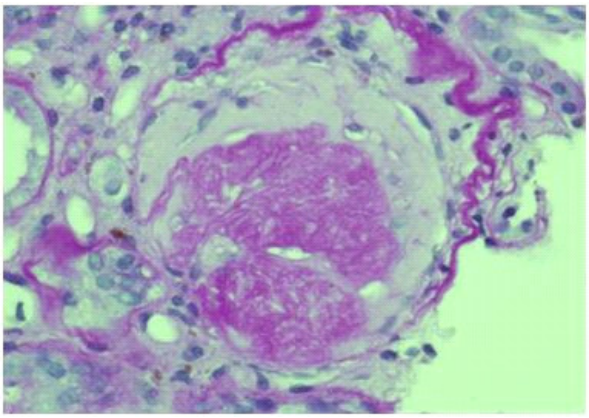
Three patients were treated by the TPI, a multi apheresis procedure at the MMA Institute of Blood Transfusion and Hemobiology, with the great help of our colleagues at the Institute. The decision to implement the TPI at the Institute of Blood Transfusion and Hemobiology was made due to a weaker response to the volume exchange performed at the Department for Hemodialysis, according to standard protocol. The efficacy was being monitored indirectly by tracking the increase in the platelet count, as well as the decrease in indirect bilirubin and LDH. The normalization of the tracked hematological parameters was achieved in the average period of 29.8 +/- 2 days. In one patient, after a consultation with a Hematologist, due to the refractory disease response to the applied therapeutic protocols combined with corticosteroids, Cyclophosphamide was administered (iv infusions every 15 days according to the creatinine clearance). In one female patient, due to the combined clinical picture of the TTP/HUS, we administered Vincristine together with corticosteroid therapy. In one female patient, due to the refractory disease response to the therapeutic plasma exchange, together with corticosteroids (88 TPI) and occasional multi apheresis procedures, Rituximab was administered at a dose of 375 mg/week according to standard protocol. Regardless of the procedures applied, there were no complications due to sepsis as a consequence of the temporary central venous catheter in any of our patients. So far, in the Republic of Serbia, Eculizumab has not been given for this diagnosis (18- 20). After monitoring the patients individually for a period ranging from 1 to 14 years, a stable remission was achieved in 5 patients (recovery without relapse, without diagnosing the SBVT and/or neoplastic processes of any etiology in the meanwhile), while a chronic renal insufficiency occurred in 3 patients (two patients with the MDRD above 40 ml/min/1.73 m2, and one patient with the MDRD 11.3 m/min/1.73 m2). A lethal outcome occurred in 6 out of 14 patients (42.86%). In 2 out of 6 patients, the fatal outcome occurred 96 hours after the admission to our Clinic, which could neither be associated with the hematologic parameters monitored (prevalently with thrombocytopenia), the time of hemodialysis commencement, the number of hemodialysis performed, nor with the number of therapeutic plasma exchanges done, but with the clinical findings of the life-threatening condition of the afore-mentioned patients at the admission. These patients were in deep somnolence/sopor, the cause of death was assumed to be the TMA, but of an unknown origin, due to the fact that the consent from their relatives for a clinical autopsy could not be obtained. In one female patient, despite our best efforts and the implementation of all treatment options (hemodialysis, 88 TPI, Rituximab), a lethal outcome occurred after 11 months, while in 3 out of those 6 patients, lethal outcomes occurred within a period of at least 24 months after discharge from our institution. The average life span of these patients was 74.8 years and there were no available data concerning the final diagnosis and cause of death. In two of our patients, a percutaneous kidney biopsy was performed with pathohistological findings described in references, with no complications in the post-biopsy recovery in terms of retroperitoneal bleeding and/or perirenal, i.e. intrarenal hematoma (see pictures 1 to 4, with a brief explanation with them, the pathohistological finding corresponds to the finding encountered at the HUS / TTP).

**Picture 1.**



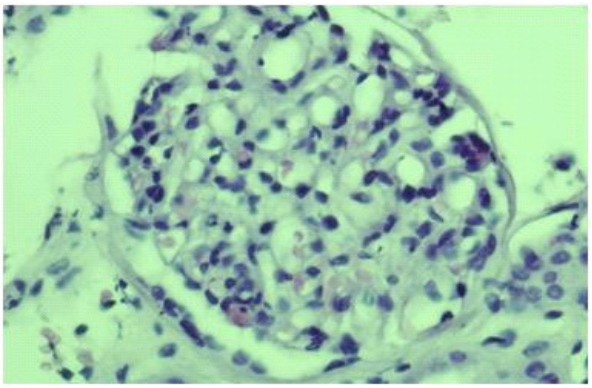
11139-15 HE 40x B.M., female,
hialini thrombus segments in the lumen
of the capillary

**Picture 2.**



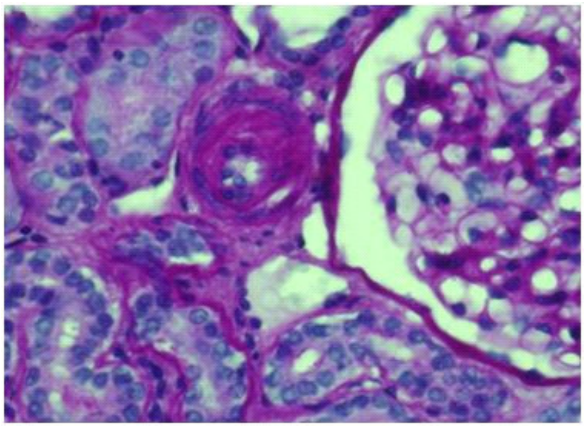
11139-15 PAS 40x B.M., female,
hyperplastic arteriolitis

**Picutre 3.**



8832-14 pas 40x Đ.L., female,
sclerotic glomerul in the form of vascular
obliteration

**Picture 4.**



8832-14 pas 20x Đ.L., female,
segmental glomerulosclerosis in the field
of ischemic lesion

## DISCUSSION

In our patients, who were monitored from 2001 to 2017, thrombocytopenia, i.e. the platelet count, could not be linked to the severity of the clinical presentation of these diseases, which coincides with the available reference data (24, 25). The number of platelets at admission could not be linked to the total number of TPIs applied individually in patients. Also, only one patient had the 24h proteinuria in reference range; while 5 patients had a nephrotic 24h proteinuria, with no prior knowledge of having a glomerular disease that could be associated with the severity of the glomerular damage. Moreover, while monitoring our patients, we observed that the maintenance of nephrotic range proteinuria for more than 3 months significantly affected the occurrence of chronic renal disease and reduced survival rate of such patients. The administration of corticosteroid therapy reduced chronic complications and significantly reduced the period of normalization of characteristic hematological parameters in these patients (an average of 29.8 days), which correlated with the data available in references (data from multicenter, non-randomized studies, with a small number of patients treated with *Eculizumab,* cumulative observation period of 95 months, 3 of the 10 patients experienced relapse within 6 weeks of discontinuation, but then immediately resumed treatment and completely recovered, but only 10 patients were observed ) (21-24). Determining the ADAMTS 13 would significantly help us assess the course of the disease and expected survival rate of these patients, as well as assess the possible necessity of applying a "more aggressive" treatment with monoclonal antibodies, currently unavailable in our institution. The possibility of opening a reference center for treating these patients in institutions that can provide a multidisciplinary approach and the TPI, a multi apheresis procedure, would be particularly encouraging. Our results in treating this serious, life-threatening disease (25-27), concerning the percentage of survival and the acute and/or chronic complications (infections, the onset and/or aggravation of renal insufficiency) correspond with the data available in references.

## CONCLUSION

Timely diagnosis (the Hemolytic-Uremic Syndrome), the initiation of therapeutic plasma exchanges, the inclusion of corticosteroids and, if necessary, hemodialysis procedures, significantly improve the survival rate of these patients. In patients admitted to our institution with the diagnosis of the TMA/HUS/TTP, most likely an atypical type, without the previous existence of an infectious and/or diarrhea syndrome, the treatment was conducted with the *Prednisone*, SSP, TPI and hemodialysis with/without therapy *(Cyclophosphamide/Vincristine/Rituximab)*. Having conducted this retrospective analysis, we can conclude that the survival rate and complications of this rare, but serious disease correspond to the available world data. Thus, we can conclude that the recognition of these patients is crucial, and so is their referral to institutions that can provide multidisciplinary treatment methods, together with sub-specialists from the Internal Disease Clinics.

Furthermore, whether this syndrome belongs to a group of diseases that Nephrologists and/or Hematologists cure stays the utter dilemma.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All research procedures were approved by the Ethics Committee Serbian Military Medical Academy Belgrade, Serbia. The study was conducted in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. Voluntary written and informed consent was obtained from each participant prior to enrollment in the study

## CONFLICT OF INTEREST

There are no conflicts of interest.


## FUNDING

None.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ORIGINAL SCIENTIFIC ARTICLE

# ROLE OF GENDER IN PHEMENON OF NON-SUICIDAL SELF-INJURIES AND SUICIDE ATTEMPT AMONG CLINICAL POPULATION OF ADOLESCENTS

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## ABSTRACT

*Non suicidal self-injury is defined as intentional self-inflicted injury without the intent to die. Suicide attempt is defined as a non-fatal, self-directed, potentially injurious behavior with the intent to die. Although distinct behaviors, they are frequently associated and possibly clinically related. The aims of this study are to explore demographic data, social-demographic differences between genders, co-occurrence of non-suicidal self-injuries with suicide attempt, their association with gender and clinical variables. Retrospective cohort study on 143 patient admitted in Clinic for mental disorders „Dr Laza Lazarevic", aged 14 to 18 years, between January 2015 and January 2016. Information were obtained from database and included two categories of variables: socio-demographic (age, gender, education level, current living situation) and clinical variables (abuse, neglect, peer violence, aggressive behavior, non-suicidal self-injuries, suicide attempt and others). The mean age of adolescents was 15.8 years, with female being more frequent in the sample (51.4%). The incidence of Mood disorders was significantly higher ($p<0.05$) in female compared to male ($\chi^2=3,96$, $df=1$, $rC=0.16$, $p=0.04$). A significantly higher incidence ($p<0.05$) of non-suicidal self-injury ($\chi^2=11.15$, $df=1$, $rC=0.27$, $p=0.001$) and suicide attempt was found in female compared to male ($\chi^2=5.38$, $df=1$, $rC=0.19$, $p=0.02$). No statistically significant difference was found in their simultaneous occurrence compared to total population of hospitalized adolescents. The findings of the present study demonstrated that non-suicidal self-injury and suicide attempt occur in clinical population of adolescent more often among female then in male adolescents.*

***Keywords***: *Non-suicidal self-injury, suicide attempt, adolescents.*

## INTRODUCTION

Self-injurious behavior (SIB) involves different forms of behavior in which an individual directly and intentionally causes harm to himself, regardless of motivation or the degree of intention to die (1, 2). One form of the SIB is the Non suicidal self-injury (NSSI) defined as intentional self-inflicted injury without the intent to die. Other form of the SIB is the Suicide attempt (SA), defined as non-fatal, self-directed, potentially injurious behavior with an intent to die (1-5). Although these forms of SIB are distinct behaviors differing in intent, form and function, they often co-occur in the same individual (6, 7). Research indicates that self-injury tends to first occur during adolescence (7). On the continuum of self-injurious behavior on which suicide is the ultimate and most severe end, the NSSI is seen as a potential predictor of future SA, which is consider to be one of the most powerful and clinically relevant predictors of eventual suicide (9-11).

NSSI has been listed as a proposed disorder in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) published by the American Psychiatric Association (APA) in 2013 under the category "Conditions for Further Study" (12). This proposal of diagnostic criteria for a future diagnosis is not an officially approved diagnosis and may not be used for clinical use but is meant for research purposes only. The disorder is defined as the intentional, self-inflicted damage to the surface of the body without suicidal intent, which is not socially sanctioned (e.g., body piercing, tattooing, part of a religious or cultural ritual and is not restricted to picking a scab or nail biting).

The most common methods of NSSI are cutting, scratching, hitting or banging, carving, and scraping. NSSI should be distinguished from some other similar forms of self-destructive behavior such as accidental self-harm in which we have an absence of intent or chronic self-destruction in cases of severe eating disorders (anorexia, bulimia) and substance abuse - which is indirect (13).

According to references adolescence is consider to be period with increased risk for the SIB (6-11). NSSI is the most common form of SIB which usually begins in puberty, between age 13 and 15. This phenomenon is reported at an earlier age among girls compared to boys. A girl is therefore at greater risk during adolescence (14-16). Among those who had the NSSI in their history, 70% attempted suicide at least once, and 55% several times. The risk of suicide is the highest in the first 6 months after the NSSI and decreases with time (10, 14, 17, 18). SA are more frequent among young girls than in boys, while the mortality rate of suicide between these groups is not that clear as in adults (17, 18). A strong correlation was found between a large number of NSSI methods, the high incidence of NSSI, and the risk of suicide attempts among adolescents. A history of past suicide attempts is one of the most powerful and clinically relevant predictors of eventual suicide (17, 18). According to references most people initially lack the ability to engage in suicide attempts. The repetition of NSSI might accordingly disrupt the pathways involved in the stress-induced analgesia that leads to the phenomenon of pain tolerance, in other words people become more „brave “to make suicide (8-10). Almost one third of all suicides occur among young people (19- 23). Suicide is the second leading cause of death among 15-29-year-olds and the second leading cause of death for females aged 15-19 (19).

A comprehensive SIB model is needed to better understand of this phenomenon and can help to identify factors that are important for their occurrence but also for its prevention. Previous research has been devoted to identifying risk factors for attempted suicide among adolescents and as well as to identify factors that increase the NSSI risk (24). There are numerous factors that can increase the risk for SIB including genotypic and neurobiological factors (25, 26), psychological and cognitive characteristics, psychiatric disorders of the adolescent (26), family factors ( 27, 28), peers and schools (29, 30). According to references, the factors that act protectively are good social skills, problem-solving abilities and an internal locus of control, enjoyment and involvement with school, playing sports, family cohesiveness (31).

International variation in data of incidence and prevalence of the NSSI exist. One of the reasons is that most of NSSI are frequently carried out in secret, wounds may be superficial and easily treated by the individual and are not reported to parents or medical professionals. Other reasons are different sociocultural norms, traditions, as well as substance use policies among countries, as well as different methodological factors use in research such as measurement errors and differences in assessment and sampling strategies (32). Provided data are based on three sources: psychiatric samples, hospital admissions and general population surveys.

In Serbia there are no data on NSSI, risk factors for this behavior or data on the relation between NSSI and SA (20). The aim of this study is to explore demographic data, social-demographic differences between genders, frequency of NSSI, co-occurrence of NSSI and SA, association of NSSI and SA with gender and clinical variables.

## PATIENTS AND METHODS

A retrospective observational cohort study was conducted at the Clinical Department for Adolescents in the Clinic for Mental Disorders ‘’Dr Laza Lazarevic“. Information regarding these patients was obtained from comprehensive database forms that are completed routinely on all inpatients. These forms contains all data about the patient completed by a Psychiatrist, a Psychologist and an Associative (a Social workers, a Defectologist and a Special pedagogue), as well as all previous medical documentation considering his somatic and mental health. All data have been entered into an electronic database and contain information regarding demographics, patient and family history, psychopathology, somatic and psychiatric diagnoses following ICD-10 criteria, objective examination and treatment. The study was conducted in

accordance with the Helsinki declaration and was approved by the Ethical Committee of the Clinic for Mental Disorders "Dr Laza Lazarevic".

A data collection instrument was designed to include two categories of variables: socio-demographic variables and clinical variables. The main socio-demographic variables included: age, gender, education level, current living situation. The main clinical variables included: previous history, duration and severity of illness, diagnosis, treatment, data of abuse, neglect, peer violence, aggressive behavior, non-suicidal self-injuries, suicide attempt, attempt of homicide, delinquent behavior, the use of alcohol and psychoactive substances.

The database was created in Microsoft Excel 2007, and the data were analyzed using the Software package SPSS for Windows v. 17.0 (SPSS Inc. Chicago, IL). For the description of data, classical methods of descriptive statistics were used.

## RESULTS

From January 1 to December 31 in the year 2015, 146 patients were admitted to the Clinical Department for Adolescents. The mean age was 15.8 years, with female being more frequent in the sample (51.4%). Majority of patients have finished 12 grades (69.3%), 7.5% have finished 8 grades while 23.2% have finished less than 8 grades. Most of the patients were living with family (78.8%), others were living in foster home (3.4%) or in the Social Welfare Institution (17, 8). Most of the patient were diagnosed with behavioral and emotional disorders with onset usually occurring in childhood and adolescence (32.2%), 26.7% were diagnosed with schizophrenia, schizotypal and delusional disorders, 15.1% mood disorders, 9.6% neurotic, stress-related and somatoform disorders. Distribution of diagnostic categories is shown in Table 1.

**Table 1.** Socio-demographic characteristics

| Characteristic | Participants (n=146) |
|---|---|
| *Gender (n)* | |
| Female | 75 |
| Male | 71 |
| *Age, years* | |
| Range | 14-18 |
| Mean | 15,78 |
| Standard deviation | 1,14 |
| *Educational level (*n) | |
| Less than 8 grades | 34 |
| 8 grades | 11 |
| 12 grades | 101 |
| *Current living situation (n)* | |
| Living with family | 115 |
| Living in foster home | 5 |
| Living in social welfare institution | 26 |
| *Diagnosis (n)* | |
| Mental and behavioral disorders due to psychoactive substance use | 7 |
| Schizophrenia, schizotypal and delusional disorders | 39 |
| Mood [affective] disorders | 22 |
| Neurotic, stress-related and somatoform disorders | 14 |
| Eating disorders | 3 |
| Mental retardation | 11 |
| Pervasive and specific developmental disorders | 3 |
| Behavioral and emotional disorders with onset usually occurring in childhood and adolescence | 47 |

No statistical significant difference was found in socio-demographic data in relation to gender. We found a statistically significant difference ($p<0.05$) in the incidence of mood disorders in females compared to males ($\chi^2=3.96$, df=1, rC=0.16, p=0.04).

**Table 2.** Social-demographic and clinical variable differences between genders

| Variable | Male | Female | Test value | p |
|---|---|---|---|---|
| Diagnosis (N/%) | | | | |
| Mental and behavioral disorders due to psychoactive substance use | 3 (2,05) | 4 (2,74) | 0,21 | 0,64 |
| Schizophrenia, schizotypal and delusional disorders | 24 (16,44) | 15 (10,27) | 2,20 | 0,13 |
| Mood [affective] disorders | 7 (4,79) | 15 (10,27) | 3,96 | 0,04 |
| Neurotic, stress-related and somatoform disorders | 5 (3,42) | 9 (6,16) | 1,51 | 0,21 |
| Eating disorders | 0 (-) | 3 (2,05) | 3,23 | 0,07 |
| Mental retardation | 7 (4,79) | 4 (2,74) | 0,71 | 0,39 |
| Pervasive and specific developmental disorders | 3 (2,05) | 0 (-) | 2,90 | 0,08 |
| Behavioral and emotional disorders with onset usually occurring in childhood and adolescence | 26 (17,80) | 21 (14,38) | 0,43 | 0,51 |
| Abuse (N/%) | 10 (6,85) | 12 (8,22) | 0,36 | 0,54 |
| Neglect (N/%) | 9 (6,16) | 11 (7,53) | 0,37 | 0,54 |
| Bulling (N/%) | 3 (2,05) | 2 (1,36) | 0,15 | 0,69 |
| NSSI (N/%) | 12 (8,22) | 29 (19,86) | 11,14 | 0,00 |
| SA (N/%) | 12 (8,22) | 23 (15,75) | 5,37 | 0,02 |
| Family history of mental disorders (N/%) | 28 (19,18) | 22 (15,07) | 0,65 | 0,41 |
| Suicide in family (N/%) | 3 (2,05) | 2 (1,36) | 0,15 | 0,69 |
| Homicide in family (N/%) | 1 (0,68) | 1 (0,68) | 0,00 | 0,96 |
| Misuse of PAS (N/%) | 30 (20,55) | 25 (17,12) | 0,35 | 0,55 |
| Misuse of alcohol (N/%) | 35 (23,97) | 29 (19,86) | 0,50 | 0,47 |

NSSI was reported in 28,1% of adolescents, more frequent among girls (70,7%) then in boys (29,3%). There is a statistically significant difference ($p<0,05$) in the occurrence of NSSI in females compared to males ($\chi^2=11$, 15, df=1, rC=0.27, p=0,001). Among adolescents who had history of NSSI 34.1% met criteria for the behavioral and emotional disorders with onset usually occurring in childhood and adolescence, 24.4% nood disorders, 12.2% schizophrenia, schizotypal and delusional disorders, 9.7% neurotic, stress-related and somatoform disorders.

In this clinical population of adolescents 15.1% reported abuse in their past and 6.2% of them reported NSSI. Further, 37.7% reported misuse of PAS and 13.7% of them reported NSSI; 43.8% misuse of alcohol and among them 14.9% had the NSSI in their past. There is no statistically significant difference in the association of these clinical variables and NSSI compared to total clinical population.

Data on family history showed that 34.1% of adolescents have family member who suffers from mental illness, and 10.3% of them were engaged in the NSSI. Suicide in family was found in 3.4% of adolescents and 2.1% of them reported NSSI. This data didn't show a statistically significant difference in the association of these clinical variables and NSSI compared to total clinical population.

The simultaneous occurrence of the NSSI and SA was found in 4.8% of adolescents, which does not indicate a statistically significant difference ($\chi^2=1.49$, df=1, rC=0.10, p=2.22) compared to the total population of hospitalized adolescents.

SA was reported in 24% of total of hospitalized adolescents, among which 32.4% in girls and 16% in boys. There is a statistically significant difference ($p<0.05$) in the occurrence of SA in the females compared to males ($\chi^2=5.38$, df=1, rC=0.19, p=0.02).

**Table 3.** Social-demographic and clinical variable differences related to the NSSI

| Variable | NSSI | | Test value | P |
|---|---|---|---|---|
| Diagnosis (N/%) | Yes | No | | |
| Mental and behavioral disorders due to psychoactive substance use | 4 (2,74) | 3 (2,05) | 3,07 | 0,08 |
| Schizophrenia, schizotypal and delusional disorders | 5 (3,42) | 34 (23,29) | 6,13 | 0,01 |
| Mood [affective] disorders | 10 (6,85) | 12 (8,22) | 3,87 | 0,04 |
| Neurotic, stress-related and somatoform disorders | 4 (2,74) | 10 (6,85) | 0,00 | 0,96 |
| Eating disorders | 2 (1,36) | 1 (0,68) | 2,25 | 1,33 |
| Mental retardation | 2 (1,36) | 9 (6,16) | 0,57 | 0,44 |
| Pervasive and specific developmental disorders | 3 (2,05) | 0 (-) | 1,19 | 0,27 |
| Behavioral and emotional disorders with onset usually occurring in childhood and adolescence | 14 (9,59) | 33 (22,60) | 0,10 | 0,75 |
| Abuse (N/%) | 9 (6,16) | 13 (8,90) | 2,11 | 0,14 |
| Neglect (N/%) | 7 (4,79) | 13 (8,90) | 0,54 | 0,45 |
| Bulling (N/%) | 0 (-) | 5 (3,42) | 2,02 | 0,15 |
| SA (N/%) | 7 (4,79) | 28 (19,18) | 1,48 | 0,22 |
| Family history of mental disorders (N/%) | 15 (10,27) | 35 (23,97) | 0,13 | 0,71 |
| Suicide in family (N/%) | 3 (2,05) | 2 (1,36) | 2,61 | 0,10 |
| Homicide in family (N/%) | 0 (-) | 2 (1,36) | 0,79 | 037 |
| Misuse of PAS (N/%) | 20 (13,70) | 35 (23,97) | 2,99 | 0,83 |
| Misuse of alcohol (N/%) | 21 (14,38) | 43 (29,45) | 1,26 | 0,26 |

## DISCUSSION

Epidemiological studies have shown that rates of the NSSI vary remarkably between countries. NSSI is present among adolescents both in community as well as in clinical settings (32-34).

The prevalence of NSSI in our study, among one year clinical population of adolescent, was 28.1%. Prevalence rates of the NSSI among adolescent samples range from 14% to 40% in the community (33) and from 40% to 61% in inpatient samples (32). In the United States, in the clinical population of hospitalized adolescents the mean prevalence of NSSI is around 35%, while its prevalence in the general population of adolescents is thought to be around 10% (35). In England data from a hospital admission monitoring study indicate that 0.3% males aged between 15 and 24 years, and 0.7% females of the same age are engaged in NSSI each year, while general population study carried out in the same country found that almost 5% of males and 8% of females aged between 13 and 15 years reported the NSSI and SA (32-34). Data from the Child and Adolescent Self-harm in Europe (CASE) Study involving seven European countries (Australia, Belgium, England, Hungary, Ireland, the Netherlands and Norway) has showed that 8.9% of females and 2.6% of males reported an episode of self-harm in the past year, and 13.5% and 4.3% respectively reported an episode sometime in their lifetime (33).

The present study confirms earlier findings that the NSSI and SA are considerably more common among female than males (14-16). According to The Centers for Disease Control and Prevention (CDC) of United States who maintains data on the estimated rate of nonfatal self-injury based on a national surveillance system of injuries treated in US Hospital Emergency Departments (the National Electronic Injury Surveillance System), in contrast to suicide mortality, rates of the NSSI and SA are consistently higher among females (35).

Previous studies have reported high rates of mental illness in adolescents engaging in both fatal and non-fatal suicidal behavior and the presence of multiple disorders is associated with especially elevated risk (24, 25) In our study among adolescents who reported the NSSI one third of them met criteria for behavioral and emotional disorders with onset usually occurring in childhood and adolescence, quarter met criteria for the mood disorders, and around 10% psychotic, neurotic and substance abuse disorders. Studies suggest that depression with hopelessness is the most prevalent mental health disorder reported in adolescent population with non-fatal suicidal behaviors. Anxiety, especially when comorbid with depression, has been identified as increasing risk for the SIB (24, 25). Panic attacks have been recognized as the high risk of suicidal ideation and the NSSI. Among adolescents with substance abuse there is also the high risk of SIB. Up to one-third of the young hospitalized after NSSI have consumed alcohol around the time of the act. Alcohol abuse is also associated with increased rates of repetition (24, 25). In our study around 14% of adolescents who had the PAS and alcohol misuse had reported the NSSI.

Abuse, sexual and physical abuse, as well as peer violence is associated with the increased risk of suicidal ideation, SIB and SA among adolescents (27-30). Exposure to

these adverse experiences increase the risk for the SIB through internalizing emotions such as shame, feelings of depression and social isolation, which affect the ability to cope with life stressors. We found out that around 6% who reported abuse in their past also have reported the NSSI. Previous studies have explained that childhood abuse and negative affect together with low emotional expressivity increase the risk of NSSI. Others have showed that there is a direct association between childhood abuse and NSSI through the low self-esteem and dissociation.

Another risk factor for the SIB is parental mental health disorders (24). Results from references show that poor parental mental health is association with the increased risk of both suicide ideation and nonfatal suicide behavior, particularly parental depression and substance abuse. Data on family history in our study showed that 10,3% of adolescents who had family member suffering from mental disorder reported the NSSI. There are also data confirming that a family history of suicide behavior is associated with the increased risk for suicide deaths and NSSI among adolescents. One of the given explanations is that adolescents copy model seen in parents when they fail in their attempt to cope with the unbearable negative feelings that overwhelm them.

In our study, we didn't find a higher incidence of the SA among adolescents with the NSSI, although research has demonstrated a strong correlation between a large number of NSSI methods, the high incidence of NSSI, and the risk of suicide attempts among adolescents (2-4). It is suggested that the NSSI can be understood as a strategy of emotional adaptation and regulation that involves reducing negative emotions, such as anger, tension, anxiety, guilt, loneliness, alienation or hatred towards oneself; to provide a sense of security or control; as punishment, as wish to die, way of communication with surrounding (36, 37). If the adolescent receives relief from the undesirable feeling or thoughts after the first NSSI or if the NSSI brings some changes as a result of the reaction of the environment which are desirable, it is very likely that the NSSI will reoccur. According to references most people initially lack the ability to engage in suicide attempts. The repetition of NSSI might accordingly disrupt the pathways involved in the stress-induced analgesia that leads to the phenomenon of pain tolerance, in other words people become braver to make suicide attempt and suicide (36, 37).

Although these data provide valuable information indicating that the NSSI exists among clinical population of adolescents in Serbia, since there were no data until now, there are several limitations of the study: Data were collected through comprehensive database completed by medical stuff, no self-report measures were used and are retrospective in nature; detailed data about methods and repetition, function of the NSSI are missing; protective factors were not taken into account; one year clinical sample.

## CONCLUSION

The findings of the present study demonstrated that the NSSI and SA occur in clinical population of adolescent more often among female then in male adolescents. Getting a better understanding of this phenomenon, its function and relationship with the SA, investigation of risk factors and psychosocial variables associated with these behaviors is a necessary step in helping Clinicians in better assessment of the risk of suicide within this group. SIB as well as suicide among adolescents is a global public health problem. Prevention of self-injury and suicide among adolescents is a challenge in which national suicide prevention strategies are essential for elevating suicide prevention on the political agenda taking into account the dangers and mortality of such behavior.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the Helsinki declaration and was approved by the Ethical Committee of the Clinic for Mental Disorders "Dr Laza Lazarevic".Voluntary written and informed consent was obtained from each participant prior to enrollment in the study.

## CONFLICT OF INTEREST

There are no conflicts of interest.


## FUNDING

None.


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ORIGINAL SCIENTIFIC ARTICLE

# RELATION BETWEEN OXIDATIVE STRESS AND CAROTID ARTERY ATHEROSCLEROSIS IN HEMODYALISIS PATIENTS

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## ABSTRACT

*Oxidative stress represents a significant risk factor for the accelerated development of atherosclerosis in a population of patients on regular hemodialysis. Oxidative stress induced by hemodialysis can be triggered with both the bioincompatibility of dialysis membrane and increased endotoxin concentration in a hemodialysis solution. The aim of this study was to investigate the correlation between the parameters of oxidative stress, microinflammation, nutrition, secondary hyperparathyroidism and carotid artery intima-media thickness in patients on regular hemodialysis.One hundred and twenty five patients treated with standard hemodialysis and on-line hemodiafiltration with "high-flux" polysulfone dialysis membrane were examined. The following parameters of oxidative stress were measured : index of lipid peroxidation - measured as TBARS, nitric oxide in the form of nitrite - $NO_2$-, super oxide anion radical - $O_2$- and hydrogen peroxide - $H_2O_2$, catalase, superoxide dismutase (SOD) and reduced glutathione activity. For statistical analysis of results, the following tests were used: the Kolmogorov-Smirnov test, the Spirman test and the Pearson correlation test. Oxidative stress affects atherosclerosis of the carotid arteries in patients treated with regular hemodialysis and online hemodiafiltration. There is a statistically significant positive correlation between $H_2O_2$ concentration and the thickness of the carotid arteries` intima-media. High statistically significant positive correlation was found between TBARS concentration and carotid arteries intima-media thickness, while a high statistically significant negative correlation was found between SOD activity and a carotid artery intima-media thickness. There is a statistically significant negative correlation between the serum albumin and prealbumin concentration and a carotid artery intima-media thickness. Oxidative stress may be a significant risk factor for the carotid artery atherosclerosis development in patients treated with regular hemodialysis.*

***Keywords***: *Oxidative stress, atherosclerosis, hemodialysis.*

## INTRODUCTION

Cardiovascular disease remained the leading cause of death in patients treated with regular hemodialysis. Atherosclerotic heart disease and congestive heart failure are two clinical conditions that are the cause of death in more than 50% of these patients. There are traditional and non-traditional risk factors for the development of cardiovascular disease in this patient population. Non-traditional risk factors are associated with impaired renal function and hemodialysis procedures and they include the oxidative stress, microinflammation, malnutrition, endothelial dysfunction, uremic toxins, hyperhomocysteinemia, anemia, hypervolemia, vitamin D deficiency and secondary hyperparathyroidism. Oxidative stress is one of the most significant non-traditional risk factors for the development of accelerated atherosclerosis in a population of patients treated with regular hemodialysis (1).

In these patients, the oxidative stress occurs due to the bioincompatibility of the hemodialysis membrane, the increased concentration of endotoxins in the hemodialysis solution, the intravenous application of iron solutions and reduced activity of enzymatic and non-enzymatic antioxidant protective mechanisms (2-4). During the hemodialysis session, activated cells of the innate immune system in peripheral blood (neutrophils, monocytes) generate reactive oxygen species (ROS) intensely and the capacity of endogenous antioxidant protective mechanisms is reduced due to loss of water-soluble antioxidants (vitamin C) and oligoelements (antioxidant enzyme cofactors). Malnutrition also contributes to reduction of antioxidant protective mechanisms activity in the population of patients undergoing hemodialysis. Main clinical consequences of oxidative stress are the development of atherosclerosis, resistance to erythropoetin and the development of cardiovascular disease in patients treated with regular hemodialysis (4-7).

In addition to oxidative stress, a significant role in the development of a carotid artery atherosclerosis in patients on regular hemodialysis has the microinflammation, hyperhomocysteinemia, and malnutrition (4-8). Main factors for the development of microinflammation in these patients are: bioincompatibility of the extracorporeal circulation, the presence of endotoxin in the hemodialysis solution (reverse diffusion), asymptomatic infection of the arteriovenous fistula for hemodialysis, the periodontal disease and dislocation of bacteria and endotoxins from intestines to circulation, as a result of disruption of the gut microbiome (7, 8). Blood contact with the synthetic material of the extracorporeal circulation during the hemodialysis session causes constant leukocyte and the complement system activation, release of elastase, myeloperoxidase, pro-inflammatory mediators and ROS (8). Increased serum neutrophil elastase concentrations have been associated with microinflammation, shortened erythrocyte lifetime (erythrocyte membrane disorder), resistance to erythropoietin and adverse outcomes in patients on regular hemodialysis (8). Oxidative stress and microinflammation are key factors for the development and progression of atherosclerotic cardiovascular disease in patients undergoing regular hemodialysis. Optimal control of the oxidative stress and microinflammation is a key step in reducing cardiovascular morbidity and mortality in this patients' population (8).

In patients who are on the regular hemodialysis program, the hyperhomocysteinemia is defined as a plasma homocysteine concentration of > 15 μmol/L, resulting from the decreased activity of enzymes crucial in homocysteine metabolism, such as 5-methyl-tetrahydrofolate reductase, methionine synthase, and γ- cistation synthase. The reduced activity of these enzymes occurs due to a deficiency of vitamins B6, B12 and folic acid, which serve as cofactors of enzymes mentioned (insufficient intake, increased loss during the hemodialysis session) (9). Increased homocysteine concentration, oxidative stress (superoxide anion radical) and microinflammation block the activity of the dimethyl diamino-hydrolase enzyme (DDHA) in endothelial cells, which degrades asymmetric dimethylarginine - ADMA (dimethylarginine) to L-citrulline and methionine. Asymmetric dimethylarginine is the most significant endogenous nitric oxide synthase blocker while the reduced nitric oxide formation in endothelial cells plays a key role in initiating the process of atherosclerosis. In addition, the superoxide anion oxidizes tetrahydrobiopterin (an endogenous NO synthase cofactor) and thus further reduces NO synthase activity and contributes to accelerating the process of atherosclerosis. Endothelial dysfunction plays a key role in the pathogenesis of atherosclerotic cardiovascular disease in a population of patients undergoing regular hemodialysis. Clinical trial results indicate a statistically significant positive association between the asymmetric dimethylarginine concentration and carotid artery intima-media thickness (10).

Malnutrition as a result of protein deficiency - PEW (Protein-Energy Wasting) is present among 30-60% of patients undergoing regular hemodialysis. According to the International Society of Renal Nutrition and Metabolism (ISRNM) guidelines, PEW exists if: the serum albumin concentration is<0.38 g/L, the serum prealbumin concentration<0.30 g/L, the total cholesterol concentration<100 mg/dL, the body mass index less than 23 kg/m2, the unintentional weight loss ≥ 5% over three months and the protein intake less than 0.8 g/kg/day. PEW results from reduced intake and increased protein catabolism. Risk factors that promote protein catabolism in patients undergoing regular hemodialysis include: the metabolic acidosis, microinflammation, and oxidative stress (11).

Carotid artery atherosclerosis is defined as an intima-media thickness (IMT) of > 0.90 mm, as measured by the ultrasound examination of carotid arteries. Based on the color Doppler ultrasonography, the atherosclerotic carotid artery disease is classified into four stages: stage I: IMT<0.9 mm, grade II: IMT > 0.9 mm, stage III: the presence of atherosclerotic plaques with a stenosis ≤ 50% and stage IV: the presence of atherosclerotic plaques with stenosis > 50%. The presence of plaque is defined as a structure that protrudes into the carotid artery lumen at least 0.5 mm (5).

Hemodialysis membranes play a key role in preventing the development of oxidative stress, microinflammation, and malnutrition in patients treated with regular hemodialysis. High-flux hemodialysis membranes have a greater number of advantages over low-flux membranes: they are more biocompatible, better remove small and medium molecular weight uremic toxins, have a less degree of neutrophil activation and complement systems (less potential to produce proinflammatory cytokines and acute phase proteins inflammation), provide less resistance to erythropoietin and better preserve residual renal function (8). High-flux membranes improve quality of life and provide a better long-term outcome for patients treated with regular hemodialysis (8). Vitamin E-coated membranes for the hemodialysis reduce microinflammation (reduce serum interleukin 6 concentration), prevent lipid peroxidation (decrease serum TBARS concentration), increase iron availability for erythrocytopoesis (reduce hepcidin concentration in erythropoietic) and reduce resistance to erythropoetin.

The aim of this study was to investigate the correlation between parameters of oxidative stress, microinflammation, malnutrition, secondary hyperparathyroidism, and carotid artery intima-media thickness in regular hemodialysis patients.

## PATIENTS AND METHODS

The study included 125 patients treated with regular hemodialysis and on-line hemodiafiltration at the Center for Nephrology and Dialysis of the Clinical Center Kragujevac. The study was conducted in accordance with the Declaration of Helsinki for Medical Research (revised in 2013.), the consent was obtained from patients and the Ethics Committee of the Clinical Center Kragujevac. Patients treated with regular hemodialysis and on-line hemodiafiltration were examined 2-3 times a week for 4 hours (8-12h per week), for a period longer than three months, with "high-flux" membranes (for regular hemodialysis: polysulfone high-flow membrane with surface area 1.4-1.7 $m^2$, for hemodiafiltration: polysulfone "high-flux" membrane with surface area 2.0-2.4 $m^2$). Dialysis machines used in study were with controlled ultrafiltration type Fresenius 5008S, Gambro Artis and BBraun, with average blood flow rate - Qb=222.80 ± 25.89 mL/min and average dialysate flow rate - Qd=500 mL/min. A standard ultrapure hemodialysis solution (endotoxin concentration - E<0.03 EU/ml) was used, with a calcium concentration of 1.75 mmol/L (PGS21), 1.50 mmol/L (PGS25) and 1.25 mmol/L (PGS27) while convective volume in patients treated with on-line hemodiafiltration was Vconv=17 liters per session. Unfractionated heparin was used for anticoagulation of the extracorporeal circulation. The average monthly dose of unfractionated heparin for single hemodialysis was 4418.00 ± 525.34 IU. The study did not include patients with the manifested active bleeding, active systemic inflammation or infection (average leukocyte count was 7.06 ± 1.56 x $10^9$/l), with uncontrolled malignancies, or patients treated with immunosuppressive and antioxidant medications.

In order to evaluate the effect of oxidative stress, microinflammation, nutrition and secondary hyperparathyroidism on the intima-media thickness of the carotid artery the following parameters were investigated: index of lipid peroxidation - measured as TBARS (thiobarbituric acid reactive substances), nitric oxide in the form of nitrite - $NO2^-$, super oxide anion radical - $O_2^-$ and hydrogen peroxide - $H_2O_2$., catalase (CAT), superoxide dismutase (SOD) and reduced glutathione activity (GSH), hemoglobin, hematocrit, iron, ferritin, transferrin saturation with iron, C-reactive protein, albumin, prealbumin, transferrin, intact parathormone (iPTH ) and vitamin D. Hemodialysis adequacy was evaluated based on the spKt/V urea index.

**Determination of serum laboratory analyzes**

Blood samples were taken before and after each hemodialysis session, before administration of heparin. Routine laboratory analyzes were determined by standard laboratory tests and calculated as an average value of three measurements over three consecutive months.

The serum ferritin concentration was determined by the turbidimetric method, on the Beckman Coulter AU680 apparatus. In patients treated with regular hemodialysis, the normal serum ferritin concentration is 100-500 ng/mL. The serum CRP concentration was determined by the turbidimetric method on the Olympus AU680 and calculated as the average value of two measurements over two consecutive months. Normal serum CRP concentration is ≤ 5 mg/L. Microinflammation is defined as the concentration of CRP in the serum of > 5 mg/L. The concentration of vitamin D in the serum was determined by electrochemiluminescence, on the Cobas e 411. Normal vitamin D concentration in the serum is 20-40 ng/mL. In patients treated with regular hemodialysis, the normal vitamin D concentration is ≥ 30 ng/mL (30-80 ng/mL). A severe deficit is defined as the concentration of vitamin D<10 ng/mL, vitamin D deficiency exists if the concentration is 10-20 ng/mL, and the insufficiency is defined as the concentration of vitamin D in the serum of 20-30 ng/mL. Concentration of an intact parathormone in the serum was determined by the immunodiathymetric method (IRMA), on the gamma counter WALLAC WIZARD 1470. Normal concentration of the intact parathormone in the serum is 11.8-64.5 pg/mL. In patients with hemodialysis the upper normal limit is 300 pg/mL.

Folate and vitamine $B_{12}$ were determined on the Access 2 Analyzer by Beckman Coulter using chemilumminescent immunoassay. Prealbumine and albumine were determined on the Abbott an Architect analyzer using prealbumin and transferrin immunoturbidimetric method. Normal concentration of prealbumine in hemodyalisis patients is ≥ 0.3 g/L ( ≥ 30 mg/dL)

**Determination of parameters of oxidative stress in plasma**

The principle of the determination of superoxide anion ($O_2^{-\cdot}$) in blood plasma samples uses the $O_2$ reaction with nitro

tetrazolium blue (Nitro Blue Tetrazolium - NBT) to nitroformase blue. The measurement was performed at a wavelength λ=550 nm (12).

Method for determining the concentration of hydrogen peroxide ($H_2O_2$) is based on the oxidation of phenol red by the hydrogen peroxide reaction, which catalyses Horse Radish Peroxidase (HRPO). The final result of this reaction is the formation of a compound with a maximum absorption λ max=610 nm (13). Determination of the lipid peroxidation index was carried out indirectly through products of the lipid peroxidation reaction with thiobarbituric acid (Thiobarbituric Acid Reactive Substances- TBARS). The principle of this method is based on the determination of lipid peroxide levels based on the reaction of one of them, malonildialdehyde (MDA) with thiobarbutyric acid (TBA). The measurement was performed at a wavelength λ=530 nm (14). The concentration of nitrogen monoxide (NO) was determined based on the amount nitrites released. The principle of this method involves the use of a Griess reagent, which builds a diazo complex with nitrites, which gives the purple color. Measurement was performed at a wavelength λ=550 nm (15).

**Determination of parameters of antioxidant defense system**

An adrenaline method was used to determine the activity of SOD. The principle of this method, which normally belongs to the group of the "negative" type is to monitor the reduction in the rate of adrenaline autoxidation in the alkaline environment, which is $O_2^-$ dependent. Considering that $O_2^-$ is removed by the present SOD, the adrenaline autoxidation reaction is inhibited. The system monitors the rate of adrenaline autoxidation change through the change in absorbance at 480 nm, which is inversely proportional to SOD activity (16). The Beutler method was used to determine the catalase activity. The principle is the spectrophotometric monitoring of the rate of the hydrogen peroxide decomposition in the presence of catalase at a wavelength of 230 nm, in which hydrogen peroxide absorbs light. For the determination of reduced glutathione (GSH) activity, the Beutler spectrophotometric method was used. The principle of the method is based on the oxidation of glutathione GSH by 5,5-dithio-bis-6,2-nitrobenzoic acid (DTNB) (17).

**Determination of parameters of hemodialysis adequacy**

Hemodialysis adequacy was assessed on the basis of the single-pool Kt/Vsp index calculated according to Daugridas second-generation formula: Kt/Vsp=-ln (C2/C1 - 0.008 x T) + (4 - 3.5 x C2/C1) x UF / W (mmol/L), T - hemodialysis duration (h), UF – interdalytic weight gain (L), W - body weight after the hemodialysis (kg). According to KDOQI guidelines, hemodialysis is adequate if Kt/Vsp is ≥ 1.2. Urea reduction rate index - URR index is calculated using the following formula: URR=(1-R) x 100%, where: R represents the ratio of urea concentration in the serum after and before the hemodialysis treatment. Hemodialysis is adequate if the URR index is=65-70%.

**Determination of blood flow and thickness of a carotid artery intima-media**

The blood flow through the vascular approach - Qavf was determined by the Color Doppler ultrasound scan, on the Logic P5 apparatus, using a 7.5 MHz probe, wherein the blood flow is calculated from the formula: $Qavf=r^2p\pi/4$ x Vmean x 60 (mL/min), r - radius of vascular access, and Vmean - mean blood flow velocity through vascular approach. The blood flow is calculated as the average of three measurements, 2-4 cm on the vein vascular approach, proximal to the anastomosis site. The blood flow through a vascular approach that provides adequate hemodialysis is 500-1000 mL/min. The thickness of a carotid artery intima-media (IMT) was determined by the Color Doppler ultrasonic examination, on the Logic P5 apparatus, using a 7.5MHz probe, as the average value of three individual measurements on the right and left carotid arteries. Measurements were performed 1-2 cm below the bifurcation of carotid arteries by the same ultrasound. The normal thickness of the intima-media is defined as a value of less than 0.9 mm.

**Statistical analysis**

Tests used for the statistical analyses of the obtained data were as follows: the Kolmogorov-Smirnov test, the Spirman test and the Pearson correlation test. Significance threshold was set on 0.05 and 0.01.

## RESULTS

A cross-sectional study was conducted at the Center for Nephrology and Dialysis of the Clinical Center Kragujevac. The study included patients treated with regular hemodialysis and on-line hemodiafiltration for a period longer than three months. Investigated population (n=125) included 78 male and 47 female patients, an average age of 62.83 ± 10.49 years with the mean dialysis treatment length 6.51 ± 6.12 years, the mean nutrition status 25.86 ± 4.60 kg/m2 and with an average dialysis adequacy index spKtV 1.24 ± 0.29. General data on patients are presented in Table 1.

For anemia treatment short-acting and long-acting erythropoietins, an intravenous iron preparation, vitamin B preparation and folic acid (per os) were used. The average monthly dose of short-acting erythropoietin was 20538.46 ± 10716.42 IU, long-acting erythropoietin 134.89 ± 71.88 μg, the average monthly dose of intravenous iron was 273.91 ± 162.38 mg, the average monthly dose of i.v. of vitamin C was 1420.00 ± 184.04 mg, the average monthly number of ampoules of Beviplex was 11.36 ± 1.47, the average monthly dose of vitamin B12 was 3060 ± 1874.70 μg and the average monthly dose of folic acid was 187.20 ± 65.04 mg. The secondary hyperparathyroidism was treated with calcium-containing phosphate binders, active vitamin D metabolites, and paricalcitol. The average monthly dose of rocaltrol was 2.56 ± 3.86 μg, and the i.v. paricalcitol 1.04 ± 6.70 μg. For the treatment of arterial hypertension, 81 (64.80%) patients used

renin-angiotensin system blockers (mainly angiotensin converting enzyme blockers), 62 (49.60%) beta blockers, 46 (36.80%) loop diuretics and 44 (35.20%) calcium channel blockers.

A hundred and one patients (80.80%) were treated with the standard intermittent high-flux hemodialysis, and 24 (19.20%) patients were treated with the postdilution on-line hemodiafiltration. For the postdilution on-line hemodiafiltration treatment, 24 (19.20%) patients were using dialysers with a high-flux polysulfone membrane of 2.0-24m$^2$, while other patients (101 patients, 80.80%) were treated with a "high-flux" hemodialysis using a high-flux polysulfone dialyser membrane 1.4-1.8m$^2$. While 113 patients used hemodialysis solution with a calcium concentration of 1.75 mmol/L (PGS21), 9 patients had $Ca^{2+}$ concentration in the hemodialysis solution was 1.50 mmol/L (PGS25), and only 3 patients used a solution with $Ca^{2+}$ concentration 1.25 mmol/L (PGS27). The $Na^+$ concentration in the hemodialysis solution was 140 mmol/L and the K+ concentration was 2.0 mmol/L.

The average values of the anemia parameters, iron status, microinflammation, nutritional status, secondary hyperparathyroidism and ultrasound examination of the carotid arteries are shown in Table 2. In order to assess the effect of oxidative stress on atherosclerosis of the carotid arteries, the following parameters were examined: the hydrogene-peroxide ($H_2O_2$), thiobarbituric acid reactive substances (TBARS), nitrites ($NO_2^-$), the superoxide dismutase (SOD), catalase (CAT), the reduced glutathione activity (GSH) and the carotid artery intima-media thickness. Average values of the investigated parameters of oxidative stress are shown in Table 2.

There was a statistically significant ($p<0.05$) positive correlation noticed between the serum $H_2O_2$ concentration and the carotid intima-media thickness. In addition, the high statistically significant ($p<0.01$) positive correlation was found between the serum TBARS concentration and the carotid artery intima-media thickness, while a significant ($p<0.01$) negative association was found between the SOD activity and intima-media thickness of carotid arteries, (Table 3). Significant ($p<0.01$) positive correlation between the prealbumin and serum albumin concentrations was found, while the high statistically significant ($p<0.01$) negative correlation was noticed between the serum albumin and prealbumin concentration and carotid intima-media thickness (Table 4). Among the other parameters tested and carotid artery intima-media thickness, no statistically significant correlation was found (Table 4).

**Table 1**. Basic characteristics of study population

| GENERAL DATA | | Statistical parameters |
|---|---|---|
| | | Xsr ± SD |
| Number (N) | | 125 |
| Gender (m/f %) | | 78/47 (62.40%/37.60%) |
| Age (years) | | 62.81 ± 10.44 |
| Hemodialysis treatment length (years) | | 6.51 ± 6.12 |
| Body mass index - BMI (kg/m$^2$) | | 25.86 ± 4.60 |
| Sistolic arterial blood pressure - SBP(mmHg) | | 127.64 ± 15.77 |
| Diastolic arterial blood pressure - DBP (mmHg) | | 76.16 ± 7.49 |
| Mean arterial blood pressure- MBP (mmHg) | | 93.32 ± 9.54 |
| Body weight - W (kg) | | 72.02 ± 14.70 |
| Interdiaytic weight gain - IDWG (kg) | | 2.44 ± 1.11 |
| Interdiaytic weight gain - IDWG (%) | | 3.45 ± 1.58 |
| Ultrafiltration rate - UFR (ml/kg/h) | | 8.64 ± 3.94 |
| Ultrafiltration rate - UF (mL/h) | | 610.93 ± 275.26 |
| Residual diuresis - RD (mL/24h) | | 652.40 ± 683.40 |
| Arteriovenous fistula flow - Qavf (mL/min) | | 845.60 ± 433.35 |
| Hemodialysis adequacy index - Kt/V | | 1.10 ± 0.24 |
| Single pool hemodialysis adequacy index - spKt/V | | 1.24 ± 0.29 |
| Urea reduction ratio - URR (%) | | 63.87 ± 8.62 |
| Primary kidney disease | Glomerulonephritis chronica | 11 (8.80%) |
| | Nephropathia hypertensiva | 40 (32.00%) |
| | Nephropathia diabetica | 19 (15.20%) |
| | Nephropathia obstructiva | 8 (6.40%) |

| | | |
|---|---|---|
| | Nephropathia chronica | 27 (21.60%) |
| | Renes polycystici | 20 (16.00%) |
| Comorbidity | | |
| Hypertensio arterialis | | 75 (60.00%) |
| Cor hypertensivum compensatum | | 20 (16.00%) |
| Cardiomyopathia dilatativa | | 5 (4.00%) |
| Hypotensio arterialis | | 5 (4.00%) |
| Diabetes mellitus complicatus | | 20 (16.00%) |

**Table 2.** General investigated final parameters of oxidative stress

| INVESTIGATED PARAMETERS | Statistical parameters |
|---|---|
| | Xsr ± SD |
| Hemoglobin - Hb (g/L) | 104.05 ± 12.28 |
| Hematocrit - Hct (%) | 31.52 ± 3.83 |
| Mean corpuscular volume - MCV (fL) | 93.89 ± 4.51 |
| Mean corpuscular hemoglobin - MCH (pg) | 30.98 ± 1.62 |
| Mean corpuscular hemoglobin concentration - MCHC (g/L) | 329.96 ± 5.09 |
| Vitamine B12 serum concentration - VitB12 (pg/mL) | 999.78 ± 516.38 |
| Folic acid serum concentration - FOL (ng/mL) | 22.48 ± 11.49 |
| Iron serum concentration - $Fe^{2+}$ (μmol/L) | 10.05 ± 4.16 |
| Transferrin saturation - TSAT (%) | 28.30 ± 11.48 |
| Feritin serum concentration - F (ng/mL) | 745.50 ± 344.60 |
| C-reactive protein - CRP (mg/L) | 10.57 ± 12.23 |
| Serum protein concentration - P (g/L) | 64.36 ± 4.66 |
| Albumine serum concentration - Alb (g/L) | 38.10 ± 3.03 |
| Prealbumina serum concentration - Palb (g/L) | 0.28 ± 0.09 |
| Transferin serum concentration - Trsf (g/L) | 1.56 ± 0.34 |
| Uric acid serum concentration - UA (μmol/L) | 366.92 ± 59.06 |
| Normalised protein catabolic rate - nPCR (g/kg/dan) | 1.76 ± 0.66 |
| Vitamine D serum concentration - VitD (ng/mL) | 17.88 ± 9.63 |
| Intact parathormon serum concentration - iPTH (pg/mL) | 175.13 ± 199.85 |
| Superoxide anion radical - $O_2^-$ (nmol/mL) | 3.58 ± 4.90 |
| Hydrogene peroxide - $H_2O_2$ (nmol/mL) | 4.65 ± 1.62 |
| Thiobarbituric acid - TBARS (μmol/mL) | 1.14 ± 0.23 |
| Nitrites - $NO_2^-$ (nmol/mL) | 3.81 ± 1.33 |
| Reduced glutathione - GSH (nmol/mL) | 119500.29 ± 17525.20 |
| Catalase - CAT (U/gHb x $10^4$) | 2.22 ± 1.88 |
| Superoxide dismutase SOD (U/gHb x $10^4$) | 32.82 ± 20.67 |
| Average intima-media thickness DKA - IMT (mm) | 1.24 ± 0.29 |
| Average intima-media thickness LKA - IMT (mm) | 1.27 ± 0.31 |
| Average intima-media thickness KA - IMT (mm) | 1.25 ± 0.28 |

**Table 3.** Correlation between carotid artery intima-media thickness and oxidative stress parameters

| Measured parameters | Basic statistical parameters | | Significance (p-value) |
|---|---|---|---|
| | Xsr ± SD | N | |
| Superoxide anion radical - $O_2^-$ (nmol/mL) | 3.58 ± 4.90 | 125 | $r_{emp}$=0.002 p=0.984 |
| Intima-media thickness- IMT (mm) | 1.25 ± 0.28 | | |
| Hydrogene peroxide - $H_2O_2$ (nmol/mL) | 4.65 ± 1.62 | 125 | $r_{emp}$=0.190 p=0.034 |
| Intima-media thickness- IMT (mm) | 1.25 ± 0.28 | | |

| Measured parameters | Basic statistical parameters | | Significance (p-value) |
|---|---|---|---|
| | Xsr ± SD | N | |
| Thiobarbituric acid - TBARS reactive substances (μmol/mL) | 1.14 ± 0.23 | 125 | $r_{emp}$=0.550 p=0.0001 |
| Intima-media thickness- IMT (mm) | 1.25 ± 0.28 | | |
| Nitrites - $NO_2^-$ (nmol/mL) | 3.81 ± 1.33 | 125 | $r_{emp}$=-0.131 p =0.144 |
| Intima-media thickness- IMT (mm) | 1.25 ± 0.28 | | |
| Reduced glutathione - GSH (nmol/mL) | 119500.29 ± 17525.20 | 125 | $r_{emp}$=0.112 p =0.214 |
| Intima-media thickness- IMT (mm) | 1.25 ± 0.28 | | |
| Catalase - CAT (U/gHb x $10^4$) | 2.22 ± 1.88 | 125 | $r_{emp}$=0.04 p =0.963 |
| Intima-media thickness- IMT (mm) | 1.25 ± 0.28 | | |
| Superoxide dismutase - SOD (U/gHb x $10^4$) | 32.82 ± 20.67 | 125 | $r_{emp}$=-0.310 p =0.0001 |
| Intima-media thickness- IMT (mm) | 1.25 ± 0.28 | | |

**Table 4.** Correlation between parameters of microinflammation, nutrition and secondary hyperparathyreoidism and carotid artery intima-media thickness

| Measured parameters | Basic statistical parameters | | Significance (p- value) |
|---|---|---|---|
| | Xsr ± SD | N | |
| C-reactive protein - CRP (mg/L) | 10.57 ± 12.23 | 125 | $r_{emp}$=0.038 p=0.673 |
| Intima-media thickness - IMT (mm) | 1.25 ± 0.28 | | |
| Albumine -ALB (g/L) | 38.00 ± 3.02 | 125 | $r_{emp}$=-0.245 p=0.006 |
| Intima-media thickness- IMT (mm) | 1.25 ± 0.28 | | |
| Prealbumine - PALB (g/L) | 0.28 ± 0.09 | 125 | $r_{emp}$=-0.243 p=0.009 |
| intima-media thickness -IMT (mm) | 1.25 ± 0.28 | | |
| Transferine - TRSF (g/L) | 1.50 ± 0.34 | 125 | $r_{emp}$=-0.139 p =0.123 |
| Intima-media thickness- IMT (mm) | 1.25 ± 0.28 | | |
| Vitamin D - VitD (ng/mL) | 17.88 ± 9.63 | 125 | $r_{emp}$=-0.148 p =0.099 |
| Intima-media thickness - IMT (mm) | 1.25 ± 0.28 | | |
| Intact parathormon - iPTH (pg/mL) | 175.13 ± 199.85 | 125 | $r_{emp}$=0.051 p =0.574 |
| Intima-media thickness - IMT (mm) | 1.25 ± 0.28 | | |
| Albumine - ALB (g/L) | 38.00 ± 3.02 | 125 | $r_{emp}$=0.479 p=0.0001 |
| Prealbumine - PALB (g/L) | 0.28 ± 0.09 | | |

## DISCUSSION

Cardiovascular diseases are the most common complications and a leading cause of death in patients with the end-stage chronic kidney disease treated with regular hemodialysis. Cardiovascular mortality in these patients is up to 20 times higher than in the general population. The first step in the prevention and treatment of these patients is good understanding of non-traditional risk factors for the development of the cardiovascular disease (18). Uremic toxins, oxidative stress, microinflammation, malnutrition and endothelial dysfunction are the most significant non-traditional risk factors for the development of atherosclerotic cardiovascular disease in patients undergoing regular hemodialysis. Uremic toxins cause enhanced leukocyte activation (increased leukocyte oxidation and proinflammatory activity), the upregulation in

leukocyte-endothelial interactions and mononuclear cell infiltration into atherosclerotic vascular lesions. Asymmetric dimethylarginine (ADMA), indoxyl sulfate (IP) and p-cresyl sulfate (pCS) are uremic toxins that exert a prooxidative and proinflammatory effect through inhibition of nitric oxide production in endothelial cells and have high atherogenic potential (via accelerating the progression of endothelial dysfunction) (18, 19).

The degree of biocompatibility of the dialysis membrane significantly impacts oxidative stress and microinflammation in patients treated with regular hemodialysis. Results of the studies that have compared the effect of two different dialysis membranes on oxidative stress have shown that the hemodialysis session with a high-flux polysulfone membrane significantly decreases the formation of oxygen free radicals during hemodialysis compared to a low-flux polysulfone membrane (21). The hemodialysis session with a high-flux polysulfone membrane provides better control of the neutrophil function than a low-flux polysulfone membrane (22). Besides the biocompatibility of the dialysis membrane and the presence of endotoxin in the hemodialysis solution, the development of uncontrolled and permanent microinflammation in the population of patients treated with regular hemodialysis is also affected by: uremic toxins, asymptomatic infection of the arteriovenous fistula for hemodialysis, periodontal disease and dislocation of bacteria and endotoxins from intestines to circulation as a result of disruption of the gut microbiome (23).

B-mode ultrasonography of the carotid arteries has been used as a non-invasive diagnostic procedure for the evaluation of atherosclerosis. Patients treated with regular hemodialysis have a significantly higher thickness of the carotid artery intima-media than the general population (absence of kidney disease) (24). Hypoalbuminemia is a good predictor of cardiovascular mortality in patients treated with the regular hemodialysis. In patients with hypoalbuminemia, microinflammatory and oxidative stress, parameters were significantly increased. Results of this study showed that there was a highly statistically significant ($p<0.01$) positive association between the serum albumin and prealbumin concentrations. A high statistically significant ($p<0.01$) negative correlation was found between the serum albumin and prealbumin and intima-media thickness, which is consistent with the results of studies to date, which also confirmed a statistically significant negative correlation with nutritional parameters in patients undergoing the regular hemodialysis (24, 25). In the population of patients undergoing the regular hemodialysis, the thickness of the carotid artery intima-media has a statistically significant positive correlation with the concentration of C-reactive protein and proinflammatory cytokines in serum (24). Results of this study showed no significant positive correlation with the carotid artery intima-media thickness, indicating the need to measure more sensitive microinflammatory parameters in a population of patients undergoing the regular hemodialysis, such as pro-inflammatory cytokines (IL-6, TNF-α). Lipid peroxidation plays a significant role in the process of atherosclerosis. Results of this study are in line with the results of other studies done so far, which also showed a statistically significant positive relationship between the concentration of thiobarbituric acid reactive substances (TBARS) and the carotid intima-media thickness in patients treated with the regular hemodialysis (26, 27). A highly statistically significant ($p<0.01$) negative association was demonstrated between the superoxide dismutase activity in erythrocytes and the carotid artery intima-media thickness, which is in accordance with results of the other authors (25). Between the parameter of oxidative DNA damage (Deoxyribonucleic Acid), 8-OhdG/dG (8-hydroxy-2-deoxyguanosine/deoxyguanosine) ratio, serum malondialdehyde concentration, and carotid artery intima-media thickness in hemodialysis patients a statistically significant positive association was found (28). Carotid artery ultrasound, measurement of cITM (Carotid Intima-Media Thickness) and detection of atherosclerotic plaques are important for assessing the health of the entire arterial vasculature in patients undergoing the regular hemodialysis (29). According to the recommendations of the EAEMP (European Agency for the Evaluation of Medicinal Products), cITM is accepted as a surrogate marker of atherosclerosis, while according to the recommendations of the National Kidney Foundation Kidney Disease Quality Initiative (NKF-DOQI), the carotid artery ultrasound is used for the accurate assessment of cardiovascular artery status in patients treated with the regular hemodialysis, and it can also be used to evaluate vascular calcifications (28, 29). Several investigations have suggested and proved that the dialysis modality and the type of hemodialysis membranes have effects on oxidative stress and microinflammation. For example, the post-dilution on-line hemodiafiltration reduces microinflammation, increases the availability of iron for erythropoesis, reduces erythropoetin resistance, improves nutritional status, the quality of life and the patient outcome compared to the conventional hemodialysis (30). Hemodialysis with a vitamin E-coated membrane over a six-month period significantly reduces the concentration of serum C-reactive protein, interleukin-6, Soluble Intercellular Adhesion Molecule-1 (slCAM1), TBARS and the oxidized LDL cholesterol - ox-LDL. During follow-up, no significant effect on the endothelial cell apoptosis was achieved (31). During the high-flux hemodialysis session and post-dilution on-line hemodiafiltration, a significant amount of vitamin C and oligoelements, which are cofactors of antioxidant enzymes are lost (32). Results of the studies so far have shown that zinc supplementation at a dose of 100 mg/day for eight weeks significantly improves the superoxide dismutase (SOD) activity and significantly reduces the serum malodialdehyde (MDA) concentration. Additionally, selenium supplementation at a dose of 200 μg/day for 12 weeks significantly enhances the glutathione peroxidase (Gpx) activity in erythrocytes (32).

## CONCLUSION

Oxidative stress and malnutrition play a significant role in the development of a carotid artery atherosclerosis in patients undergoing the regular hemodialysis. Statistically significant positive correlation was found between the thickness of carotid arteries intima-media and TBARS plasma concentration, while a high statistically significant negative correlation was found between the SOD activity in erythrocytes and the thickness of carotid arteries intima-media. This indicates the importance of lipid peroxidation in the development of atherosclerosis and atherosclerotic cardiovascular disease in a population of patients treated with the regular hemodialysis. A high statistically significant positive correlation between the serum prealbumin and albumin concentration and the carotid artery intima-media thickness highlights the importance of malnutrition in development. Thus, oxidative stress and malnutrition, both individually and together, significantly contribute to the development and acceleration of atherosclerosis and increase the risk of cardiovascular morbidity and mortality in patients undergoing the regular hemodialysis. A high-flux hemodialysis and on-line post-dilution hemodiafiltration could ensure an optimal control of oxidative stress and malnutrition. The control of oxidative stress might be better when using membranes for hemodialysis coated with antioxidants such as vitamin E, along with the substitution of vitamin C and oligoelements, zinc and selenium.

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## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the Declaration of Helsinki for Medical Research (revised in 2013.). The consent was obtained from patients and the Ethics Committee of the Clinical Center Kragujevac.

## CONFLICT OF INTEREST

There are no conflicts of interest.

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ORIGINAL SCIENTIFIC ARTICLE

# ANTIOXIDANT SUPPLEMENTATION WITH N-ACETYLCYSTEINE AS A PROTECTION AGAINST CISPLATIN-INDUCED MOTOR IMPAIRMENT IN RATS

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## ABSTRACT

*The aim of this study was to estimate a potential beneficial influence of N-acetylcysteine (NAC) in the treatment of cisplatin-induced motor impairment. We included 32 male Wistar albino rats, divided into 4 equal groups: control (received saline on the 1st and 5th day), cisplatin – CIS (received saline on the 1st and cisplatin 7.5 mg/kg i.p. on the 5th day), NAC (received NAC on the 1st and 5th day, 500 mg/kg i.p.), and CIS+NAC (received NAC on the 1st and 5th day, 500 mg/kg i.p. and cisplatin 7.5 mg/kg i.p. on the 5th day) group. Motor performance was estimated by rotarod, grip wire, open field (OF), elevated plus maze (EPM) and beam-walking (BW) tests on the 10th day. Cisplatin administration resulted in decreased motor performance in all tests, except for BW test, compared to the control group. NAC supplementation on its own had no significant effect on motor performance parameters. However, simultaneous administration of NAC along with cisplatin reversed negative impact of cisplatin in rotarod, OF and EPM tests, with no significant effect on the results obtained in grip wire test. The results of this study confirmed numerous motoric manifestations of cisplatin-induced neurotoxicity in rats. However, the decline in most of the estimated parameters was successfully prevented by antioxidant supplementation with NAC.*

***Keywords***: *Cisplatin, N-acetylcysteine, motor performance, rats.*

## INTRODUCTION

Since the almost accidental discovery of its biological effects in 1965 (1), and the first results of clinical trials in 1978 followed by FDA approval, cisplatin (cis-diamminetetrachloroplatinum(IV)) has been pronounced as "the drug of the 20th century" and became the frontline therapy in the treatment of various malignancies (2). Unfortunately, the therapy with platinum-based compounds has very serious limitations including numerous adverse effects.

Although they have not been evaluated as some other adverse effects of cisplatin therapy such as nephrotoxicity (3) and hepatotoxicity (4), clinical manifestations of cisplatin-induced neurotoxicity are very common and represent a serious limitation in therapeutic protocols that include cisplatin. Platinum-based therapy induces neurotoxicity that usually appears in clinical features that are manifested both in the form of peripheral and central neuropathy. The peripheral neuropathy induced by platinum compounds is believed to be the consequence of the initial accumulation of platinum compounds and their metabolites in the dorsal root ganglia after their systemic administration. Formation of platinum-DNA adducts are considered to be a key step in neurotoxicity development (5). The most pronounced effects are observed on large-diameter sensory nerve fibers that appear to be the most affected by platinum drugs. That condition is usually leading to symmetrical glove and stocking type of sensory loss, numbness, tingling, pain and burning sensation (6). Besides, platinum-based therapy also significantly affects central nervous system. The most frequent manifestations of this specific adverse effect are classified as ototoxicity (frequently with permanent hearing loss), nausea and vomiting (7). Also, there are numerous reports about various mood disorders accompanied with platinum-based therapy (8). Many of those clinical symptoms that are characteristic for platinum-based therapy have been evaluated using animal experimental models. Although considered rare, clinical manifestations of motor impairment induced by platinum-based therapy are very intriguing since they may appear as the symptoms that resemble the combined consequences of both peripheral and central neurotoxicity.

Despite the fact that adverse effects of cisplatin toxicity are diverse, it is well-known that underlying mechanisms that are common for cisplatin-induced toxicities are of the same origin. The most commonly described pathophysiological base for adverse effects of cisplatin can be categorized in a few interconnected processes that include oxidative damage, mitochondrial dysfunction, apoptosis and inflammation. Each of those processes may be the target that should be solved in order to reduce cisplatin-induced toxicities and, therefore, achieve the breakthrough in the principle limitations of cisplatin therapy.

The aim of this study was to estimate the potential beneficial influence of N-acetylcysteine (NAC) in the treatment of cisplatin-induced motor impairment, since NAC is established as safe for clinical use (9).

## MATERIAL AND METHODS

A total of 32 male Wistar albino rats, 12-14 weeks old (250–300 g), purchased from the Military Medical Academy (Serbia), were randomly divided into four equal groups. The animals were housed in transparent cages (four per cage) under standard environmental conditions, which include maintaining the constant temperature at 23 ± 1 °C and humidity at 50 ± 5%, with a light/dark cycle (12/12h). All animals were allowed food and tap water intake ad libitum.

The animals were treated in previously defined conditions, according to predefined groups, as follows: control, cisplatin (CIS), NAC and CIS+NAC group. Rats in the control and cisplatin groups received saline (approximately 2 mL i.p.) at the start of the experimental protocol (day 1). At the same time, at the start of trials (day 1), NAC and CIS+NAC group rats were administered with NAC (500 mg/kg i.p., Sigma-Aldrich, Germany). On the fifth day of the trials, the animals in the control group were treated with saline (approximately 2 mL i.p.), while the rats in the CIS group received a single dose of cisplatin (7.5 mg/kg i.p., Merck, France). Also, the animals in the CIS+NAC group, besides the single dose of cisplatin (7.5 mg/kg i.p.), were administered with NAC (500 mg/kg i.p.). Finally, the animals in NAC group were treated with NAC again in a dose of 500 mg/kg i.p.

All animals were exposed to the training protocols for rotarod and beam-walking tests. Training protocols for those tests consisted of 5-7 attempts for each animal, on an adequate apparatus, on the 4th and 8th day of the trial.

Behavioral testing was performed after completing pretreatment trials, on the 10th day. In order to allow accommodation, the animals spent 1-2 hours in the testing room (at approximately 9 a.m.), prior to the testing.

### Rotarod test

Rotarod test is one of the most commonly used tests for the evaluation of motor coordination and balance in the experimental behavioral animal models, especially in rodents (10, 11). The apparatus for this test consisted of rubber coated rotating rod elevated 50 cm above surface. The space below the rod was appropriately equipped (for the prevention of injury following animals' falls) with soft pad. Starting rotation speed was 5 rounds per minute, and then it was gradually increased for 5 rpm each 30 seconds, up to the maximal speed of 60 rpm. On the final testing day, the rats had three attempts and the best score was taken into consideration. The time spent on the rod was expressed in seconds.

### Open field (OF) test

The equipment and basic methodology for OF test, as one of the principle tests for mood disorders testing in animal experimental models, is very common and minutely described in literature (12). The apparatus consisted of the black painted wooden square arena (60×60×30 cm). The testing, that lasted 5 minutes, had begun by placing the animal in the

center of the square arena. Spontaneous activity was allowed throughout the test. As an indicator of the overall motor activity, we estimated the mean velocity. This parameter was calculated by dividing the total distance and test duration, expressed in cm/s.

**Elevated plus maze (EPM) test**

The elevated plus maze test is usually considered the most sensitive behavioral test for the estimation of anxiety state level (13). Like in OF test, spontaneous activity of the animals was evaluated in the apparatus that consisted of two open (50×20 cm) and two enclosed (50×20×30 cm) opposite arms elevated 100 cm above the floor. Again, the test was initiated by placing the animal in the center of the maze (facing the open arm), and each animal was individually allowed 5 minutes for free exploration. As an indicator of the overall motor activity, we estimated the mean velocity. This parameter was calculated by dividing the total distance and test duration, expressed in cm/s.

**Grip wire test**

Grip wire test is used for the estimation of muscle strength and balance in rodents (14). In this test, we used square metal frame (15x15 cm) with wire thickness of 5 mm. The rats were allowed to grasp the wire of metal frame (elevated 30 cm above the floor) with their forepaws. The space below the apparatus was adequately equipped to prevent the fall of animals. On the testing day, the rats were allowed three attempts, and the best score was evaluated for further analysis. In this test, we quantified the time on the wire (expressed in seconds).

**Beam-walking (BW) test**

Beam-walking test is used for the estimation of motor coordination, integration, balance and motor skills (15, 16). The apparatus consisted of the stainless steel beam (100x3x2 cm). The beam had rubber pad in order to reduce animals' slip down, fixed between two vertical steel columns heightened 60 (start point) and 100 cm. Escape wooden box (20x20x20 cm), with the hole (the opening is adapted to thesize of the animal) was located on the highest side of the beam, and represented the safe place for the escape of rats. The cushions were placed below the whole apparatus in order to prevent the possible injury.

In order to remove possible interfering scents, the equipment for behavioral testing was thoroughly cleaned with the water solution of ethanol after each animal testing.

All tests were recorded using a digital video camera mounted above the mazes. Interpretation of video files was conducted by Ethovision software XT 12 (Noldus Information Technology, the Netherlands).

All research procedures were carried out in accordance with the European Directive for the welfare of laboratory animals No 86/609/EEC, the principles of Good Laboratory Practice (GLP), 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.

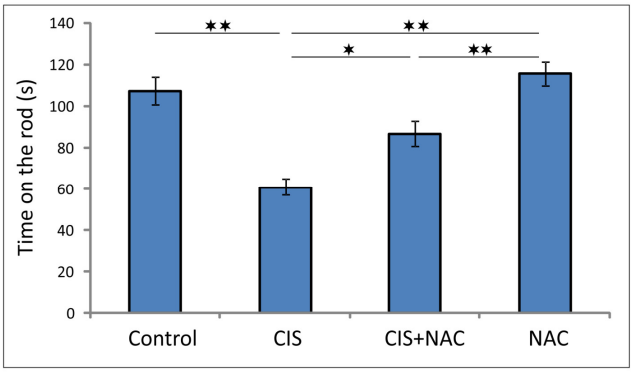
**Statistical Analysis**

The data were presented as means ± S.E.M. After completing the tests for homogeneity (Levene's) and normality (Shapiro-Wilk), comparisons between groups were performed using One-way ANOVA, followed by Bonferroni post hoc analysis. Significance was determined at $p<0.05$. Statistical analysis was performed with SPSS version 20.0 statistical package (IBM SPSS Statistics 20).

## RESULTS

As shown in Figure 1, the applied protocols significantly altered time on the rod estimated in rotarod test (df=3, F=19.127). Cisplatin administration in a single dose (7.5 mg/kg i.p.) markedly reduced this parameter often used as an indicator of endurance performance when compared to control values ($p<0.01$). This effect of cisplatin was successfully attenuated with NAC supplementation ($p<0.05$) reversing the values of this parameter back to control values. NAC itself did not significantly alter the time on the rod when compared to control values.

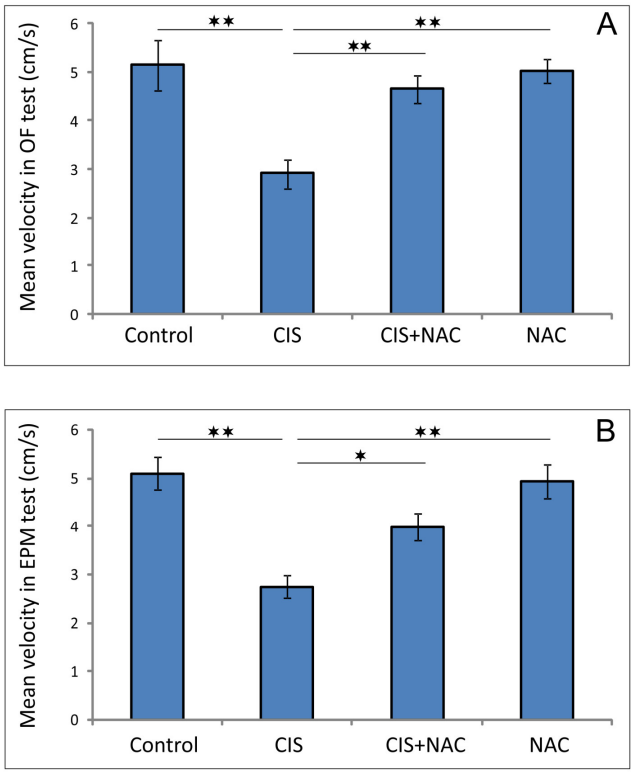
**Figure 1.** The effects of cisplatin and NAC in the rotarod test. The values are mean ± standard error of the mean (SEM), *denotes a significant difference $p<0.05$, **denotes a significant difference $p<0.01$.




The motor performance was also evaluated in this study by means of two behavioral tests, originally used for the estimation of mood disorders (OF and EPM test, Figure 2A and B, respectively). The same parameter, a mean velocity during five-minute testing, was determined in both tests, in order to analyze the influence of the applied protocols. Both indicators of motor activity in OF and EPM test were significantly altered in this experimental design (df=3, F=8.552 and 12.827, respectively). Cisplatin application resulted in significant increase in MV in both OF and EPM test ($p<0.01$) compared to control. However, although NAC administration on its own showed no significant effect when compared to control values, simultaneous administration with cisplatin

resulted in diminishing effect on cisplatin-induced decline of overall motor activity in OF ($p<0.01$) and EPM test ($p<0.05$). The attenuation of cisplatin-induced motor impairment by simultaneous administration of NAC was sufficient to increase the mean velocity observed in both tests back to control values.

**Figure 2.** The effects of cisplatin and NAC on the mean velocity in the open field (A) and the elevated plus maze (B) 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 evaluation of motor performance by means of muscle strength estimation in this study was performed in grip wire test (Figure 3). The applied protocols significantly influenced the time on the wire ($df=3$, $F=14.893$). A single dose of cisplatin significantly lowered this parameter of muscle strength ($p<0.01$). Unlike previously described tests, the administration of NAC along with cisplatin treatment failed to reverse the diminishing effect of cisplatin on muscle strength. The time on the wire, in this (combined) group remained below the control values ($p<0.05$). On the other hand, when applied solely, NAC produced no significant effect on this parameter when compared to control group.

The testing of motor abilities by means of motor coordination in this study was performed in beam-walking test (Figure 4). However, neither of applied protocols had shown significant impact on the time to cross the beam ($df=3$, $F=2.372$).

**Figure 3.** The effects of cisplatin and NAC in the grip wire test. The values are mean ± standard error of the mean (SEM), *denotes a significant difference $p<0.05$, **denotes a significant difference $p<0.01$.

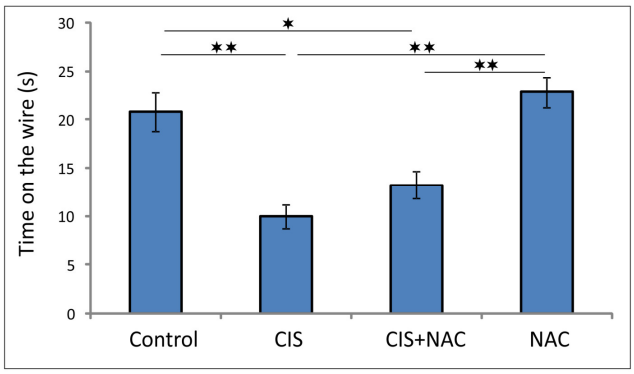
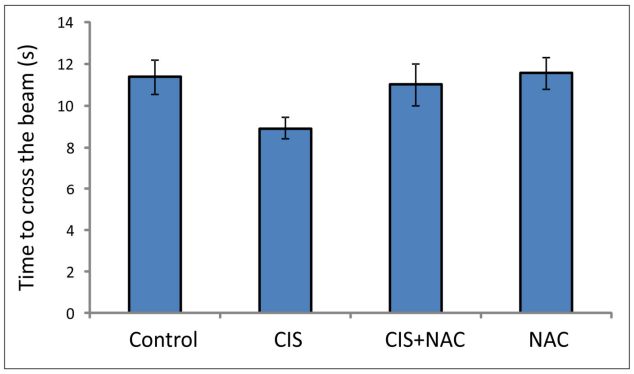



**Figure 4.** The effects of cisplatin and NAC in the beam-walking test. The values are mean ± standard error of the mean (SEM).




## DISCUSSION

Apart from the unquestionable benefits of cisplatin usage in the treatment of various malignancies, we are still facing some major difficulties in this therapeutic approach. Predominantly, they are connected with numerous adverse effects that are often reported during cisplatin therapy. However, the numerous side effects of cisplatin usually have similar pathophysiological background that includes oxidative damage, mitochondrial dysfunction, apoptosis and inflammation. Therefore, it seems worth to investigate the potential therapeutic approaches that can reduce the processes that usually limit platinum-based therapy.

According to the results obtained in this study, it seems that even initial administration of cisplatin produces respectable effects on motor abilities in rats. However, the response to cisplatin in an early phase of treatment is very complex, with strong variations depending on the specific aspects of motor performance. The highest (negative) impact of cisplatin was observed in tests used for estimation of continuous, prolonged motor activity. Namely, a single dose of cisplatin in just a few days was sufficient for significant reduction of

motor performance observed in rotarod test (Figure 1). Our results are in line with previously reported data regarding the reduction of the time on the rod following chronic treatment with cisplatin (17). This test is usually considered a good indicator of endurance performance. Therefore, this kind of exercise, in order to achieve results at the physiological level, aside of optimal functioning of neuromuscular units with an adequate reflex coordination at the spinal cord level, requires undisturbed function of supporting systems, such as cardiovascular and respiratory system. So, the basic mechanism of adverse effect of cisplatin should be addressed both to affection of basic neuromuscular units involved in this characteristic motor pattern continually repeated in this test, and, at the same time, to systemic action of cisplatin that includes the adverse effects on cardiovascular system. Indeed, it has been reported that platinum-based compounds (oxaliplatin) produced significant motor impairment by means of endurance performance that was accompanied with verified signs of peripheral neuropathy (18). Nevertheless, it has been also previously noticed that cisplatin administration produced deleterious effects on cardiovascular system, even after the treatment in a single dose (19, 20).

The similar effects of cisplatin in this study were also observed in other tests for continual motor activity, only this was spontaneous (explorative) activity in OF and EPM test, instead of forced (endurance) performance in rotarod test. Again, a single dose of cisplatin significantly diminished overall motor activity, expressed by means of decline in mean velocity in both tests (Figure 2). Besides the potential explanation for negative impact of cisplatin on motor performance presented for rotarod test, the influence of cisplatin on motor action in OF and EPM tests may require additional analysis based on the specific, original nature of those two tests. As a matter of a fact, both tests are considered sensitive for estimation of mood disorders, such as increased anxiety level. Since our previous report (21) confirmed clear and strong anxiogenic effects following a single dose of cisplatin (the same dose as applied in this study), and due to the fact that increased anxiety has been proposed as a reason for decreased motor performance in both OF and EPM tests (22), it seems that, at least, the part of observed reduction in mean velocity of moving in OF and EPM tests should be addressed to anxiogenic effect of cisplatin that takes place concomitantly to affection of motor units. Even more, as those two tests do not exert forced (extreme) motor performance (like rotarod test) that is sufficient to reveal the decreased abilities of cardiovascular system following cisplatin treatment, it is likely that cisplatin-induced motor impairment observed in OF and EPM tests may appear as a result of combined motor-disturbing and anxiogenic effects of cisplatin. The results for an overall motor activity in primary behavioral tests following cisplatin treatment are in accordance with previously published reports (17, 23).

The results obtained in grip-wire test (Figure 3) offer the conformation that cisplatin also had a negative impact on other specific feature of physical performance, such as strength and/or power. Besides the previously described adverse effect on neuromuscular units and the regulation of their function, the observed decline in principal strength performance following cisplatin administration could be also accompanied by previously described disturbances in body composition. Namely, it has been reported that cisplatin treatment resulted in significant decrease in body weight by 27.5% after the three-week administration protocol with a total dose of 15 mg/kg (24). The reduction of body weight as a result of cisplatin treatment is very likely to be accompanied by simultaneous reduction of skeletal muscle mass, which can lead to proportional decline of motor performance by means of strength and/or power. This is in accordance with previously published study with a single dose of cisplatin (16 mg/kg), which resulted in significant muscle atrophy in female rats of the same age as in this study (25). The proposed explanation can even be augmented by the fact that all animals evaluated in this study were male, so the reduction of skeletal muscle mass following body weight decline should be potentiated to a higher level. This conclusion is in line with reported muscle mass wasting characterized by decline in myofiber diameter and cross-sectional fiber areas in male rats (24).

Unlike the other test that estimated other features of physical performance, the results obtained in the beam-walking test revealed that a single dose of cisplatin failed to significantly affect specific motoric elements (Figure 4), such as balance and coordination. On the other hand, the literature data describes the motor impairment following cisplatin treatment that was accompanied with specific cerebellar injury manifested by degenerative changes including shrunken nuclei and eosinophilic cytoplasm in Purkinje cells (23). The observed differences can be addressed to different experimental protocols, including the duration (5 weeks *vs*. single dose) and the total applied dose (25 *vs*.7.5 mg/kg), when compared to experimental protocol performed in this study. It seems that adverse impact of cisplatin on principally cerebellar-controlled functions may appear later when compared to spinal cord level and systemic responses to cisplatin administration.

The results of this study also showed the beneficial effects of antioxidant supplementation with NAC in the treatment of cisplatin-induced motor impairment. This protective effect of NAC was significantly manifested in several tests. For example, NAC supplementation along with cisplatin treatment attenuated cisplatin-induced decline in endurance performance estimated in rotarod test (Figure 1). This is in line with previously reported protective effect of new NAC-based product (N-acetylcysteine amide) that also improved performance in rotarod test following iatrogenic-induced neurotoxicity (26). Also, it should be mentioned that NAC administration showed beneficial effects in pathophysiological events characterized by neurodegeneration (27) that is similar to manifestations of cisplatin-induced neurotoxicity.

Furthermore, according to the results of this study, NAC supplementation showed beneficial effects on the impairment of strength/power feature of physical performance. Our

results obtained in grip wire test are in accordance with the results of the studies where the cisplatin-induced (20 mg/kg in two doses and 25 mg/kg in five doses, respectively) adverse effects in grip wire test were prevented by antioxidant supplementation with natural products from tomato (28) and antioxidant rich nuts (17). The results obtained in OF and EPM test also confirmed the protective effect of NAC supplementation by means of correcting of cisplatin-induced decline in overall motor activity. This manifestation of beneficial effect of NAC, besides previously commented action of NAC, should be also analyzed on the basis of corrective role of NAC in specific mood disorders that could be estimated in those two tests. In general, it has been confirmed that antioxidant supplementation can diminish anxiogenic effect of cisplatin that, in turn, may lead to decreased motor performance in OF and EPM tests (21). Obviously, NAC supplementation had no impact on the motor performance observed in BW test. This can be explained by the fact that applied experimental protocols were not sufficient to produce initial motor impairment that could be estimated in BW test. Therefore, the trials performed in this study do not seem suitable for the evaluation of the NAC impact on cisplatin-induced cerebellar dysfunction.

## CONCLUSION

The results of this study confirmed numerous motoric manifestations of cisplatin-induced neurotoxicity in rats. However, the decline in most of the estimated parameters was successfully prevented by antioxidant supplementation with NAC.

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## ETHICS APPROVAL

All research procedures were carried out in accordance with the European Directive for the welfare of laboratory animals No 86/609/EEC, the principles of Good Laboratory Practice (GLP), 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

## CONFLICT OF INTEREST

There are no conflicts of interest.

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REVIEW PAPER

# ROLE OF METASTATIC LYMPH NODES TO TOTALLY REMOVED LYMPH NODES RATIO IN BREAST CANCER?

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## ABSTRACT

*To determine prognostic ratio of metastatic lymph nodes to totally removed lymph nodes (MLN/TRLN) on overall and progression-free survival with diagnosis of breast cancer. Radiation Oncology department of Kayseri Training and Research Hospital, relationship of MLN/TRLN between prognosis and other prognostic factors was evaluated in T1-3 and N1-3 non-metastatic breast cancer patients. Two hundred female patients and 5 male patients with an average age of 56 years were enrolled in this study. Of all patients, 63.4% of the patients were postmenopausal and modified radical mastectomy was performed in 96.6% of them. While 93.2% of the patients were diagnosed with invasive ductal carcinoma, 52.7% of them had clinical N1 disease, 62% of them were staged as T2, 94% of them received chemotherapy and 57.1% of them received hormonal therapy. Metastatic lymph node ratio below 0.2 was 48.8%, between 0.21-0.65 it was 35.1% and above 0.65% it was 16.1%. Five-year Overall survival and progression-free survival rates were 76% and 58% respectively. Statistically significant difference was found between MLN/TRLN and age (p=0.044), chemotherapy (p=0.039), pathological lymph nodes (p <0.001) according to Pearson's Chi-Squared test. Factor affecting overall survival was Progesterone receptor status (p=0.021) and for progression-free survival they were gender (p=0.003) and human epidermal growth factor receptor 2 (p=0.018). Univariate and multivariate analysis found that gender (p=0.04, OR 5.9, CI: 1.7-19.6) and lymph node (p=0.05, OR: 1.4, CI: 0.9-2.1) were significant factors affecting progression-free survival.*
*MLN/TRLN was shown to have no effect on prognosis in non-metastatic breast cancer patients due to small number of patients and short follow-up period.*

***Keywords***: *Breast cancer, metastatic lymph nodes, removed lymph nodes.*

## INTRODUCTION

There have been many studies in the treatment of breast cancer starting from the discovery of anti-cancer drugs and radiotherapy (RT). Given that breast cancer show wide variety of biological difference, heterogeneity and consists of subgroups with different prognosis, there is still no consensus on the optimal treatment. Therefore, in order to individualize treatment, it is required to identify patients with good or poor prognosis (1,2).

While lymph node metastasis is an indicator of poor prognosis, it is still the most powerful prognostic factor for disease-free survival and overall survival. Also axillary lymph node involvement is still considered the most important sign of progressive disease. Therefore axillary lymph node dissection besides providing locoregional control, has huge contribution to accurate staging and to the decision-making process of which patient should be given adjuvant therapy (1-4). In the literature, in cases with node-negative axillary dissection, 5-year survival was reported to be 88% and 10-year survival, 65%. In cases with node-positive axillary dissection, when 4 or more axillary lymph node metastases were present, 5-year survival was reported to be 32% and 10-year survival, 13% (3).

Pathological staging after axillary dissection is gold standard to determine if chemotherapy and/or radiotherapy will be useful. Removal of numerous lymph nodes and excision of metastases provide better prognosis. In many studies, it was observed that number of metastatic lymph nodes were increased in parallel to the number of dissected lymph nodes. In some studies 10 or more dissected lymph nodes, and in some of them 16 and more dissected lymph nodes were shown to represent axillary status in a reliable manner. However, there are reports saying that removal of 4 to 6 lymph nodes with more limited dissection would be enough. Yet, the optimal number of lymph nodes and extension of axillary dissection to provide axillary control and adequate prognostic value haven't been clarified.

Radiotherapy has an important role in the breast cancer treatment. Mastectomy, large tumors, tumors with lymph node metastases and breast-conserving surgery are indications for adjuvant RT. In the treatment planning, target volume is determined by evaluation of patient, tumor, and treatment characteristics. Radiotherapy after mastectomy is often recommended for T3-4 cases with high risk of local recurrence or patients with 4 and more axillary lymph node metastasis. However, in the recent years, patients with 1 to 3 lymph node metastasis have been shown to be sensitive to radiotherapy and it has been increasingly indicated in these patients. Currently the indication for adjuvant RT and determination of RT region are decided according to the number of metastatic lymph nodes (1).

In the recent studies, ratio of metastatic lymph nodes to totally removed lymph nodes (MLR) has been reported to provide better prognostic information while grouping nodal disease according to number of metastatic lymph nodes and making adjuvant treatment plan decisions.

In this study, relationship of MLR between prognosis and other prognostic factors was evaluated in T 1-3 and N 1-3 non-metastatic breast cancer patients. Two hundred female patients and 5 male patients with an average age of 56 years were enrolled in this study. Of all patients, 63.4% of the patients were postmenopausal and modified radical mastectomy was performed in 96.6% of them. While 93.2% of the patients were diagnosed with invasive ductal carcinoma, 52.7% of them had clinical N1 disease, 62% of them were staged as T2, 94% of them received chemotherapy and 57.1% of them received hormonal therapy. Metastatic lymph node ratio below 0.2 was 48.8%, between 0.21-0.65 it was 35.1% and above 0.65% it was 16.1%.

The median follow-up time was 20 months (range: 1-112 months). Five-year overall survival and progression-free survival rates were 76% and 58%, respectively. Twenty nine patients died due to either cancer related or other causes. In 43 patients local recurrence or distant metastasis were detected. For age ($p=0.044$), chemotherapy ($p=0.039$) and pathological lymph nodes ($p<0.001$), statistically significant results were observed with MLR according to Pearson's Chi-Squared test. Factor affecting overall survival was Progesterone receptor status ($p=0.021$) and for progression-free survival they were gender ($p=0.003$) and human epidermal growth factor receptor 2 ($p=0.018$) (Table 1). According to univariate and multivariate analysis, gender ($p=0.04$, OR 5.9, CI: 1.7-19.6) and lymph nodes ($p=0.05$, OR: 1.4, CI: 0.9-2.1) were significant factors affecting progression-free survival.

In our study, in the light of literature, the threshold for MLR was taken as 0.20. Although overall survival and progression-free survival were observed better in patients with MLR of less than 0.2 than in MLR of 0.21-0.65, and above 0.65, it wasn't statistically significant. When MLR was examined for relation to other parameters, age ($p=0.044$), chemotherapy ($p=0.039$) and pathological lymph nodes ($p<0.001$) were found to be significant. Vinh-Hung et al stated that in node-positive breast cancer patients MLR had prognostic importance and it was an alternative to pN stage (6). In the meta-analysis of Liu et al, it was reported that in breast cancer patients, MLR had prognostic significance when the threshold (cut-off) values were taken as 0.2 and 0.65(4). There are also reports using 0.25 and 0.7. These differences were mostly result of varied number of patients and treatment modalities (e.g., surgical approach, adjuvant therapy, histological type, size, grade and hormone receptor status of the tumor) (2,4,5,6). In the study of Altundag et al, they evaluated the importance of pathologic node (pN) stages and MLR classification in predicting prognosis. They analyzed N1, N2 and N3 clinical disease by dividing them into subgroups and reported that both pN stage and MLR classification were independent prognostic factors for overall survival; MLR classification was found to show prognostic distinction for overall survival more accurate and clearer thus it could give more accurate results in both staging and adjuvant

treatment planning (2). Fortin et al worked with T1-2 stage node-positive patients and suggested axillary radiotherapy to patients who had 1 to 3 lymph nodes metastasis with MLR of 0.4 and above and to patients who had 4 or more lymph node metastasis with MLR above 0.5 (5). In the study of Truong and colleagues which was published in 2005, they evaluated patients who had no radiotherapy treatment before and who had 1 to 39 lymph nodes removed. Most significant MLR threshold was found as 0.25 to predict locoregional recurrence, distant metastasis and overall survival. Radiotherapy was suggested to patients with MLR more than 0.25 after mastectomy. In 2007, in their other work, to predict locoregional recurrence, they reported that MLR was more significant than pN stage and also because 10-year locoregional recurrence risk was above 20% in patients with MLR more than 0.2, they recommended radiotherapy to these patients after mastectomy (8).

Karlsson et al found that in node-positive patients who did not receive radiotherapy, with the increase in number of non-metastatic lymph nodes, locoregional recurrence was significantly reduced and in the group of patients who had 1 to 3 positive lymph nodes and less than 10 non-metastatic lymph nodes, it was indicated that a 10-year cumulative incidence of locoregional recurrence was over 20% and these patients should receive post-mastectomy radiotherapy (9).

## CONCLUSIONS

In conclusion, MLR was shown to have no effect on prognosis in non-metastatic breast cancer patients due to small number of patients and short follow-up period. These studies should be supported by more extensive researches to elucidate the role of MLR in prognosis and in adjuvant radiotherapy indication.

**Table 1.** Overall (OS) and progression-free survival (PFS) and p value (p<0.05)

| Variable | No. of patients | Mean OS (months) | *P* value (<0.05) | Mean PFS (months) | *P* value (<0.05) |
|---|---|---|---|---|---|
| **Gender** | | | **0.242** | | **0.003** |
| male | 5 | 29.5 (29.1-29.7) | | 22.4 (14.5-30.3) | |
| female | 201 | 84.4 (73.3-95.4) | | 78.4 (69.1-87.6) | |
| **Age (years)** | | | **0.885** | | **0.988** |
| <60 | 128 | 83.5 (68.8-98.2) | | 77.2 (65.9-88.4) | |
| =>60 | 77 | 80.1 (63.8-94.7) | | 70.7 (53.9-87.5) | |
| **Menopausal status** | | | **0.804** | | **0.318** |
| premenopausal | 68 | 65.3 (57.1-73.5) | | 55.3 (46.1-64.5) | |
| postmenopausa | 131 | 83.0 (69.8-96.2) | | 82.1 (71.2-39.6) | |
| unknown | 6 | - | | 30.2 (20.8-39.6) | |
| **Performance status** | | | **0.199** | | **0.685** |
| ECOG 0 | 120 | 93.4 (83.9-103.0) | | 77.0 (64.4-89.6) | |
| ECOG 1 | 85 | 74.0 (72.6-94.7) | | 71.6 (57.7-85.5) | |
| **Pathology** | | | **0.980** | | **0.694** |
| invasive ductal | 191 | 86.2 (75.7-96.7) | | 78.4 (68.8-88.0) | |
| inflematuar | 9 | 68.3 (51.8-84.8) | | 56.9 (31.1-82.7) | |
| the other | 5 | 70.9 (35.9-1058) | | 54.8 (19.9-98.7) | |
| **Tumor size** | | | **0.883** | | **0.147** |
| I | 43 | 86.9 (71.6-102.3) | | 93.3 (79.5-107.1) | |
| II | 127 | 59.3 (53.1-65.6) | | 53.1 (46.1-60.1) | |
| III | 35 | 45.6 (40.0-51.1) | | 40.5 (34.0-47.0) | |
| **Lymph node status** | | | **0.275** | | **0.079** |
| I | 108 | 85.2 (72.1-98.3) | | 78.3 (66.0-90.6) | |
| II | 51 | 68.1 (59.3-76.9) | | 61.6 (52.7-70.5) | |
| III | 46 | 34.3 (30.0-38.5) | | 51.1 (20.0-82.2) | |
| **Histologic grade** | | | **0.699** | | **0.719** |
| I | 48 | 94.3 (78.4-110.2) | | 85.8 (69.4-101.5) | |
| II | 90 | 76.3 (63.6-92.1) | | 69.5 (57.5-81.4) | |
| III | 53 | 63.8 (52.7-53.5) | | 60.5 (48.0-72.9) | |
| unknown | 14 | 47.2 (38.9-55.4) | | 44.0 (34.8-53.1) | |
| **Perinodal involvement** | | | **0.349** | | **0.676** |
| no | 70 | 89.9 (73.1-106.8) | | 76.2 (61.2-91.3) | |

| | | | | | |
|---|---|---|---|---|---|
| yes | 135 | 77.2 (64.8-89.6) | | 74.5 (63.8-85.2) | |
| **Lymphovascular invasion** | | | **0.121** | | **0.596** |
| no | 87 | 94.2 (82.7-105.7) | | 72.1 (57.7-87.5) | |
| yes | 118 | 66.7 (72.6-94.7) | | 65.7 (57.7-74.7) | |
| **ER status** | | | **0.808** | | **0.114** |
| positive | 120 | 86.4 (74.8-98.0) | | 81.8 (70.2-93.4) | |
| negative | 68 | 66.5 (58.4-74.6) | | 66.3 (55.6-77.1) | |
| unknown | 17 | 82.2 (72.6-94.7) | | 52.0 (29.6-74.5) | |
| **PR status** | | | **0.021** | | **0.484** |
| positive | 102 | 83.0 (67.5-98.6) | | 82.2 (70.6-93.9) | |
| negative | 99 | 72.1(62.3-81.9) | | 59.6 (48.7-70.4) | |
| **unknown** | 4 | 21.6 (72.6-94.7) | | 26.2 (11.5-40.8) | |
| **HER2** | | | **0.761** | | **0.018** |
| negative | 123 | 88.4 (77.0-99.7) | | 86.2 (76.2-96.2) | |
| positive | 65 | 47.1 (39.2-54.9) | | 39.4 (32.2-46.6) | |
| unknown | 17 | 68.6 (57.2-80.0) | | 68.0 (47.6-88.4) | |
| Number of removed lymph nodes | | | **0.423** | | **0.764** |
| **<16** | 100 | 81.0 (69.5-92.5) | | 74.2 (61.2-87.2) | |
| **=>16** | 105 | 80.8 (72.6-94.7) | | 76.5 (64.4-88.6) | |
| **MLR score** | | | **0.915** | | **0.526** |
| **0.0-0.2** | 100 | 83.1 (70.2-96.0) | | 77.5 (64.9-90.0) | |
| **0.21-0.65** | 72 | 82.1( 62.9-101.39 | | 72.9 (58.0-87.7) | |
| **=>0.65** | 33 | 57.4 (45.3-69.5) | | 57.2 (45.2-69.2) | |
| **Surgery** | | | **0.273** | | **0.234** |
| mastectomy | 196 | 83.5 (71.9-95.1) | | 76.2 (66.6-85.8) | |
| lumpectomy | 9 | 77.5 ( 71.9-95.1) | | 75.5 (62.9-88.0) | |
| **Chemotherapy** | | | **0.307** | | **0.610** |
| yes | 193 | 86.1 (75.3-96.9) | | 75.8 (65.9-85.7) | |
| no | 12 | 60.2 (36.7-83.7) | | 58.4 (30.7-86.3) | |
| **Hormonotherapy** | | | **0.450** | | **0.204** |
| yes | 117 | 86.0 (71.8-100.1) | | 72.2 (60.3-84.1) | |
| no | 88 | 64.4 (55.3-73.5) | | 66.6 (57.8-75.3) | |

## ABBREVIATIONS

**ECOG**: Eastern Cooperative Oncology Group;
**MLR**: metastatic lymph nodes to totally removed lymph nodes ratio;
**N** : lymph node;
**OS :** overall survival;
**PFS** : progression-free survival;
**T** : tumor size.

## CONFLICT OF INTERESTS

The authors declare no conflicts of interest.


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# MUCOCELE OF THE APENDIX - A CASE REPORT AND REVIEW OF THE REFERENCES

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## ABSTRACT

*Mucocele of the apendix is a rare clinical and pathological entity with a higher incidence in the female population, and non-specific symptomatology that includes more diagnoses including neoplastic and non-neoplastic causes of the apendix dilation and mucin proliferation. With obtained numerous variations in symptomatology, clinical picture, intraoperative presentation, surgical treatment and histopathological findings, the aim of this paper is an inspection into current knowledge of this disease, its histopathological characteristics, differential diagnosis and current treatment modalities. We present a patient with a clinical picture of the acute abdomen and numerous comorbidities. Ultrasound diagnostics indicated the presence of thin-walled dilatation of the small intestine, especially in the lower right quadrant, as well as the presence of free fluid in the peritoneal cavity. Apendectomy and removal of the mucocele and resection of the gangrenous sigmoid colon according to Hartmann were done. A definitive histopathological finding suggests ischemic colitis and retention mucocele of the apendix with normal mucosa. Histopathologically, four types of mucocele apendixes are distinguished, namely the retention mucinous cyst, mucosal hyperplasia, mucinous cystadenoma and mucinous cystadenocarcinoma. Surgical treatment is the method of choice in treating these tumors, either open or laparoscopic. Imperative is the preservation of the integrity of the entire mucocele, due to the possible dissemination of contents of the cyst and the subsequent development of pseudomyxoma peritonei.*

***Keywords***:*Mucocele of the apendix, surgical treatment, pseudomyxoma peritonei.*

## INTRODUCTION

Mucocele of the apendix is a rare but well-known clinical and pathological entity with non-specific symptomatology which includes more diagnoses, including neoplastic and non-neoplastic causes of the apendix dilation and mucin proliferation. This disease is characterized by dilatation of a lumen as a result of an accumulation of a large amount of mucus (1, 2). A patient's clinical symptoms may include pain in the right lower quadrant of the abdomen, palpable abdominal mass, nausea, vomiting, weight loss, gastrointestinal bleeding, and signs of intussusception of the intestines (1, 3). Although with the incidence of pathological changes of 0.2 to 0.3% in the general population, with a slightly more frequent incidence in women and people after the age of 50, usually can be detected during abdominal surgery, routine radiological diagnosis or during endoscopy (4-6). Approximately 23–50% of patients are asymptomatic (4, 7).

Ultrasound findings usually show a cystic mass that is filled with dense content and marginal calcification. The CT is the most accurate method of diagnostics. The CT can be used to discover the signs specific to mucocele with high accuracy: appendix lumen more than 1.3 cm, its cystic dilatation, and wall calcification (8).

In the case of appendiceal mucocele, resection is recommended for possible malignancy, and the imperative is to maintain an integrity of the mucocele wall during surgery to prevent peritoneal pseudomyxoma (9-11). However, although laparoscopic intervention is traditionally contraindicated in mucocele apendix due to the perforation risk, some studies indicate that possible benefits of the minimally invasive surgical treatment over classical appendectomy (12, 13). Histopathologically, four types of this entity are distinguished, namely retention mucinous cyst, mucosal hyperplasia, mucinous cystadenoma and mucinous cystadenocarcinoma (1, 3, 14).

With obtained numerous variations in symptomatology, clinical picture, surgical treatment and histopathological findings in the case of this clinical-pathological entity, the aim of this paper was to present the case of mucocele of the appendix, and to explain more closely the problems that may arise in clinical practice.

## CASE REPORT

A patient was first referred to a surgeon due to abdominal pain and diarrhea. At the reception the patient's follower (son) states that the patient did not vomit, had a normal miction control, and that he had a stroke 4 months ago and that he was immobile and poorly communicative ever since. The companion also states that the patient operated the abdominal hernia 30 years ago. He was regularly used acetylsalicylic acid for cardiological and neurological purposes, and the follower negates patient's allergy to medicines and food.

Objectively, the patient on reception was conscious, noncommunicative, afebrile, eupneic and with normal skin color. Locally, anterior abdominal wall was at the chest level, palpatory painfully sensitive on the deep palpation to peritoneal reaction and with rigidity. External inguinal openings are free, and kidney boxes are insensitive to succusion. Laboratory findings showed that patient has granulocytosis (17.81) in the differential blood count, and the ultrasound of the abdomen because of meteorism was insufficient. The liver is seen in a minimal part, which is insufficient for interpretation, and the pancreas and spleen are covered by gases. The right kidney does not visualize, while the left is without a path. Urinary bladder was empty. The dilated intestinal convoluts are seen with the linear probe, at the right ilica fossa with diameter up to 38 mm. Also, on the right, perivesically, between the intestinal bruises a smaller amount of free liquid is observed. Based on the clinical picture and laboratory findings, the patient was hospitalized and initially treated conservatively.

As an adequate clinical response did not follow, the patient was prompted for surgical treatment on the same day. After a short and immediate operative preparation, the patient undergone general endotracheal anesthesia. After the opening of the abdomen, a gangrenally-altered part of the sigmoid column (length of about 20 cm) was discovered by exploration, with a slightly cloudy liquid paracolic and in the Douglas space. Further exploration found a cystic change of about 4x2 cm (a cystic expansion of the distal two-thirds of appendix), which was in close contact with the terminal ileum. After exploration, appendectomy (Figure 1) was performed, with complete removal of the cyst without perforation, and resection of the gangrenous part of the sigmoid colon by Hartmann procedure (Figure 2). Then the distal part of the colon was closed with the GIA stapler, and the proximal part of the left colon was derived on the lateral left abdominal wall – in the form of an unipolar colostomy. Local toilet and haemostasis were made. Douglas's pouch was clean. An abdominal drain was placed in the Douglas space, followed by the closure of the surgical space by layers. Appendix with cyst (Figure 3) and sigma were sent to a pathohistological analysis with a clinical diagnosis: Abdomen acutum pp gangrenae coli sigmoidei, Mucocela appendicis, Collitis, and as an operative procedure: Laparotomia mediana supra et infraumbilicalis, Appendectomia et extirpatio mucocoellae appendicis, Resectio coli sigmoidei sec Hartmann, Drainage cavi Douglasi. The post-operative course worsened, due to current comorbidities, and the next day patient was prolonged further treatment in an adequate ICU.

A pathohistological examination has shown that it is ischemic colitis (peritonitis fibrinospurulent acuta focalis) and appendiceal mucocele, i.e. retention type of mucocele with normal mucosa (Figure 4).

**Figures 1. i 2.** Intraoperative finding of mucocele of the apendix. An intact cyst is seen as well as a partially altered gangrenous part of the sigmoid colon

**Figure 1.**

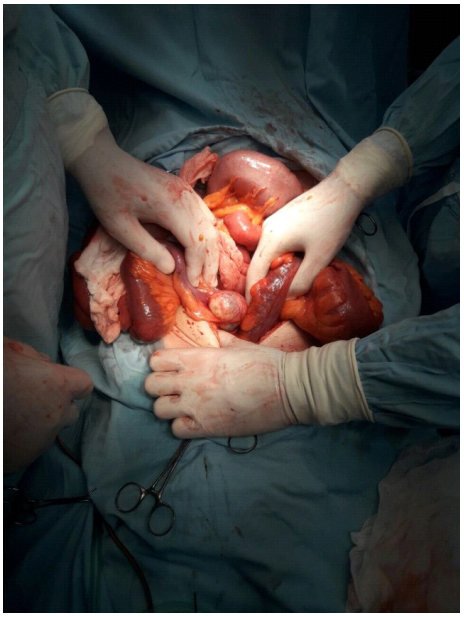


**Figure 2.**

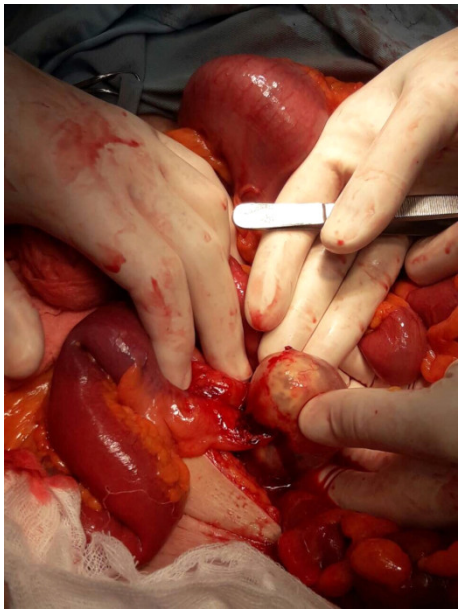


**Figure 3.** Material for biopsy - preparation of mucocele of the apendix (gross appearence)

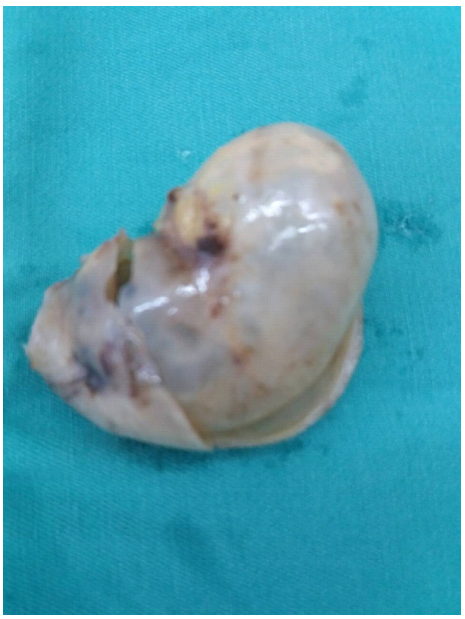
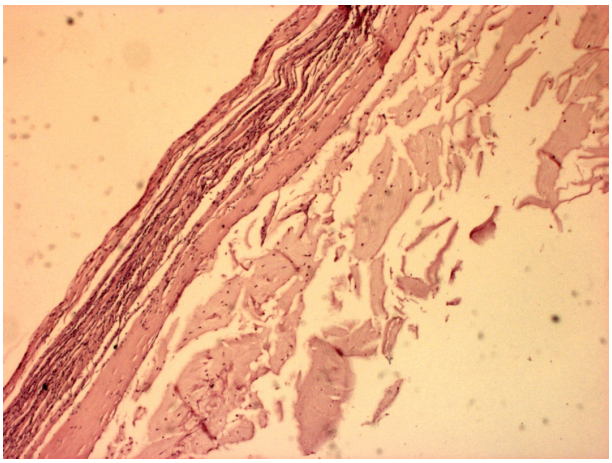


**Figure 4.** Histopathological finding (microscopic slide) of mucocele of the apendix with normal mucosa



## DISCUSSION

Mucocele of the appendix was recognized first by Karl Freiherr von Rokitansky in 1842. (4, 15,16) and was described by Virchow in 1863 (16). Feré applied the name "mucocele" in 1877 (16). The major pathological mechanisms that are responsible for the formation of appendiceal mucocele are elevated appendiceal pressures as sequelae of luminal obstruction caused by prior inflammation, mucosal hyperplasia, or appendiceal lesions (fecaliths, endometriosis, diverticulae, polyps) and tumors of the appendix (carcinoid, cystadenoma, cystadenocarcinoma) (3, 7). Mucinous cystadenocarcinomas are less common than mucinous cystadenomas. This neoplasm rarely spreads through the lymphatic or vascular routes, but has an atypical tendency of penetration and spread well beyond the appendix to the peritoneum (3, 10). Moreover, in advanced cases the whole peritoneal cavity becomes distended with adhesive semi-solid mucous; a

condition termed pseudomyxoma peritonei (17, 18). Complications of appendicular mucocele include: the intestinal bleeding/obstruction, melena, pyonephrosis, intussusception, perforation, peritonitis and pseudomyxoma peritonei (7, 19).

One of basic principles of the patients' surgical treatment with mucocele of the appendix is that an intact mucocele is not a danger to the patient. However, if it is perforated, and consequently its content is spread into the peritoneum, that finally leads to the development of peritoneal pseudomyxoma, whose surgical treatment is difficult, and the prognosis for the patient is not always favorable (9, 20). However, in this case, the patient was primarily operated due to an advanced gangrenous process on the sigmoidal part of the colon, while the appendiceal mucocele was an incidental finding. For that reason we did not perform any extensive surgical procedure on the right colon, but only appendectomy with intact cyst excision.

In the references it has traditionally been indicated that in the case of mucocele apendix the advantage is given to open surgical techniques, in comparison with laparoscopic methods. If, however, a laparoscopic technique is applied and an appendiceal mucocele is detected, it is necessary to continue the surgery with an open surgical technique, for the following: 1) prevention of perforation of the cyst during surgery, and preventing the spillage of content in the peritoneal cavity; 2) an open surgical method, the transparency of the operating field is better, and palpation is enabled, as well as a direct examination of the site in the abdomen where the mucocele is localized to (18, 20, 21). However, in recent years, there have been numerous studies that have indicated the safety of laparoscopy in the surgical treatment of this disease (13, 22-25). Very important algorithm for the selection of the type of surgery has been proposed by Dhage-Ivatury and Sugarbaker (26). It pointed out three main factors: (1) Is there or not a perforation of a mucocele i.e. wall integrity; (2) Involvement of the base of an appendix (margins of resection); (3) Is there any positive lymph nodes of mesoappendix and ileocolic. These criteria determine further treatment modalities: appendectomy to the right colectomy, including cytoreductive surgery, heated intraoperative intraperitoneal chemotherapy, early postoperative intraperitoneal chemotherapy. Simple appendectomy is the choice for patients with benign mucocele as suggested by the presence of a normal caecum and appendicular base and no evidence of perforation (20, 27). Right hemicolectomy is recommended when malignant mucocele is suspected by the presence of a perforated mucocele, enlarged mesenteric lymph node or a positive cytology. Due to the well-known association between the appendicular mucocele and other mucin-secreting cells cancers, such as colon and ovarian cancers an accurate exploration of the abdomen is advised (4, 28).

In the case of mucocele of the appendix, a histologopathological finding is of a great importance. Namely, histopathologically, there are four types of this rare entity – the retention mucinous cyst or lumen occlusion by carcinoid tumor (18%), mucosal hyperplasia (20%), mucinous cystadenoma (52%) and mucinous cystadenocarcinoma (10%) (3, 14, 17). In this specific case, retention mucocele with a normal mucosa was obtained histopathologically. The reference data indicate that appendiceal mucocele represent about 8% of apendiceal tumors, because if there is a condition of pseudomyxoma peritonei, there is probably a malignant component, or cystadenocarcinoma of appendix (1, 29). Most of these tumors are detected incidentally, presenting as the acute appendicitis in as much as 40% of cases, and a definitive diagnosis is made by the postoperative pathohistological finding. Patients with benign mucinous cystadenomas have an excellent with 5-year survival rates of those greater than 90%. Taking into account that malignant cystadenocarcinoma peritonei and pseudomyxoma have a 5-year survival rate of early 25%, despite recent advances in treatment modalities, including cytoreductive surgery, heated intraoperative intraperitoneal chemotherapy, early postoperative intraperitoneal chemotherapy, it is obvious that there is need for recognition, management and follow-up (11, 21). In conclusion, mucocele of the appendix are treated exclusively surgically, and preoperative diagnosis assists in planning the careful mobilization and resection, in order to prevent the occurrence of possible peritoneal contamination. If there is a suspicion of malignancy, the method of choice is the right hemicolectomy. In the concrete case, there was no suspicion of malignancy, and this entity was an incidental finding. However, since the risk of colorectal adenocarcinoma is a significantly higher in patients with apendiceal mucocele compared to those who do not have it, regular controls are needed in the postoperative period.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

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

## CONFLICT OF INTERESTS

The authors declare no conflict of interest.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CASE REPORT

# CONSERVATIVE TREATMENT OF PUERPERA WITH SEVERE HEMORRHAGIC SHOCK AND SECONDARY COAGULOPATHY

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## ABSTRACT

*Aim: The main aim is to show that the life of a patient depends on the decisions the doctor makes as well as the proper assessment of the case. The decision to avoid a surgical procedure and continuewith the conservative treatment following the vital parameters, was beneficial for the patient who was later discharged recovered. Case report:The case is about a patient who was in labor for the fifth time with the delivery complicated by severe postpartum hemorrhage in the secondary health care institution. Despite all conservative measures taken, the hemorrhage hasn't stopped, therefore, the subtotal hysterectomy was performed, after which the patient was directed to the Clinical Centre Kragujevac. Regarding the fact that the postpartum hemorrhage hasn't stopped and abdominal hematoma as well as intracranial hemorrhagewere diagnosed, the main dilemma was if the surgical procedure should be redone or if the conservative treatment should be continued. By applying the conservative treatment andcontinued consultations of the multidisciplinary team, the patient was discharged from the Clinical Centre Kragujevac. Conclusion: The main issue with severe cases like this one, is to define and direct the treatment towards the lower risk rate – repeated surgery could be fatal with the current state of the patient. The estimation was correct, at the end, the patient was released after thelengthy treatment, recovered.*

***Keywords***: *Conservative treatment, hemorrhagic shock, secondary coagulopathy*

## INTRODUCTION

Postpartum hemorrhage (PPH) is the leading cause of maternal mortality, and is responsible for the loss of over half of million women at childbirth. The vast majority ofdeaths during childbirth caused by PPH is in the low or middle developed countries (1,2). The postpartum hemorrhage is commonly defined as blood loss over 500ml after the vaginal delivery, or loss over 1000ml after the caesarean section. However, the exact blood loss during or after the delivery is very hard to estimate and severeness of the situation depends on the accoucheur's predicament, as well as on the health status of the mother prior to the labour (3-5). The most common reason for PPH is bleeding from the placental site as the result ofuterine atony, that persists shortly after the delivery (6,7). Thus, it is highly recommended to apply the active management for the third stage of the labour, in order to prevent the occurrence of uterine atony and PPH (8-10). In some cases, the applied techniques and measures are insufficient, so the operative treatment is necessary (11,12). The surgical treatment is the last step, and it is done only in the case of persistent PPH when all other measures are exhausted (the application of the uterotonics, bimanual uterine compression, uterine curettage, uterine artery embolisation), when the puerperal hysterectomy presents the last choice to stop the hemorrhage. Right alongside with the actions to stop PPH, measures for sustaining the patient's optimal conditions are applied in order to prevent development of the disseminated intravascular coagulopathy (DIC) and stop the patient from entering the irreversible stage of the hemorrhagic shock. In this case report, we present the patient with severe PPH in the second stage of the hemorrhagic shock.

## CASE REPORT

The patient, thirty three years old, who had given birth five times, was urgently transferred from the Gynecological Department of the General Hospital Kraljevo because of persistent postpartum hemorrhage. The patient had a delivery by applying the vacuum extraction because of the stasis during expulsion of fetus. The hemorrhage occurred postpartum during the uterine atony which hadn't stopped after the application of syntocinon or other uterotonics. The uterine hemorrhage was present combined with the hemorrhage from episiotomy and the back fornix of the vagina as a result of the soft tissue lacerations. The patient was submitted to an urgent surgery, which impliedthe subtotal hysterectomy, drainage of the Douglas pouch and suturingof the soft tissue injuries . The hemorrhage however didn't stop afterthe surgery. At the end, the compression was applied to try to stop further bleeding by placing iodine pads and suturingthem to the labia majora, after which the patient was transported to the Clinical Centre of Kragujevac. Upon theadmission to the Intensive Care Unit (ICU), the patient was intubated, hypotensive TA 85/35mmHg, tachycardic (pulse over 125 per min) with persistent hemorrhage which appeared through the pads and sutures. Complete urgent laboratory blood analyses with simultaneously rotational thromboelastometry (ROTEM) had been done which showed a decrease in the blood counts and a decrease in the coagulation factors (Table 1)

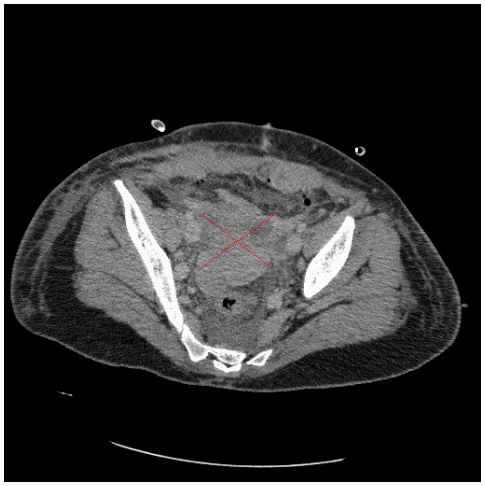
**Table 1.** Initial Laboratory values

| | | | |
|---|---|---|---|
| Hemoglobin | 49g/dl | RBC | 1,8 x $10^9$/L |
| HTC | 0,155 | PLT | 73 x $10^9$/L |
| WBC | 24,9 x $10^9$/L | APTT | 110 s |
| INR | 3,02 | FIB | 0,34 g/L |
| Albumins | 20 g/L | | |

The urgent radiological diagnosis showed only a smaller amount of the free fluid in the Douglas pouch.The initial multi sequential computerised tomography (MSCT) of the endocranium didn't indicate any pathological alterations. MSCT angiography of the major pelvic arteries didn't show extravasation of the contrast agents. After the consulting examinations, which included an anesthesiologist, gynecologist, surgeon and vascular surgeon, neurologist and transfusiologist, the decision was made to continue the treatment conservatively. Cardiopulmonary resuscitation wasinitially performed with volume recompense, crystalloides and colloids like Ringer lactate, 0.9% NaCl, 20% albumins were given, blood transfusion and blood components (9 doses of deplasmatic erythrocytes, 5 doses of fresh frozen plasma, 5 doses of platelets and 2 doses of apheresis platelets ,tranexamic acid, 17 doses of cryoprecipitate) after that inotropic dopamine stimulation was applied together with antibiotic and analgesic therapy. The patient was continually sedated and observed and the control of blood values was repeated. The therapeutic response was adequate, which was confirmed afterwards by blood test results.

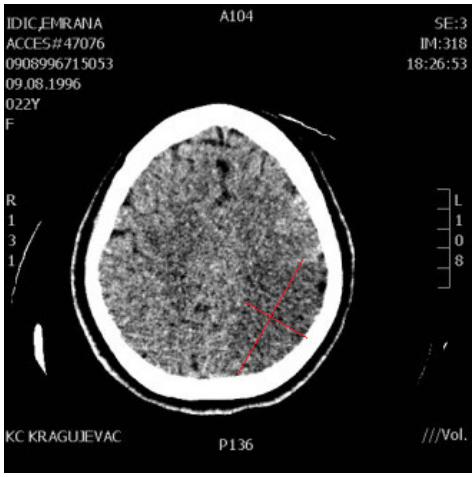
On the second day of the hospitalization, despite the previously mentioned therapy, general condition worsened followed up by high fever (over 39°C), as well as blood tests results. The ultrasound and X ray (abdominal and pelvis EHO and MSCT) were redone and they showed hematoma 89x69x68 mm in diameter localized in the uterine segment with a small quantity of serohemorragic fluid in the Douglas pouch (Figure 1). After the repeated consulting examinations which included a gynecologist, surgeon, transfusiologist and pharmacologist, it was decided to continue with backup antibiotics (Meronem and Vancomycin) instead of previously used (Longacef and Metronidasole).

**Figure 1.** CT scan of the pelvis. Markedly hypervascular mass in Recto-Uterine pouch



In the following days, the general condition of the patient was getting better, the patient was no longer on inotropic stimulation or mechanical ventilation and general nutrition was given. After extubation, the patient was somnolent, uncommunicative with a deviation of the eye on the left side. A neurologist ordered MSCT of the endocranium, which showed hyperdensity in the left parietal zone (hemorragia) with laucnar ischemic zone (Figure 2), while MSCT of the abdomen and pelvis showed regression of the aforementioned hematoma (Figure 3).

**Figure 2.** CT scan of the brain




After consulting the neurologist, the antiedema, anticonvulsant and anticoagulant therapy was administered. On the 10th day of the hospitalization, the patient was communicative, hemodynamically stable, neurologically recovered, with the following blood results (Table 2). After the last consultation with the neurologist, the rehabilitation was indicated because of a minor neurological disorder. The patient was discharged home fully recovered after one week of the physical therapy.

**Fugure 3.** CT scan of the pelvis shows regression of hematoma

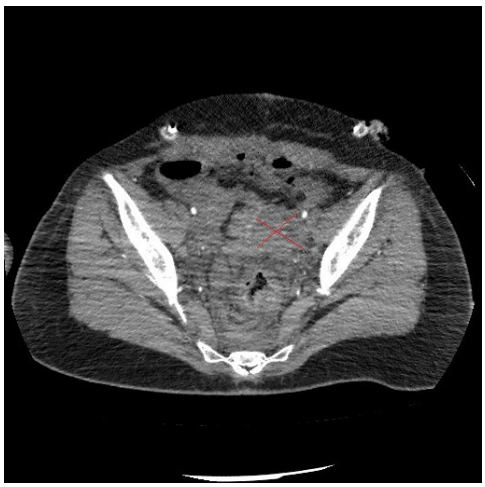


**Table 2.** Laboratory values after conservative treatment

| | | | |
|---|---|---|---|
| Hemoglobin | 98g/dl | RBC | 3,54 x $10^9$/L |
| HTC | 0,298 | PLT | 76,8 x $10^9$/L |
| WBC | 10,71 x $10^9$/L | APTT | 43 s |
| INR | 1,51 | FIB | 5,47 g/L |
| Albumins | 32 g/L | | |

## DISCUSSION

Disseminated intravascular coagulation (DIC) occurs in obstetrics usually after the massive postpartum bleeding (13,14) In the case reported, surgical measurements in order to stop the hemorrhagewere partially depleted, because of the fact that the postpartum hysterectomy was already done and minor injuries of the soft tissue were handledPelvic hematoma conjoined with the intracranial hemorrhage was a repercussion of secondary coagulopathy, and thus barely mentioned in the case reports so far (15,16). The treatment of coagulopathy with aggressive blood transfusion, fresh frozen plasma, cryoprecipitate and tranexamic acid was crucial in the conservative treatment of the patient. The initial doubt over the surgical treatment was overruled with repeated consulting examinations and continuous monitoring of the patient. This case report shows that secondary coagulopathy might be a risk for hematoma occurrence as well as for intracranial hemorrhage and the prompt surgical procedure is not always the solution.

## CONCLUSION

Evaluating the risks and following the ethical code '*Primum non nocere'* were the reasons to treat the patient conservatively despite the presence of secondary hemorrhage. The evaluation was correct and the patient was discharged fully recovered.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

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

## CONFLICT OF INTEREST

There are no conflicts of interest.


## FUNDING

None.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# INFORMATION FOR AUTHORS

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