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TABLE OF CONTENTS

ELECTROCARDIOGRAPHIC FINDINGS IN SYSTEMIC SCLEROSIS

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ABSTRACT

Systemic sclerosis (SSc) is an autoimmune connective tissue disease which affects various tissues and organs, including skin, lungs, kidneys, gastrointestinal tract and cardiovascular system. Cardiac involvement is the most commonly recognized problem and a significant cause of morbidity. Abnormal ECG is present in 25-75% of patients with SSc and is considered to be an independent redictor of mortality. It is known that the supraventricular arrhythmias are considered as more common in SSc patients, occurring in about two-thirds of the cases, and more often than ventricular tachyarrhythmias. It has been established that right bundle branch block is associated with an increased risk of mortality and that it is an independent predictor of mortality, and should be considered as a marker of the severity of the disease in SSc. The prolonged QTc interval is an independent risk factor for a sudden cardiac death reflecting the instability of repolarization and predisposing the onset of cardiac arrhythmias.The prognosis of the disease depends on the SSc subtype and the involvement of internal organs. SSc is a lifelong disease and cannot be cured, but knowing that cardiac dysfunction significantly worsens the prognosis, early detection of cardiac complications and appropriate therapy can influence its progress and improve the patients' quality of life.

Keywords: *Systemic sclerosis, electrocardiogram, right bundle branch block,ventricular arrhythmias, supraventricular arrhythmias.*

INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune connective tis-sue disease which affects various tissues and organs, in-clud-ing skin, lungs, kidneys, gastrointestinal tract and cardi-ovas-cular system (1).

The clinical picture SSc is different. The most common clinical manifestations are presented by Raunayd's phenom-enon, skin changes and changes in the internal organs (2).

CARDIAC INVOLVEMENT

Cardiac involvement is the most commonly recognized problem and a significant cause of morbidity (3). The pres-ence of cardiac events is a bad prognostic sign and is one of the leading causes of mortality in patients with SSc (4-6). The most frequent changes in the registered electrocardiogram are ventricular and supraventricular arrhythmias, heart block rhythms, ventricular hypertrophy (7-9).Cardiac involvement can be primary and secondary. The primary cardiac involve-ment, which develops as a direct consequence of SSc, may manifest as myocardial involvement, fibrosis of the conduc-tion system, pericardial and less frequently, as valvular dis-ease (10). The secondary cardiac involvement may manifest as a consequence of pulmonary arterial hypertension (PAH), interstitial lung disease and kidney disease (11, 12). Cardiac involvment in patients with SSc, according to clinical stud-ies, is found in about 20-25% of patients (12). The primary cardiac involvement involves all layers of the heart: endo-cardium, myocardium, and pericardium, separately or sim-ulta-neously. As a consequence, there may be ventricular and su-praventricular arrhythmias, heart block rhythms, ventric-ular hypertrophy, myocardial ischaemia, pericardial effusion and cardiac insufficiency. In addition to a direct damage, car-diac involvement is a consequence of the involvement of the lungs and pulmonary hypertension, leading to the right ven-tri-cle hypertrophy and dilatation and the right atrial hyper-tro-phy and dilatation (10).

ELECTROCARDIOGRAPHIC ABNORMALITIES

Several studies have described electrocardiographic ab-normalities in SSc (13). Abnormal ECG is present in 25-75% of patients with SSc and is considered to be an independent predictor of mortality (13). Arrhythmias and conduction ab-normalities are thought to be the result of fibrosis of the con-ductive system and myocardial fibrosis.14 Atrial and ven-tricular tachyarrhythmias are a consequence of myocardial fi-brosis, while the conduction defects and bradyarrhythmi-as are the consequence of the fibrosis of the conductive sys-tem (11). Arrhythmias may be associated with a poor out-come and represent 6% of total causes of death in The Euro-pean League Against Rheumatism (EULAR) and European Scle-roderma Trials and Research group (EUSTAR) (15). Ven-tricular ectopia and tachyarrhythmias recorded on the ECG are associated with the increased mortality. In the study deal-ing with the cause of death in patients with SSc, 128 patients were involved , out of whom 33 (26%) died of cardiovascular disease, of which about half died from malignant arrhythmias (15). Ferri et al. were following resting ECG abnormalities in SSc patients. In 22 (42%) patients, out of 53 patients, some of the abnormalities in the ECG were reported (16). Some of the types of rhythm disturbances were reported in 30% of pa-tients. At the 24-hour Holter

ECG, supraventricular arrhythmias were registered in 66% of SSc patients, and ventricular arrhythmias were found in 90% of SSc patients (16). It is commonly known that ven-tricular arrhythmias have a poor prognosis when associated with miocardial ischemia. Despite very frequent occurrence of ventricular arrhythmias, a sudden cardiac death is not very common in SSc (17). A large observational study showed a sudden cardiac death in 18 (5%) of 391 deaths occurring in 1258 SSc patients, and severe cardiac arrhythmias with the poor prognosis were significantly more common in the pa-tients with skeletal and cardiac muscles involvement (17). It is known that the supraventricular arrhythmias are consid-ered to be more common in SSc patients, occurring in about two-thirds of cases, and more often than ventricular tach-yarrhythmias (18). Ferry et al. were followed 53 patients with SSc in their study usinng 24h Holter ECG monitoring 34 with diffuse scleroderma and 19 with calcinosis, Rainaud's phe-nomenon, esophageal disorders, Telangiectasia syndrome (CREST). They registered rhythm and conductive abnor-mali-ties in most SSc patients (eg Conduction defects, supra-ventricular or ventricular arrhythmias and ST-T changes), but in only 42 % (22/53) were found rhythm and conductive ab-normalities on resting electrocardiogram. In a study, left bun-dle branch block and and right bundle branch block with left anterior fascicular block were associated with abnormal left ventricular function, while isolated right bundle branch block or left anterior fascicular block were found in the pa-tients with normal left ventricular function (10). In a prospec-tive study, 16 out of 50 SSC patients (32%) had conduction ab-normalities in resting ECG. The most common abnormali-ties were left bundle branch block (16%), followed by the first-degree atrioventricular block (8%), while the second and third degree atrioventricular block was rare (<2%) (13). Hilda T. Draeger et al. demonstrated that the right bundle branch block was associated with an increased risk of mor-tality. These findings indicate that right bundle branch block is an early predictor of the severity of the disease and should be in-cluded in the modified version of the severity index (19). It was established that right bundle branch block was associated with an increased risk of mortality and that it was an inde-pendent predictor of mortality, and should be consid-ered as a marker of the severity of the disease in SSc (20). Accord-ing to the findings of a study, it can be assumed that pa-tients with SSc in the active phase maesured by capil-larosco-py, who have incomplete right bundle branch block have a predisposition to develop complications, such as dig-ital ul-ceration and possible cardiovascular complications (10). Holter monitoring is, therefore, recommended in the pa-tients with symptoms of palpitations, obesity, dizziness or syncope, regardless of the normal resting electrocardiogram. Exercise electrocardiogram can be helpful in identifying an

exertional type arrhythmias. In all cases, a correlation with echocardiographic findings should be sought. Treatment protocols should respect general guidelines in cardiology to manage different forms of arrhythmias (11). Some studies have found that cardiac manifestations occur in diffuse and limited angular forms of SSC, whereas, in others, the prevalence is higher in diffuse cortical form of the disease, and DSSc is associated with significant morbidity and mortality (21). It has been known that the prolonged QTc interval was registered in the patients with SSC without clinical miocardial involvement and the absence of echocardiographical abnormalities (22). The prolonged QTc interval is an independent risk factor for a sudden cardiac death reflecting the instability of repolarization and predisposing the onset of cardiac ar-rhythmias (23). In a study, it was shown that prolongation of the QTc interval was observed in the patients with SSc and that the prolonged QTc interval correlated with ca-pillary examination, with digital ulcers and with skin thickening , i.e. increased Rodnan Scores (22). In a re-cent study, it has been proven that the QTc interval is prolonged in the patients with SSC. It is known that the QTc interval is prolonged in the patients with myocardial ischemia, cardiomyopathies, complete heart block and other diseases. The prolongation of the QTc inteval in the patients with SSc is associated with interstitial myo-cardial fibrosis and autonomic dysfunction (23). Draeger HT at al. in their study has proven that ECG abnormali-ties are common in the patients with early SSC and are associated with the severity lung and miocardial in-volvement. Since electrocardiography parameters are useful for detecting early cardiac complications, it was recommended, in one study, that ECG be performed as a routine diagnostic procedure in clinical practice (24).

TREATMENT

The cause of systemic sclerosis is not yet well known, so there is no effective etiological treatment. The patients with SSc are mostly treated symptomatically, depending on the involvement of internal organs. The choice of treatment should be personalized to the indi-vidual patient because the patients with SSC may have multiple organs involved and may take more drugs at the same time.

THE COURSE AND PROGNOSIS OF THE DISEASE

SSc has a chronic and progressive course of the dis-ease. The prognosis of the disease depends on the SSc subtype and the involvement of internal organs. The mortality is higher in the patients with dcSSc, particular-ly in kidney, lung and cardiac involvement, especially within the first year. The main causes of death in the pa-tients with SSC are interstitial lung disease and pulmo-nary arterial hypertension (25). The studies based on au-topsy findings have shown that the percentage is signifi-cantly higher and reaches up to 80% (26, 27). The pres-ence of cardiac events is a poor prognostic factor and is one of the leading causes of mortality in the patients with SSc (26-28). The pa-tients with cardiac manifestations can therefore be left without diagnosis, which potentially allows the disease to progress quietly. Early diagnosis is therefore very im-portant (26).

CONCLUSION

Notwithstanding the great progress in the treatment of patients with systemic sclerosis, the percentage of cardiovascular involvement in these patients is still rela-tively high. Early detection of SSc in the period before significant manifestation to certain organs or systems is in focus of many researchers. SSc is a lifelong disease and cannot be cured, but knowing that cardiac dysfunc-tion significantly worsens the prognosis, early detection of cardiac complications and appropriate therapy can in-fluence its progress and improve the patients' quality of life.

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CONFLICT OF INTEREST

None.

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DOES AN ALTERATION IN NOCICEPTIVE RESPONSE TO MINERAL COMPONENTS OF DENTAL COMPOSITES INVOLVE CHANGES IN OXIDATIVE STATUS? A BRIEF REPORT

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ABSTRACT

Since that use of bioactive mineral components of dental composites have been accompanied with various toxicities, including neurotoxicity, the aim of the study was to examine the effect of chronic application of hydroxyapatite, tricalcium phosphate and amorphous calcium phosphate in nanoparticles (nHA, nTCP, nACP) to parameters of sensitivity to thermal pain stimuli. Although the systemic toxicity of those compounds is frequently attributed to an oxidative damage, we also decided to examine the potential effects of Filipendula ulmaria extract on nociception alterations induced by the nano-sized mineral components of dental composites. Forty-two Wistar albino rats were divided into control and six experimental (equal) groups that orally received either nHA, nTCP, nACP alone, or simultaneously with FU extract for 30 days. Nociceptive alterations were quantified in the hot plate and tail flick test. The chronic administration of nHA and nACP resulted in significant increase in reactivity to thermal stimulus, with no significant change observed in nTCP group when compared to the control in the hot plate test, while simultaneous application of FU extract prevented any significant alteration of time to respond. The reaction time in the tail flick test for all three groups that received only nano calcium phosphates was reduced, with no changes in the groups treated with FU extract. The results of this study confirmed that calcium phosphates of mineral components of dental composites produced hyperalgesic effects, and this side effect were significantly attenuated by antioxidant supplementation.

Keywords: *Nanoparticles of calcium phosphates, filipendula ulmaria, nociception, oxidative status, rat.*

INTRODUCTION

Although the use of dental composite has began in the 1960s, today they represent the gold standard in tooth restoration (1). Their complex structure, composed of organic resin and mineral filler, provides good aesthetic and physical properties, very similar to dental tissues, but without any influence on the remaining enamel and dentin (2). The application of calcium phosphate, such as hydroxyapatite (HA), tricalcium phosphate (TCP) and amorphous calcium phosphate (ACP) in the bone tissue replacement and confirmation of its bioactivity is very well confirmed (3). In order to achieve bioactive effect in the physiological environment, the development of dental composites has been moved to integration of HA, and other more soluble forms of calcium phosphates to mineral component (4,5). The basic mechanism of composite bioactivity is the partial dissolution of calcium phosphate molecules in a thin layer of saliva, located between solid dental tissues and fillings, which lead to increase in local concentrations of Ca^{2+} and PO_4^{3-} ions with subsequent deposition of HA layer on the surface of dental tissues and remineralization of solid tissue (6). With the expansive development of nanotechnology and its application in medicine, during the time, nanoparticles were included in prophylactic and therapeutic procedures in dentistry (7). Novel dental composites contain nano-sized calcium phosphates with large specific surface area, which provide a high degree of dissolution, while on the other hand, the solubility is determined by the chemical structure of compound (8). To achieve adequate bioactive effect, it is necessary to ensure that calcium phosphate release and dissolute for a long time period (9). However, the prolonged effect of dissolved compounds, carried by the physiological path of saliva, sets the importance of toxic effects to other oral tissues and the organism in general as a high priority issue.

Calcium phosphates have been considered as biocompatible materials for decades, while the recent investigation of these compounds toxicity, when applied as nano-sized particles, still offer contradictory results. Systemic toxicity of calcium phosphates has been demonstrated through several *in vivo* experiments using nanoparticles of hydroxyapatite (nHA). Thus Liu et al. showed a toxic effect of nHA parenteral administration confirmed hepatotoxicity by means of an increase in AST, ALT and alkaline phosphatase (10). Wang et al. reported that low doses of nHA, after intraperitoneal administration in rats, causes apoptosis in liver and kidney cells, but still without necrosis (11). Although the numerous results of other metallic nanoparticles, such as ZnO, MgO, CuO and CeO, include the neurotoxicity of those compounds manifested by alterations in nociception (12-17), to our knowledge this behavioral pattern that may also be affected by calcium phosphate nanoparticles has not been evaluated yet.

Evaluating the mechanisms of metallic nanoparticles toxicities, Mosu et al. suggested that systemic administration of nHA induces generation of reactive oxygen species (ROS) that leads to an increase in p53 transmitters and activation of apoptosis in renal cells (18). Also, the study on C6 cell culture showed that cell damage was mediated by an oxidative mechanism with consequent apoptosis (19). As the recent investigations have shown that the oxidative state disturbance may be the basic mechanism of cell damage, it seems reasonable that treatment with antioxidants may be beneficial in reducing side effects following the application of dental composites with nano-sized calcium phosphates, as the active substance. Also, the natural products with high antioxidant potential have a wider application, and become more and more interesting to the scientific community. Among the natural supplements with the confirmed antioxidant potential is the extract of *Filipendula ulmaria* - FU (20).

Filipendula ulmaria (L.) Maxim., better known as meadowsweet, is a perennial herbaceous plant that is widespread in Europe and Asia with a long history of medicinal use (21). In addition to flowers, leaves and roots are also used for healing purposes, especially in the therapy of various inflammatory processes (22). The extract of this plant is rich in phenolic compounds and shows a wide range of pharmacological activities (23). Numerous studies have shown the mechanisms of action of FU, whereby Katanic and coworkers showing that FU have a strong antioxidant and antimicrobial potential (23,24). In addition to those described, it also shows antihypertensive, neuroprotective and many other pharmacological activities, so there are numerous indications for its use (24).

Based on the lack of data for the action of calcium phosphate nanoparticles to the central nervous system, the aim of the study was to examine the effect of chronic application of HA, TCP and ACP in the form of nanoparticles (nHA, nTCP, nACP) on parameters of sensitivity to thermal pain stimuli. Following the trend and the extent of research of the antioxidant effects of natural products, and according to the suggested mechanism of cell damage induced by nano-sized calcium phosphates, we also chose to examine the potential effects of FU extract on nociception alterations induced by the nano-sized mineral component of dental composites.

MATERIALS AND METHODS

Animals and treatment

Two-month-old male Wistar rats (180-220 g, n=42) were purchased from the Military Medical Academy, Serbia. The animals were kept in transparent cages (three animals per cage) in controlled standard environmental conditions of temperature 23±1 °C, humidity 50±5 %, with light/dark cycle 12/12h, and were provided standard chow and water ad libitum for the duration of the study. All protocols lasted for 30 days.

The animals were randomly divided into seven equal groups (6 animals per group) as follows:

1. Control group;
2. HA group, orally received nHA (17.8 mg/kg b.w.);
3. HA+FU group, orally received nHA (17.8 mg/kg b.w.) and FU extract (100 mg/kg b.w.);
4. TCP group, orally received nTCP (11 mg/kg b.w.);
5. TCP+FU group, orally received nTCP (11 mg/kg b.w.) and FU (100 mg/kg b.w.);
6. ACP group, orally received nACP (9.65 mg/kg b.w.); and
7. ACP+FU group, orally received nACP (9.65 mg/kg b.w.) and FU (100 mg/kg b.w.).

The mineral components of dental composites in nanoparticles were purchased from the Sigma-Aldrich, Germany: Hydroxyapatite nanopowder, <200 nm particle size (BET), ≥97%, synthetic; Tricalcium phosphate hydrate nanopowder, <200 nm particle size (BET); Calcium phosphate, amorphous nanopowder, <150 nm particle size (BET). The FU extract preparation was performed according to previously established procedure (23). The doses of mineral components used in this study were selected to equimolary equalize the lowest dose of nHA that showed the toxic effects in the previous report for the experiments performed in vivo with nHA (11). It should be noted that the doses administered are similar to the concentrations of mineral components released from dental composites in vitro (25), while the oral administration was chosen in order to mimic the authentic route of application in humans. The dose of FU extract was selected based on our previous study which confirmed the biological efficacy of this natural product (22), and the final concentration of all applied substances was calculated based on the average water intake in the previous 24 hours, dissolved in tap water.

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

Behavioral testing

The behavioral testing was performed 24 h after completing the protocols. Approximately at 8 a.m., rats were placed in the testing room, and allowed to accommodate for 1 h before behavioural testing. The nociception assessment was performed in hot plate test and tail flick test under appropriate conditions (23). In order to remove potential interfering odours, the apparatus for both tests were cleaned with water and ethanol (70%) for each animal.

Hot plate test

The hot plate test was conducted according to algorithm previously defined in our lab (26). The appliance consisted of a square metal plate measuring (43 x 43 cm) and glass walls (30 cm) (Figure 1). Each animal was placed in the central part of the plate and the temperature was maintained at 51.5 ± 0.5°C. The duration of the test was individual and defined by the appearance of a specific reaction to a thermal stimulus - in the form of licking the hind paw, shaking the hind paw or bouncing off the ground with all 4 limbs at the same time. To prevent burns, the test time was limited to 180 seconds. The parameter monitored in this test is the reaction time expressed in seconds.

Tail flick test

The tail flick test is the nociception test in which a high-intensity heat stimulus is directed at the rat's tail according to the procedure described by Bannon et al. (27). The animals were placed on a raised grid and covered with an appropriately sized tube to disable movement (Figure 2). After achieving a temperature of 75°C, a heat stimulus was placed in the middle of the tail and the reaction of the experimental animal was monitored. A strong enough stimulus was necessary to provoke the expected reaction of the animal - the tail flick. By measuring the time from the initiation of the painful stimulus to the manifested form of the expected reaction, the results of this test were quantified and expressed in seconds.

Statistical analysis

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

RESULTS

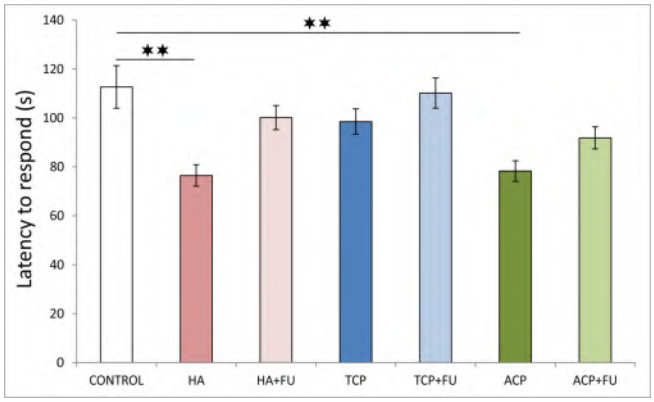
All applied protocols significantly affected the reaction time in the hot plate test (Figure 3, dF=6, F=6.321). The chronic administration of nHA and nACP resulted in significant increase in reactivity to thermal stimulus ($p<0.01$), with no significant change of observed in nTCP group when compared to the control. At the same time, simultaneous application of FU extract prevented any significant alteration of time to respond in the hot plate test.

As represented in Figure 4, the results of the tail flick test showed the significant alterations in time to respond following the applied protocols (dF=6, F=10.863). The reaction time for all three groups that received only nano calcium phosphates was reduced ($p<0.01$ for nHA and nACP; $p<0.05$ for nTCP), while in the groups that received nano calcium phosphate along with FU extract no changes in the reaction time were observed when compared to the control. Furthermore, antioxidant supplementation with FU extract resulted in significant elongation of reaction time when compared to groups with single nano-sized calcium phosphate administration ($p<0.01$).

Figure 1. Apparatus used for the hot plate test.

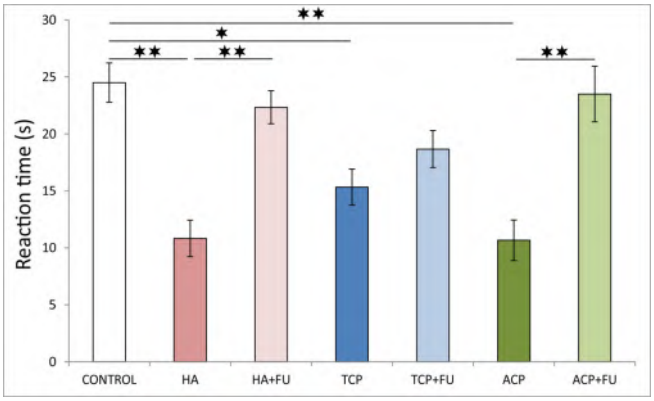
Figure 2. Apparatus used for the tail flick test.

Figure 3. Hot plate test.



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

Figure 4. Tail flick test.



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

DISCUSSION

The results of this study indicate that calcium phosphate nanoparticles significantly affect nociceptive mechanism, thus altering the response to thermal pain stimuli. Although there is no data of nano calcium phosphates effects of central nervous system, previous studies indicate that systemic application of other metals nanoparticles could significantly affect both peripheral and central nervous system, also including the nociception control (28). Kesmati and co-workers showed that ZnO nanoparticles act analgesic through the central mechanisms of pain regulation, without dose dependence at high concentrations (12). It was later explained by the high rate of receptor saturation (13). Further research concluded that both conventional ZnO and ZnO in nanoparticles (with more pronounced effect of its nanoparticle form), may be effective in reducing acute pain in animal experimental models, with the analgesic mechanism by ZnO applied in nanoparticles could influence antinociceptive action by activation of opioidergic system (14). Also, it was shown that MgO and ZnO nanoparticles directly affect pain perception through changes in glutamate levels, while changes in ion levels, after injection of these nanoparticles, may be effective in altering gene expression in the hippocampus, with overall hypoalgesic effect (15). There is also evidence in the literature that CuO produced antinociceptive effects (16). In contrast, it was reported that CeO reduced the latency time in the hot plate test and that hyperalgesic effect was accompanied with increased oxidative stress expressed by means of cyclooxygenase-2 (17), which almost resembles the results obtained in this study.

Beside the known distribution of calcium phosphate nanoparticles after the systemic application (29), it was already shown that calcium phosphate nanoparticles pass through the blood-brain barrier and enter numerous parts of the brain (30). The results of previous studies indicate that parenteral administration of nHA induced changes in liver function demonstrated through changes in liver enzymes levels (10),

while oral administration of the same compound induced nephrotoxicity of various levels, including renal tissue damage, changes in biochemical parameters, as well as enhancement of proinflammatory cytokine production (18).

Some authors have shown that basic mechanism of cell damage induced by nHA is through oxidative imbalance as the result of the increased production of hydroxyl radicals (31), as well as impaired antioxidant capacity via down-regulation of SOD activity *in vitro* (19). The particle characteristics (size and chemical structure) of calcium phosphate nanoparticles have decisive impact on the inflammatory and apoptotic mechanisms underlying calcium phosphate toxicities in various tissues. The reduction of nHA particle size has been reported to increase the release of NO and pro-inflammatory factors, such as tumor necrosis factor alfa, by the activated microglia in cell culture (31). Analyzing the proapoptotic action (as a mechanism that result in toxic effect), calcium phosphate in form of nHA have shown increase of p53 and decrease of bcl-2 activation, which lead to DNA damage in rat kidney cells (18), while nACP caused apoptosis by selective action on the G1 phase in the cell cycle in leukemia p388 cells (32). Also, other mechanisms of apoptosis induction have been suggested, such as an increase in intracellular Ca^{2+}, probably originating from nanoparticles (33), a modulation of mitochondrial membrane potential (34) and an increase in intracellular PO_4^{3-} (35).

The dose of FU extract used in this study was selected on the basis of previously confirmed antioxidant effect in the same species (23). Therefore, the neuroprotective action of FU extract (by means of the enhanced latency to respond in both tests) could be attributed to the attenuation of previously discussed oxidative damage following the application of calcium phosphate nanoparticles.

Slightly more pronounced response to thermal pain stimuli observed in the tail flick test, when compared to hot plate test, may be considered as confirmation that neurotoxic effect of nano calcium phosphates was more convincing on nociceptive elements that include predominantly peripheral nerves. In addition, according to the results of our study, it seems that antioxidative supplementation with FU extract has shown more beneficial antioxidative effects at the level of nociception control up to the level of spinal cord. At the same time, although it prevented hyperalgesic effect of nano calcium phosphates in hot plate test (when compared to control), FU extract administration did not include significant influence on nociception when compared to the groups that received calcium phosphate nanoparticles solely. The possible explanation for the observed phenomenon may be found in the fact that central mechanisms for pain control (that could be evaluated only in hot plate test) remained less affected to oxidative damage induced by calcium phosphate nanoparticles.

CONCLUSION

In summary, the results of our study may be considered as an experimental confirmation that mineral components of calcium phosphates (commonly used in dentistry), may produce hyperalgesic effects themselves, and this side effect of therapy that includes calcium phosphates may be significantly attenuated by antioxidant supplementation.

CONFLICTS OF INTEREST

The authors declare no financial or commercial conflicts of interest.

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CLINICAL FEATURES AND DISEASE COURSE OF CANCER PATIENTS INFECTED WITH SARS-COV-2 DURING ANTICANCER TREATMENTS

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ABSTRACT

Cancer patients infected with SARS-CoV-2 during their active anticancer treatment represent a highly vulnerable population. We aimed this investigation to show clinical features and outcomes of the patients who had mild to moderate COVID-19 symptoms or were asymptomatic at the admission to the COVID Center. The retrospective study included 25 cancer patients confirmed with SARS-CoV-2 within seven days of their last anticancer treatment. Clinical data were collected from medical records and processed by methods of descriptive and inferential statistics. Patients' mean age was 68.1±10.4 years. More than 2/3 of the patients were with ECOG PS 0 and 1, and about 4/5 of patients were in III or IV cancer stage. The most frequently applied types of therapy were radiotherapy and combined radio/chemotherapy. Eleven (44.0%) patients had bilateral while 4 (16%) had unilateral pneumonia. The most frequent symptoms were fever (72%), fatigue (72%), dyspnea (32%), and cough (32%). 1/5 of the patients needed oxygen support. Mean neutrophil (2.6±1.2), lymphocyte (0.9±0.6) and platelets (200.1±88.1) number significantly increased from admission to discharge ($p=0.004$, $p=0.005$, $p<0.001$). Median CRP significantly decreased from 40.4 (6.2-96.2) at admission to 11.35 (3.75-27.65) at discharge ($p=0.008$). Twenty-four patients were cured, and one patient died. Naso-pharyngeal SARS-CoV-2 clearance time was 19.4±6.9 days; the minimum was seven, and the maximum was 39 days. Cancer patients infected with SARS-CoV-2 during active anticancer treatment can successfully overcome COVID-19 without developing further respiratory or other complications during hospitalization. An increase in lymphocyte and neutrophil counts, with a decrease in CRP, may be markers of a favorable prognosis.

Keywords: *SARS-CoV-2, COVID-19, cancer, chemotherapy, radiotherapy.*

INTRODUCTION

In December 2019. China was experiencing an outbreak of a novel beta coronavirus known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1). On January 30, 2020, the WHO declared the coronavirus disease 2019 (COVID-19) outbreak a public health emergency of international concern and, in March 2020, began to characterize it as a pandemic (2).

Cancer patients are regarded as a highly vulnerable group in the COVID-19 pandemic due to immunosuppression caused by malignancy and systemic anticancer therapies. The potential to cause harm by SARS-CoV-2 is at least three-fold larger in oncology patients (3). Delivering cancer care during this crisis is challenging, given the competing risks of death from cancer versus death or serious complications from COVID-19 (4).

Cancer patients with “active disease” who are receiving chemotherapy or extensive radiotherapy or who have received chemotherapy in the last three months are at higher risk of infection. The specific risk groups are those with an impaired immune system such as leukocytopenia, low immunoglobulin levels, and long-lasting immunosuppression (steroids, antibodies) (5).

In general, all adults with COVID-19 can be grouped into the several severities of illness categories from asymptomatic infection to critical illness, although the criteria in each category may overlap or vary across guidelines and clinical trials (6). The most common symptoms are fever, cough, sore throat, malaise, fatigue, headache, and muscle pain. The symptoms of lower respiratory tract infectious disease are diagnosed in patients with tracheitis and bronchitis, whereby dyspnea and/or declining oxygen saturation at ambient air often predict bi-lateral ground-glass infiltrates seen earliest on computed tomography (CT) scans, hence identifying viral pneumonia (7).

The first investigations of cancer patients infected with SARS-CoV-2 showed the fatality rate in this population was 5,6% compared with 2,3% in the general population (8). Zhang at al. (9) showed that cancer patients develop deteriorating conditions and poor outcomes with high mortality. They also found that within 14 days, anti-tumor therapies were significantly associated with the occurrence of severe clinical events in SARS-CoV-2 infection. On the other hand, Lee at al. analyzed 800 patients with a diagnosis of cancer and symptomatic for COVID-19 and could not identify evidence that cancer patients on cytotoxic chemotherapy or other anticancer treatment were at an increased risk of mortality from COVID-19 compared with those not on active treatment (10).

Due to contradictory and insufficient data related to cancer patients infected with SARS-CoV-2 during the period of their cancer treatment, we aimed this investigation to show our clinical experience related to this vulnerable population and to gather new information on their clinical and laboratory features, and disease course.

METHODS

Study design and participants

The retrospective study included 25 cancer patients diagnosed with SARS-CoV-2 infection, referred from the Institute of Oncology and Radiology of Serbia (IORS) to COVID Center Zemun. Patients with different types of solid cancers were tested for SARS-CoV-2 infection on the day of the scheduled continuation of their ongoing anticancer treatment, or during the hospitalization in IORS due to the presence of symptoms or data about risk contacts with persons tested positive for SARS-CoV-2. A confirmed case of SARS-CoV-2 positive patient was defined as a positive result on real-time reverse-transcriptase-polymerase-chain-reaction (RT-PCR) assay of nasal and pharyngeal swab specimens. Only laboratory-confirmed cases were included in the analysis. The patients were admitted to the COVID Center Zemun between March 07, 2020, and April 18, 2020. on the day of the confirmation of SARS-CoV-2 infection. This study included only cancer patients with mild or moderate COVID-19 severity or were asymptomatic at hospital admission. Disease severity was assessed according to the WHO summary of typical features of COVID-19 severity (11). All patients received the last dose of anticancer therapy within seven days before admission to COVID Center Zemun. We obtained data from the medical records for hospitalized patients. Radiologic assessments included chest radiography, and all laboratory testing was performed at hospital admission and further according to the patients' clinical care needs. The patients were discharged from the hospital after they achieved criteria according to Guidance for discharge and ending the isolation in the context of widespread community transmission of SARS-CoV-2 of the European Centre for Disease Prevention and Control (ECDC) (12). A COVID-19 patient is considered 'fully discharged' if no symptoms are related to acute COVID-19 for ≥48 hours AND two negative tests at 24-hour intervals from nasal and oropharyngeal swabs.

Statistical analysis

Descriptive statistics were calculated for the baseline demographic and clinical features. Categorical variables were presented as number and percentage. Continuous data distribution was tested with mathematical and graphical methods. Continuous variables were presented as mean with standard deviation (SD) or median with 25-75 th percentile, according to data distribution. Differences between admission and discharge were analyzed using Student’s paired t-test (or Wilcoxon signed rank test) for continuous variables. For all statistical calculations, the significance level (α) was 0.05. For statistical processing of the obtained results, we used the SPSS software package (version 23.0, SRSS Inc., Chicago, IL).

RESULTS

This study included 25 cancer patients with confirmed COVID-19 diagnosis or SARS-CoV-2 infection, mean age 68.1±10.4 years. Baseline clinical characteristics are presented in table 1. Male patients were dominant, and the gastrointestinal system was the most affected system, with ECOG PS 0 and 1 in more than 2/3 of the patients. Most of the patients were in III or IV stages of cancer, and radiotherapy or combined radio/chemotherapy were the most frequently applied types of therapy.

In 3/4 of the patients, the main symptoms at the hospital admission were fever and fatigue (table 2**)**. Eleven (44.0%) patients had bilateral pneumonia (figure 1), while 4 (16) had unilateral pneumonia. At admission, 2/3 of the patients had leucopenia and lymphopenia, without leukocytosis and lymphocytosis in any patient. Neutropenia was present in less than half of the SARS-CoV-2 positive cancer patents. A high level of CRP was present in 2/3 of the patients. Thrombocytopenia was present in 1/3 of the patients and anemia in more than 80%. A high level of D-Dimer was present in all, except for 2 patients with referent values. More than half of the patients had low proteins and albumin levels. The level of LDH was increased in one-third of the patents. Sodium levels were low in 5, while potassium level was low in 6 patients. Low calcium level was present in more than half of the SARS-CoV-2 positive patients. The iron level was low in 70%, and ferritin was high in more than half of the patents. Urea and creatinine levels were mostly in reference ranges. Liver enzymes were sporadically increased in some patients (table 3).

Mean neutrophils number was 2.6 ± 1.2 at admission and significantly increased 3.1 ± 1.1 at discharge p=0.004 (figure 2). Mean lymphocytes number also significantly increased from 0.9 ± 0.6 to 1 ± 0.5 at discharge (p=0.005), (figure 2). NLR and PLR increased at discharge, but change was not significant (NLR 2.83 (1.66-6) to 3.7 (1.81-5.35), p=0.346; PLR 195 (131.17-453.33) to 323.9 (175.75-552.5), p=0.607). Median CRP significantly decreased from 40.4 (6.2-96.2) at admission to 11.35 (3.75-27.65) at discharge (p=0.008) (figure 2). Mean number of platelets significantly increased from 200.1 ± 88.1 to 300.3 ± 124.6, p<0.001, while mean hemoglobin remained unchanged (113.4 ± 19.7 to 111.4 ± 17.9, p=0.686).

All patients were treated with hydroxychloroquine. Sixteen (64.0%) patients were treated with Azithromycin. The most often applied antibiotics were Azithromycin and Ceftriaxone. In patients with pneumonia, Ceftazidime, Meropenem, Metronidazole, and Levofloxacin were applied depending on the clinical symptoms, CRP values, and performance status of the patients' (table 4**)**. Twenty-four patients were cured, and one patient had a deadly outcome. Negative results of the PCR test were present after 19.4 ± 6.9 days; the minimum was seven days and maximal after 39 days.

Table 1. Baseline clinical characteristics of SARS-CoV-2 positive cancer patients with mild to moderate SARS-CoV-2 symptoms at the admission to the Covid center

		n (%)
Gender	male	18 (72)
	female	7 (28)
Age*		68.1±10.4
Localization of the primary tumor	Gastrointestinal tract (GIT)	9 (36)
	Urogenital tract (UGT)	5 (20)
	Respiratory tract (RT)	6 (24)
	Other	5 (20)
ECOG PS [a]	0	4 (16)
	1	16 (64)
	2	1 (4)
	3	3 (12)
	4	1 (4)

		n (%)
Cancer stage	II	3 (12)
	IIA	1 (4)
	III	9 (36)
	IIIB	1 (4)
	IV	6 (24)
	IVA	5 (20)
Type of current cancer therapies	HT [b]	2 (8)
	CT [c]	4 (16)
	CT/Biological therapy	1 (4)
	RT [d]	9 (36)
	HT/RT	1 (4)
	CT/RT	5 (20)
	Not started	3 (12)
Comorbidities		16 (64)
Hypertension		13 (52)
Diabetes mellitus		2 (8)
Unilateral pneumonia		11 (44)
Bilateral pneumonia		4 (16)

*data are expressed as mean±sd;
a) ECOG PS - Eastern Cooperative Oncology Group (ECOG) Performance Status
b) HT - hormone therapy;
c) CT- chemotherapy;
d) RT - radiotherapy

Table 2. SARS-CoV-2 symptoms of infected cancer patients on a day of admission to COVID Center

Symptom	Yes	No
Fever	18 (72%)	7 (28%)
Cough	8 (32%)	17 (68%)
Dyspnea	8 (32%)	17 (68%)
Need for oxygen support	5 (20%)	20 (80%)
Fatigue	18 (72%)	7 (28%)

Table 3. Laboratory findings of SARS-CoV-2 positive cancer patients with mild to moderate SARS-CoV-2 symptoms at the admission to the COVID Center

Laboratory findings	Values	N (%)
Leucocytes	Leucopenia	18 (72)
	referent values	7 (28)
Neutrophils	Neutropenia	11 (44)
	reference values	14 (56)

Laboratory findings	Values	N (%)
Lymphocytes	Lymphopenia	17 (68)
	reference values	8 (32)
Platelets	Thrombocytopenia	9 (36)
	reference values	16 (64)
Hemoglobin	low	22 (88)
	reference values	3 (12)
CRP	reference values	7 (28)
	high	18 (72)
D-Dimer	low	0 (0)
	reference values	2 (9.1)
	high	20 (90.9)
Proteins	low	15 (60)
	reference values	10 (40)
	high	0 (0)
Albumins	low	16 (64)
	reference values	9 (36)
	high	0 (0)
LDH	low	0 (0)
	reference values	16 (66.7)
	high	8 (33.3)
Sodium	low	5 (20.8)
	reference values	19 (79.2)
	high	0 (0)
Calcium	low	14 (60.9)
	reference values	9 (39.1)
	high	0 (0)
Potassium	low	6 (25)
	reference values	13 (54.2)
	high	5 (20.8)
Iron	low	14 (70)
	reference values	6 (30)
	high	0 (0)
Ferritin	low	1 (4.8)
	reference values	9 (42.9)
	high	11 (52.4)
Urea	low	0 (0)
	reference values	21 (84)
	high	4 (16)
Creatinine	low	0 (0)
	reference values	23 (92)
	high	2 (8)
AST	low	0 (0)

Laboratory findings	Values	N (%)
	reference values	15 (68.2)
	high	7 (31.8)
ALT	low	0 (0)
	reference values	20 (90.9)
	high	2 (9.1)
GGT	low	0 (0)
	reference values	15 (75)
	high	5 (25)

Table 3. Laboratory findings of SARS-CoV-2 positive cancer patients with mild to moderate SARS-CoV-2 symptoms at the admission to the COVID Center

Laboratory findings	Values	N (%)
Leucocytes	Leucopenia	18 (72)
	referent values	7 (28)
Neutrophils	Neutropenia	11 (44)
	reference values	14 (56)
Lymphocytes	Lymphopenia	17 (68)
	reference values	8 (32)
Platelets	Thrombocytopenia	9 (36)
	reference values	16 (64)
Hemoglobin	low	22 (88)
	reference values	3 (12)
CRP	reference values	7 (28)
	high	18 (72)
D-Dimer	low	0 (0)
	reference values	2 (9.1)
	high	20 (90.9)
Proteins	low	15 (60)
	reference values	10 (40)
	high	0 (0)
Albumins	low	16 (64)
	reference values	9 (36)
	high	0 (0)
LDH	low	0 (0)
	reference values	16 (66.7)
	high	8 (33.3)
Sodium	low	5 (20.8)
	reference values	19 (79.2)
	high	0 (0)
Calcium	low	14 (60.9)

Laboratory findings	Values	N (%)
	reference values	9 (39.1)
	high	0 (0)
Potassium	low	6 (25)
	reference values	13 (54.2)
	high	5 (20.8)
Iron	low	14 (70)
	reference values	6 (30)
	high	0 (0)
Ferritin	low	1 (4.8)
	reference values	9 (42.9)
	high	11 (52.4)
Urea	low	0 (0)
	reference values	21 (84)
	high	4 (16)
Creatinine	low	0 (0)
	reference values	23 (92)
	high	2 (8)
AST	low	0 (0)
	reference values	15 (68.2)
	high	7 (31.8)
ALT	low	0 (0)
	reference values	20 (90.9)
	high	2 (9.1)
GGT	low	0 (0)
	reference values	15 (75)
	high	5 (25)

Table 4. The list of applied therapy and the outcome of SARS-CoV-2 positive cancer patients with mild to moderate SARS-CoV-2 symptoms at the admission to the Covid center

Type of therapy	Aplication of therapy	n (%)
Hydroxychloroquine	No	0 (0)
	Yes	25 (100)
Azithromycin	No	9 (36)
	Yes	16 (64)
Ceftriaxone	No	12 (48)
	Yes	13 (52)
Ceftazidime	No	24 (96)
	Yes	1 (4)
Meropenem	No	24 (96)
	Yes	1 (4)
Metronidazole	No	22 (88)

Type of therapy	Aplication of therapy	n (%)
	Yes	3 (12)
Levofloxacin	No	24 (96)
	Yes	1 (4)
Outcome	Death	1 (4)
	Cured	24 (96)

Figure 1. Changes of neutrophil, lymphocyte, and CRP counts from admission to discharge of SARS-CoV-2 positive cancer patients with mild to moderate SARS-CoV-2 symptoms: significantly increase of mean neutrophils and lymphocytes number and decrease of median CRP value at discharge

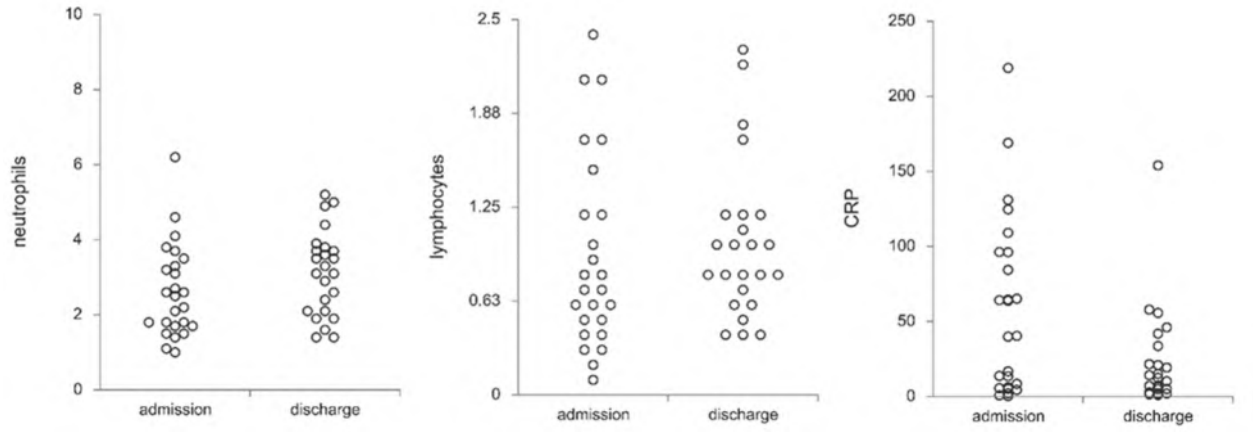
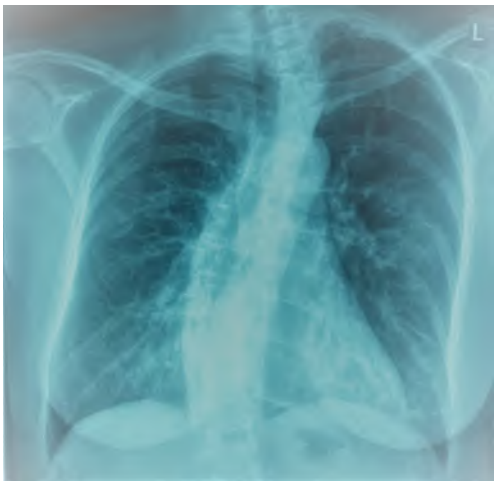
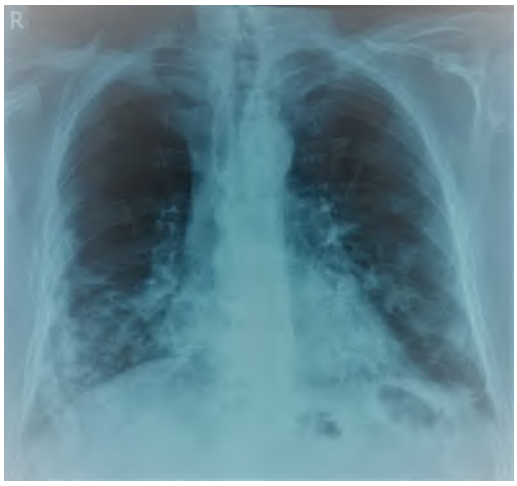


Figure 2. Chest X-ray findings in cancer patients infected with COVID-19 during the cancer treatment: four examples of bilateral pneumonia show patchy or diffuse ground-glass opacity with small fields of consolidation and reticular areas of increased opacity. Patients had no diagnosed lung metastases.

A)



B)



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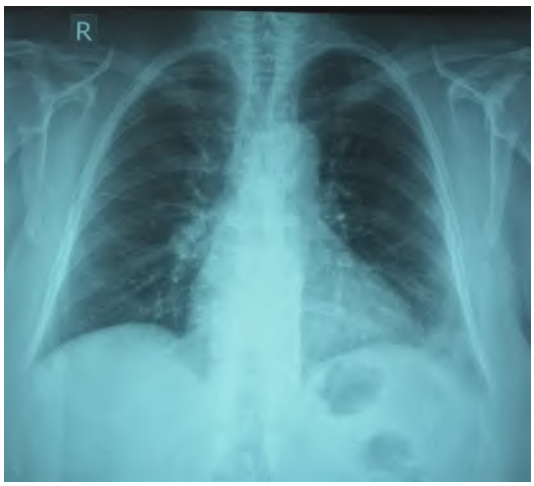
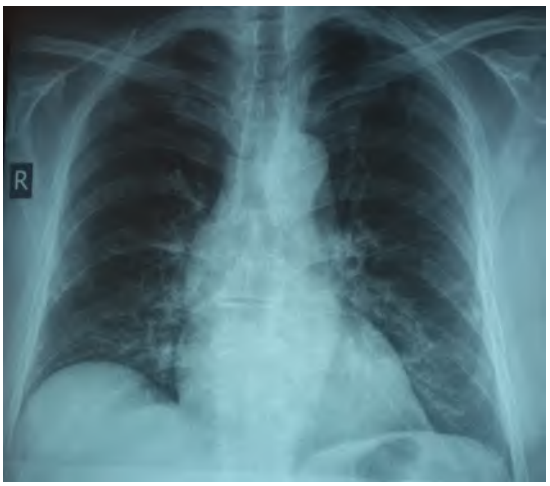


D)



DISCUSSION

We present the clinical course and laboratory characteristics of 25 cancer patients infected with SARS-CoV-2 during their active anticancer treatment. At presentation, all patients had mild or moderate symptoms or were asymptomatic, and most of them successfully overcame SARS-CoV-2 infection and were discharged from the hospital able to continue their further oncology treatment.

The most common symptoms were typical for COVID-19 patients and most frequently included fever, fatigue, cough, and dyspnea. Oxygen support was needed by 1/5 of the patients. The most frequent initial laboratory findings were leukopenia, lymphopenia, neutropenia, and thrombocytopenia. Wang at al. (13) shown that COVID-19 patients had significantly lower total number of lymphocytes and NK cells. They also documented that in responsive patients, total lymphocytes, CD8+ T cells, and B cells increased significantly after treatment, and no significant change was detected in CD4+T cells, CD4+/CD8+ ratio. In nonresponsive patients, no significant change was detected. Our study showed that the levels of lymphocytes, neutrophils, and thrombocytes significantly increased at discharge, which could have a positive effect on the outcome of the disease.

At the hospital admission, about 2/5 of patients had bilateral and less than 1/5 unilateral pneumonia. The diagnosis of pneumonia was established by clinical and laboratory examination and confirmed by chest X-ray (CXR) of the lungs. The fact was that some incipient pneumonia might have been remained unrecognized because CXR is insensitive in the detection of early disease (14), but, on the other hand, it was useful for establishing a baseline and as follow-up imaging for disease progression (15). Compared to chest CT, CXR appears to have lower sensitivity and might have higher specificity (16).

Anemia was present in 4/5 of patients, and 1/5 of them needed a blood transfusion, mean hemoglobin remained unchanged. The levels of CRP significantly decreased at discharge. The other laboratory findings showed high D-Dimer, hypoproteinemia, and hypoalbuminemia and were in line with the experiences of other authors (9).

Most of our cancer patients infected with SARS-CoV-2 continued to be respiratory stable during the hospitalization and successfully defeated infection. Our experience was completely different from Zhang et al. (9) reported that 53% of the 28 cancer patients developed severe events, 21,4% were admitted to intensive care units (ICU), 35,7% had life-threatening complications, and 28,6% of the patients died. Yang et al. (17) also showed that receiving chemotherapy within 4 weeks before symptom onset and male sex were risk factors for death during admission to hospital. According to our research, one patient developed a severe respiratory event and was admitted to the intensive care unit (ICU) for non-invasive mechanical ventilation, and one patient died in the terminal stage of metastatic colorectal cancer with mild respiratory symptoms. Of course, the fact that 80% of the patients were in good performance status (ECOG 0 or 1) could positively contribute to this outcome. Also, large COVID-19 cancer cohorts of predominantly solid organ tumors have shown no significant excess mortality risk from recent chemotherapy (18,19).

Despite the large use of antiviral and/or anti-inflammatory drugs, until now, no proven treatment is available for the current COVID-19 pandemic (18,20). Our patients were treated with hydroxychloroquine and one or more antibiotics, depending on the severity of their symptoms and the presence of pneumonia. They also received multivitamin therapy, adequate rehydration, and all needed symptomatic and supportive therapy. No antiviral agents were used. It remains unclear

whether the use of any applied treatment was crucial for the clinical course of the disease and the patients' recovery.

The patients could be discharged from the hospital after two consecutively negative test results plus the absence of respiratory symptoms for at least two consecutive days before testing after prescribed therapy was finished. The therapy was prescribed on the day of confirmation of SARS-CoV-2 infection. Two consecutive negative RT-PCR results were presented after 7 to 39 days (19.4 ± 6.9) after the confirmation of infection. Most patients (76%) took more than 14 days to meet the discharge criteria. Xu at al. (21) observed that time to nasopharyngeal SARS-CoV-2 RNA clearance in their oncology patients was substantially longer than the approximately 17-20 days previously reported in the general population. In their retrospective cohort study, the median time to SARS-CoV-2 clearance was 50 days using the ASCO/CDC criteria of 2 negative RT-PCR assays >24 hours apart, and the virus clearance times differed substantially depending on criteria.

The study's limitation is reflected in patients' small sample size and heterogeneity of cancer types. The study is retrospective and non-randomized, which could not have been avoided given the patients' admission circumstances to our center. However, the study's value is reflected in the fact that clinical features and disease courses were monitored in patients infected with SARS-CoV-2 during their active anticancer treatment when their vulnerability to infections is greatest due to the possible toxicity of oncology therapies.

CONCLUSION

Cancer patients with mild or moderate COVID-19 symptoms or asymptomatic for SARS-CoV-2 infection at presentation can successfully overcome disease without developing any further respiratory or other complications even though the infection occurred during their active anticancer treatment. An increase in neutrophil and lymphocyte counts and a decrease in CRP may be markers of a favorable prognosis.

CONFLICTS OF INTEREST

The authors declare no financial or commercial conflicts of interest.

FUNDING

None.

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FETUIN-A AS A MARKER OF INSULIN RESISTANCE

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ABSTRACT

Fetuin-A is a glycoprotein which helps in the regulation of metabolism. It is an early marker of insulin resistance (IR). The aim of this study was to evaluate the role of Fetuin-A as a predictive biomarker in cases of newly detected type 2 diabetes (NDD). The study involved 60 NDD and 60 Normal Healthy Controls (NHC). All the demographics and anthropological characteristics were noted. Fasting blood samples were drawn and various biochemical parameters were analyzed. The homeostatic model assessment of insulin resistance (HOMA-IR) and the quantitative insulin sensitivity check index (QUICKI) score was calculated. Chi-square, student T-test and Mann Whitney U tests were employed to associate and compare the mean and median between the NDD and NHC groups. Pearson's and Spearman's correlation analysis were employed to examine the relationship of Fetuin-A levels with parametric and nonparametric variables. The independent predictors of Fetuin-A was determined by employing multiple forward linear regression. Fetuin-A was significantly high in NDD compared to NHC (1323 vs. 306.98 mcg/mL; p<0.001). Majority of NDD patients demonstrated IR based on the HOMA-IR (88.33% vs. 66.67%) and QUICKI score (96.67% vs. 85%). The multiple linear regression analysis showed that systolic blood pressure, age and QUICKI score were independently associated with Fetuin-A (p value <0.01). Fetuin-A may be used as a biomarker to detect NDD. Therefore, early detection of Fetuin-A levels in NDD gives an opportunity for suitable patient management.

Keywords: *Alpha-2-HS-Glycoprotein, biomarkers, endocrinology, insulin resistance, type 2 diabetes mellitus.*

INTRODUCTION

The global incidence of diabetes mellitus was 463 million (9.3%) in 2019 and is expected reach to 700 million (10.9%) by 2045 (1). Type 2 diabetes mellitus (T2DM) is a major public health issue, caused by either insulin resistance (IR) or insulin deficiency (2). Insulin is the primary regulator of glucose homeostasis and its deficiency occurs due to damaged β cells of the islets of Langerhans, thereby stopping insulin production (2). However, IR is due to the decreased response or sensitivity towards insulin and plays a crucial role in T2DM pathophysiology (3). IR is associated with prediabetes and can be preferably treated using biguanides and thiazolidinediones (4,5).

Various inflammatory and metabolic biomarkers, such as Fetuin-A, glycated hemoglobin A1c (HbA1c), adiponectin, retinol binding protein-4, myostatin, IL-6, fibroblast growth factor-21, irisin chemerin, adipocyte fatty acid-binding protein have been studied to identify T2DM and IR (6,7). Previous studies have documented a relatively higher level of serum Fetuin-A in newly detected type 2 diabetes (NDD), cardiovascular disease, metabolic syndrome and obesity patients and is an emerging novel biomarker of IR (8-10).

Fetuin-A is a multifunctional glycoprotein secreted by the liver, also known as α-2 Heremans Schmid glycoprotein (AHSG). The gene encoding locus of Fetuin-A is located on chromosome 3q27 region and is identified as the T2DM susceptibility locus (11). Fetuin-A can induce IR by inhibiting insulin receptor autophosphorylation in the liver and skeletal muscle (12). It is a major carrier of free fatty acids (FFA) in the circulation and is required for FFA interaction with toll-like protein receptor 4 (TLR4) in adipocytes, thereby triggering pro-inflammatory adipokine expression and IR (13). Thus, Fetuin-A promotes lipid-induced IR via inflammatory signaling pathway, causing inflammatory cytokines production. Chronic inflammation caused by free radicals is thought to be the reason for IR (14). Therefore, this study was aimed to elucidate the clinical relevance of Fetuin-A, to identify individuals at increased risk of developing T2DM and for therapeutic purposes.

PARTICIPANTS AND METHODS

This two-year cross-sectional study was conducted at the endocrinology department of a multispecialty hospital, from January 2017 to December 2018. Ethical clearance was procured from the institutional review board and participants were enrolled after obtaining written informed consent.

A total of 120 participants, aged between 18-80 years, were included in this study. They were divided into two groups - normal healthy controls (NHC, n=60) and NDD (case, n=60). Participants with normal fasting blood sugar (FBS) and fasting serum insulin levels were considered as NHC. Participants who were euthyroid, met the 2017 American Diabetic Association (ADA) criteria, with HbA1c <12%, were considered as NDD.

Exclusion criteria considered for this study were pregnant women, patients with secondary diabetes, Type 1 DM, known T2DM patients receiving insulin or oral antidiabetic drugs/not on treatment, patients with BMI >30 kg/m^2, smokers, drinkers, alcohol consumers of ≥20 g/d for a year prior to study, patients with hepatitis B, hepatitis C and other causes of liver diseases (hemochromatosis, α1antitrypsin deficiency, Wilson's disease, primary sclerosing cholangitis or primary biliary cirrhosis), serum creatinine >1.5 mg/dl and any chronic or acute inflammatory disease (leukocyte count >10,000/mm^3) with any clinical signs of infection or any other major diseases, including advanced malignant diseases.

Sample size

A previous study reported serum Fetuin-A levels of 337±96 and 291±63 mcg/ml in cases and controls, respectively (15). Based on the above-mentioned findings and considering the power of the study at 80%, α error of 5% and effect size of 0.53, a minimum of 59 subjects were required per group. Hence, sample size of 120 patients in total was justified for this study.

Patient demographic data (age and gender) was collected by direct interview method. All patients underwent further clinical examination, and all findings were recorded on a predesigned and pretested proforma. Anthropometric details such as height, weight, waist and hip circumferences were measured according to the standard procedures and body mass index (BMI), waist to hip ratio were calculated. Whole blood samples were withdrawn under aseptic precautions after an 8 hour overnight fasting from participants, and were evaluated for various hematological and biochemical parameters, such as complete blood count, glycated HbA1c, FBS, fasting insulin and Fetuin-A levels. After blood collection, serum was separated within 1 hour and stored at -80^0 C.

Serum Fetuin-A and serum insulin levels were measured using Quantikine ELISA Human Fetuin-A Immunoassay kit (R&D Systems, Inc.) and DRG Insulin Elisa kit (DRG Instruments, GmbH, Germany), respectively. All participants were divided on the basis of four quartile range of Fetuin-A (quartile 1: 0-<305 µg/ml; quartile 2: 305 - 757 µg/ml; quartile 3: 758 - 1328 µg/ml; quartile 4: >1328 µg/ml). The IR was calculated using homeostatic model assessment of insulin resistance (HOMA-IR) and patients were considered as insulin resistant if HOMA-IR (fasting insulin x fasting glucose) was ≥2 (16). Quantitative insulin sensitivity check index (QUICKI) was calculated using the formula QUICKI=1/ [log (fasting insulin (µU/L) + log (fasting glucose (mg/dL)] and patients were considered as insulin resistant if QUICKI was ≤0.36 (16).

Statistical analysis

Statistical analysis was performed using SPSS statistical software version 18. Shapiro test was employed to check normality of the data. Normally distributed data are represented as mean ± standard deviation (SD) and the means between the two groups were compared by performing student T-test. Non-normalized data are represented as median (interquartile range, [IOR]). Mann Whitney U test was employed to evaluate the difference in the serum Fetuin-A levels among the groups in case of non-normalized data. Chi-square test was employed to assess the association between the Fetuin-A level and various variables for categorical data. Pearson's and Spearman's correlation analysis was employed to examine the relationship of Fetuin-A levels with parametric and non-parametric variables, respectively. Multiple forward linear regression was employed to determine the independent predictors of Fetuin-A. Multivariate logistic regression was employed to calculate the odds ratio (OR) and 95% confidence interval (CI). Level of significance was set at $p \leq 0.05$.

RESULTS

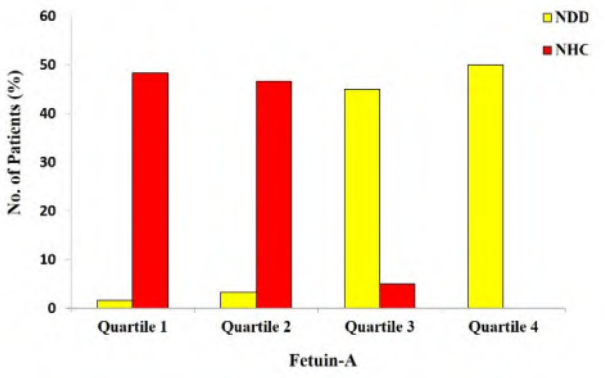
The mean age of patients in the NDD and NHC groups were 51.63±12.01 and 48.72±11.87 years respectively, with male predominance, with male to female ratio of 1.72:1 in NDD and 1.60:1 in NHC groups. On comparing the anthropometry variables, such as waist circumference (WC) and waist to hip ratio, a significantly higher WC (98 cm vs. 89.6 cm; p=0.01) and waist to hip ratio was noted (0.99 vs. 0.87; p=0.009) in NDD compared to the NHC group. However, BMI was found to change insignificantly between both the groups. The difference in the biochemical variables between NDD and NHC groups were significant ($p<0.05$) along with platelet count except for the serum albumin levels. NDD group had a significantly higher systolic (125 mmHg vs. 116 mmHg) and diastolic blood pressure (80 mmHg vs. 77 mmHg), HbA1c (8.4 vs. 5.4), FBS (157.5 mg/dL vs. 92.5 md/dL), fasting serum insulin (16.08 µIU/mL vs. 10.98 µIU/mL), fetuin-A (1322.98 µg/mL vs. 306.97 µg/mL) and platelet count (256.38 ± 74.26 mm^3 vs. 246.73 ± 83.25 mm^3) as compared to NHC group. The NDD group also had significantly higher IR based on HOMA-IR (8.07 vs. 2.39) and QUICKI score (0.29 ± 0.03 vs. 0.33 ± 0.02) compared to NHC group ($p<0.05$) (Table 1).

Table 2 represents the distribution of patients based on the different quartile of Fetuin-A and IR as per HOMA-IR and QUICKI. Majority of patients in the NDD group demonstrated IR based on the HOMA-IR (88.33% vs. 66.67%) and QUICKI score (96.67% vs. 85%) and belonged to the Fetuin-A 3rd and 4th quartile compared to NHC group.

Patients in the NDD group belonging to Fetuin-A 3rd quartile had a significantly higher value of HOMA-IR (median: 7.04; IQR: 3.37-11.01) compared to NHC group (median: 4.7; IQR: 4.18-5.23) with p=0.038 (Table 3).

Percentage of patients with NDD was highest in the 4th quartile than the 1st quartile (50% vs 1.66%, $p<0.001$) (Figure 1).

Figure 1. Comparing different quartiles of Fetuin-A amongst NDD and NHC groups.

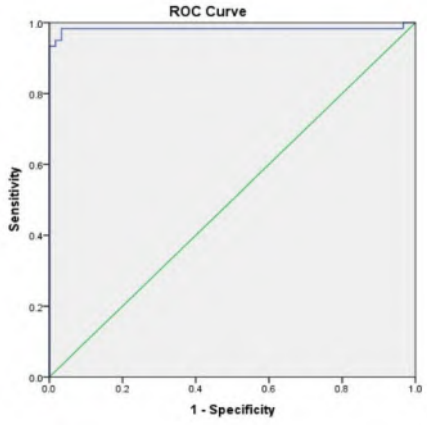


NDD - newly detected type 2 diabetes;
NHC - normal healthy control.

The correlation of Fetuin-A levels with various clinical and biochemical parameters for entire population are represented in Table 4. A significant weak positive correlation of Fetuin-A was noted with age, systolic blood pressure (SBP), diastolic blood pressure (DBP), FBS, HbA1c, fasting insulin and HOMA-IR score ($p<0.01$).

Fetuin-A cut-off value of 733.6296 mcg/ml yielded maximum sensitivity of 98.3% and specificity of 96.7% to differentiate NDD patients from NHC with an AUC of 0.983 (Figure 2).

Figure 2. ROC curve for Fetuin-A.



Multiple regression models were employed to choose the best fit model for selecting independent marker of Fetuin-A. The multiple linear regression analysis showed that QUICKI, SBP and age were independently associated with Fetuin-A ($p<0.01$; adjusted $R^2=0.368$), indicating these may serve as independent predictor of Fetuin-A. Age and SBP were positively associated where as QUICKI was negatively associated with Fetuin-A (Table 5).

Table 1. Baseline characteristics of NDD and NHC patients.

Parameters		NDD group (n=60)	NHC group (n=60)	p value
Demographic				
Gender [a]	Male	38 (63.3)	37 (61.6)	0.85
	Female	22 (36.6)	23 (38.3)	
Age (Years) [b]		51.63 ± 12.01	48.72 ± 11.87	0.119
Anthropometry				
BMI (Kg/m^2) [c]		26.35 (24.4-28.3)	26.25 (22.64-27.89)	0.277
WC (cm) [c]		98 (85.5-106)	89.6 (82.5-100.7)	0.010*
Waist to hip ratio [c]		0.99 (0.88-1.04)	0.87 (0.82-1.01)	0.009*
SBP (mmHg) [c]		125 (118-134)	116 (110-124)	0.000***
DBP (mmHg) [c]		80 (75-87)	77 (71-80)	0.001**
Biochemical				
Platelet count (mm^3)		256.38 ± 74.26	246.73 ± 83.25	0.038*
Albumin (g/dL)		4.2 (4-4.4)	4.5 (4.35-4.8)	0.246
HbA1c (%) [c]		8.4 (7.1-9.85)	5.4 (5.3-5.5)	0.000***
FBS (mg/dL) [c]		157.5 (131-226.5)	92.5 (85-97)	0.000***
Fetuin-A (µg/mL) [c]		1322.98 (1133.11-1575.15)	306.97 (260.84-530.05)	0.000***
Insulin (µIU/mL) [c]		16.08 (8.55-27.58)	10.98 (8.02-17.6)	0.000***
HOMA-IR [c]		8.07 (3.62-12.19)	2.39 (1.84-4.12)	0.0286*
QUICKI score [b]		0.29 ± 0.03	0.33 ± 0.02	0.000***

BMI - body mass index; DBP - diastolic blood pressure; FBS - fasting blood sugar; HOMA-IR - homeostatic model assessment of insulin resistance; NDD - newly detected type 2 diabetes; NHC - normal healthy control; QUICKI - quantitative insulin sensitivity check index; SBP - systolic blood pressure; WC - waist circumference.
*, **, and *** indicates statistically significant P value of ≤0.05, ≤0.001 and ≤0.0001, respectively.
[a] data represented as frequency (%); [b] data represented as mean ± SD; [c] data represented as median (IQR).
Chi-square test was used for categorical data analysis.
Student T-test and Mann Whitney U test were used for normalized and non-normalized data analysis.

Table 2. Distribution of patients based on Fetuin-A quartile and insulin resistance as per HOMA-IR and QUICKI.

Fetuin-A (IQR)	Groups - No. of Patients (%)							
	NDD (n=60)				NHC (n=60)			
	IR based on HOMA-IR		IR based on QUICKI Score		IR based on HOMA-IR		IR based on QUICKI Score	
	No	Yes	No	Yes	No	Yes	No	Yes
1 (0-304.94)	0	1 (1.67)	0	1 (1.67)	12 (20)	17 (28.33)	4 (6.67)	25 (41.67)
2 (305 - 757.4576)	1 (1.67)	0 (0)	0	1 (1.67)	8 (13.33)	21 (35)	5 (8.33)	24 (40)
3 (757.5 - 1327.5)	2 (3.33)	26 (43.33)	1 (1.67)	27 (45)	0 (0)	2 (3.33)	0	2 (3.33)
4 (>1328 - 00)	4 (6.67)	26 (43.33)	1 (1.67)	29 (48.33)	0 (0)	0 (0)	0	0
Total	7 (11.67)	53 (88.33)	2 (3.33)	58 (96.67)	20 (33.33)	40 (66.67)	9 (15)	51 (85)
p value [¥]	0.039 *		0.995		0.32		0.779	

IR - insulin resistance; HOMA-IR - homeostatic model assessment of insulin resistance; IQR - inter quartile range; NDD - newly detected type 2 diabetes; NHC - normal healthy control; QUICKI - quantitative insulin sensitivity check index.
[¥] denotes Chi-square p values; * indicates statistically significant p value of ≤0.05.

Table 3. Comparison of insulin resistance as per HOMA-IR and QUICKI score among Fetuin-A quartiles in NDD and NHC groups.

Fetuin-A (IQR)	IR in Patients					
	Based on HOMA-IR [Median (IQR)]			Based on QUICKI Score [Mean ± SD]		
	NDD	NHC	p value	NDD	NHC	p value
1 (0-304.94)	12.31 (12.31-12.31)	2.24 (1.74-2.63)	0.149	0.27±0	0.34±0.167	-
2 (305 - 757.4576)	1.53 (1.53-1.53)	3.28 (1.99-5.17)	0.094	0.36±0	0.32±0.032	-
3 (757.5 - 1327.5)	7.04 (3.37-11.01)	4.7 (4.18-5.23)	0.038*	0.29±0.03	0.31±0.01	0.283
4 (>1328 - 00)	8.29 (4.07-13.31)	0	-	0.29±0.033	0	-

IR - insulin resistance; HOMA-IR - homeostatic model assessment of insulin resistance; IQR - inter quartile range; NDD - newly detected type 2 diabetes; NHC - normal healthy control; QUICKI - quantitative insulin sensitivity check index; SD - standard deviation.
* indicates statistically significant p value of ≤0.05.
'-' represents analysis could not be performed.

Table 4. Correlation of Fetuin-A with various clinical parameters (n=120).

Parameters	Correlation Coefficient	p value
Age	0.1684	0.066
BMI (kg/m2)	0.0077	0.934
Waist circumference (cm)	0.0904	0.326
Waist hip ratio	0.1255	0.172
SBP (mmHg)	0.3754	0.000***
DBP (mmHg)	0.2504	0.002**
Platelet count (mm3)	0.1582	0.282
Albumin (g/dL)	-0.4025	0.000***
HbA1c (%)	0.7679	0.000***
Fasting Blood Sugar (mg/dL)	0.7247	0.000***
Insulin (µIU/mL)	0.2426	0.008**
HOMA-IR	0.5193	0.000***
QUICKI score	-0.5257	0.000***

BMI - body mass index; DBP - diastolic blood pressure; HOMA-IR - homeostatic model assessment of insulin resistance; QUICKI - quantitative insulin sensitivity check index; SBP - systolic blood pressure.
** and *** refer to <0.01 and <0.001 level of significance respectively.

Table 5. Multiple linear regression analysis showing independent predictors of Fetuin-A.

Parameters	OR (95% CI)	p value	Adj. R^2 value
***QUICKI	-7073.65 (-9341.22, -4806.07)	0.000***	0.368
*SBP	13.25 (6.53, 19.98)	0.004**	
*Age	6.56 (0.11, 13.01)	0.005**	

Adj - adjusted; CI - confidence interval; OR - odds ratio; QUICKI - quantitative insulin sensitivity check index; SBP - systolic blood pressure.
and * refer to <0.01 and <0.001 level of significance respectively.

DISCUSSION

Fetuin-A is a natural inhibitor of insulin receptor tyrosine kinase along with terminating the downstream signal cascades, thereby resulting in IR and the onset of T2DM (17, 18). Fetuin-A binds to tandem fibronectin type 3 (Fn3) domains of the insulin receptor-subunit. Insulin and Fetuin-A exhibit affinity towards the same insulin receptor ectodomain, with insulin activating the receptor's intrinsic tyrosine kinase activity through its binding to the α-subunit, causing a conformational change that promotes fetuin binding to the β-subunit, thereby resulting in the receptor inactivation (19). Fetuin-A also promotes lipid-induced IR by binding to TLR4 through an endogenous ligand function that is mediated by its terminal galactoside moiety, being able to directly bind the Leu100-Gly123 and Thr493-Thr516 residues in TLR4, causing adipose tissue inflammation and subsequent onset of IR (13,20).

The emergence of Fetuin-A as a biomarker was demonstrated by many studies (10, 11, 21, 22). However, only few evaluated the correlation between serum Fetuin-A levels with T2DM patients' characteristics (23). Therefore, this study was attempted to pitch some light on the potential role of Fetuin-A as a biomarker and a therapeutic target in IR.

This study demonstrated that NDD group significantly differed from NHC group for various baseline characteristics, such as WC, waist to hip ratio, SBP, DBP, platelet count, HbA1c, FBS, Fetuin-A, fasting insulin levels, HOMA-IR and QUICKI score ($p<0.05$) except age, gender BMI and albumin ($p>0.05$). Similar findings were reported in a previously conducted study (24). Sharma and colleagues (24) suggested that WC can be better predictor of diabetes than BMI and reported that a strong correlation between WC and FBS (R=0.813) compared to BMI and FBS (R=0.539). Demographic factors, such age and gender were not significantly associated in this study ($p>0.05$) and supported by the findings of previous researchers (25-27). Dabrowska and colleagues (25) and Vörös and colleagues (26) stated that Fetuin-A concentrations were independent of gender and Sun and colleagues (27) showed no association between age and Fetuin-A levels.

The NDD group had higher levels of Fetuin-A levels compared to NHC group ($p<0.001$) and was concurred with Ou and colleagues (15) and Song and colleagues (28). The current study also showed that NDD patients majorly distributed in the Fetuin-A 3rd and 4th quartile in comparison to the NHC group, who were majorly distributed in the 1st and 2nd quartile. On comparing the HOMA-IR and QUICKI score among the four quartiles of Fetuin-A levels, HOMA-IR was significantly higher in the NDD compared to the NHC group ($p<0.05$). This finding was in accordance with the study conducted by Song and colleagues (28). Whereas QUICKI score varied insignificantly among both the groups ($p>0.05$).

The independent predictors of Fetuin-A by multiple linear regression analysis were QUICKI, SBP and age ($p<0.01$) and were in contrast with findings of Yin and colleagues (23) and Song and colleagues (28). Yin and colleagues (23) reported fasting plasma glucose, 2 h oral glucose tolerance test, HOMA-beta-cell insulin secretion index, triglycerides and carotid intima media thickness as independent associated predictors of Fetuin-A. Song and colleagues (28) reported the association of Fetuin-A levels with fasting serum insulin and HOMA-IR. Our findings suggested that Fetuin-A was positively associated with SBP and age, but negatively with QUICKI and were independent predictors of Fetuin-A. This finding suggested that Fetuin-A and QUICKI represents a counteracting mechanism. Higher Fetuin-A were related to IR indicated as QICIKI score and might lead to development of T2DM and act as predictive marker for NDD cases that may be used as a therapeutic target in IR cases.

Limitations of this study was the small sample size considered. Since this study was not prospective in design, it did not allow for causal inference between serum Fetuin-A concentrations and the progression of T2DM. Moreover, as the study cases were mostly men, our results may not be applicable to women.

CONCLUSION

High levels of Fetuin-A were associated with NDD compared to NHC group, which in turn associated independently with SBP, albumin and HbA1c. Thus, Fetuin-A may be used as a biomarker for detection of NDD. This can help in for accurate diagnosis, thereby facilitating appropriate treatment for NDD and helps in managing patients with the risk of T2DM.

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CONFLICTS OF INTEREST

The authors declare that there are no competing interests associated with the manuscript.

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CORRELATION BETWEEN ULTRASOUND BI-RADS 4 BREAST LESIONS AND FINE NEEDLE CYTOLOGY CATEGORIES IN A SAMPLE OF IRAQI FEMALE PATIENTS

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ABSTRACT

Breast cancer is the most common malignancy in female and the most registered cause of women's mortality worldwide. BI-RADS 4 breast lesions are associated with an exceptionally high rate of benign breast pathology and breast cancer, so BI-RADS 4 is subdivided into 4A, 4B and 4C to standardize the risk estimation of breast lesions. The aim of the study: to evaluate the correlation between BI-RADS 4 subdivisions 4A, 4B & 4C and the categories of reporting FNA cytology results. A case series study was conducted in the Oncology Teaching Hospital in Baghdad from September 2018 to September 2019. Included patients had suspicious breast findings and given BI-RADS 4 (4A, 4B, or 4C) in the radiological report accordingly. Fine needle aspiration was performed under the ultrasound guide and the results were classified into five categories. The biopsy was performed for suspicious, malignant or equivocal FNA findings. This study included 158 women with BI-RADS 4 breast lesions with the mean age of (44.6 years); There was a highly significant association between BI-RADS 4 breast lesion and FNA results ($p<0.001$); 51.9% of BI-RADS IV-C had C5 FNA results. There was a highly significant association between BI-RADS 4 lesion and the final diagnosis ($p<0.001$); 41.2% of BI-RADS 4 B had a malignant breast lesion, while 37.3% of BI-RADS 4 C had a malignant lesion. A clear relationship was observed between BI-RADS 4 subcategories and the fine needle aspiration cytology subgroups. BI-RADS 4-B is helpful in the discrimination between benign and malignant breast lesions; furthermore BI-RADS 4C has more acceptable validity in the diagnosis of breast malignancy. Therefore, BI-RADS subcategories are encouraged to be included and mentioned in the ultrasound report for more accurate estimation of the lesion nature.

Keywords: *BIRADS 4 breast lesion, ultrasound, fine needle aspiration cytology.*

INTRODUCTION

Breast cancer is the most common malignancy in females and the most registered cause of women's mortality worldwide (1,2). Ultrasound has been an essential imaging modality for evaluation and characterization of breast lesions.

The fifth edition of the Breast Imaging Report and Data System (BI-RADS) was delivered by the American College of Radiology (ACR) (3) in order to standardize the risk estimation of breast lesions. In the ACR BI-RADS US lexicon, breast lesions of the category 4 have a 3-94% rate of malignancy and were subdivided into 4A, 4B and 4C. The category 4A may be used for a breast lesion that needs an intervention but has a low risk of malignancy (3-10%), the category 4B lesions has a moderate risk of malignancy (11-50%), The findings in this category need careful radiologic and pathologic correlation. Suggested follow-up with a benign result will rely on concordance. The category 4C lesions has a high probability for malignancy (51-94%) but it is not highly suggestive of breast cancer. This implies that BI-RADS 4 breast lesions are associated with a greatly variable risk of benign and malignant breast lesions (4), which might lead to a high rate of unnecessary biopsies.

US is the main diagnostic and screening tool for breast lesions. However, breast lesions with BI-RADS 4 assessment category tend to have an overlapping appearance and are sometimes difficult to identify (5,6), which may result in a high false positive rate, and subsequently unneeded biopsies and treatment (7).

There aren't many literature data evaluating the Correlation of BI-RADS 4 subcategories with FNA cytology results categories but there are many studies evaluating the correlation in general. A recent study by Zonderland et al showed that breast cancer was the biopsy result in (52.7%) of BI-RADS 4 lesions (8). Another study by Orel et al. revealed that 34% of BI-RADS 4 breast lesions proved to be cancer by guided biopsy (9). The PPV of BI-RADS 4 lesions in the previous literature data ranged from 6.2% to 52.7% (8,10,11).

By subdividing the category 4 lesions into 4A, 4B and 4C, it becomes possible to demonstrate significant probabilities for malignancy within this category so the patient and her physician can give an optimal decision regarding the management (12).

Fine needle aspiration cytology (FNAC) has become an important tool in the preoperative evaluation of breast pathology, and it has revealed high sensitivity, specificity and accuracy. It became popular and frequently needed due to its easy and quick approach, being cheap, and can be done without complications. The main indication for FNA cytology is to differentiate malignant from benign lesions (13). However, in some situations; the differentiation of benign and malignant breast lesions is difficult and sometimes not possible in FNA cytology. This difficulty arises when a specimen is deficient or if there is overlapping in the morphological features of benign and malignant lesions (for example, low-grade carcinoma in situ and atypical ductal hyperplasia). Consequently, the cytological reporting was classified for further standardization and the characteristic features are demonstrated in cytological terms in order to overcome these challenging situations. The five-tier system was the most commonly used categorization, which includes five categories from insufficient materials (C1), benign category (C2), atypical finding (C3), suspicious for malignancy (C4), or frankly malignant (C5) (14). This cytological reporting system enables the cytopathologists to define the indeterminate areas, and the referring clinicians to recommend an additional investigation for example, excisional biopsy.

In the study of Sehgal et al ,37.5% of C3 cases were found to be malignant during the histopathological evaluation ("false negative") (15) . Also in other literature data, C3 category revealed a malignant diagnosis in 8.6-52% cases. Sehgal et al reported that 87.5% of C4 category cases had a malignant diagnosis, and 12.5% cases revealed a benign diagnosis during the histopathological examination. Many other studies showed that C4 breast lesions were found to have a malignant diagnosis during the histopathological study in the range of 81% to 97% (16, 17, 18).

The aim of the study is to evaluate the correlation between BI-RADS IV subdivisions 4A, 4B & 4C and the categories of reporting FNA cytology results**.**

PATIENTS AND METHODS

Study design, location and patient selection

This case series study was conducted in the Oncology Teaching Hospital /medical city from September 2018 to September 2019. The study included 158 female patients who attended the Breast Clinic in the Oncology Teaching Hospital either for screening or diagnostic purposes. Those patients had suspicious findings and given BI-RADS 4 in the breast ultrasound report.

Sonography technique and lesion characterization

Each patient underwent the breast ultrasound using GE machine (Voluson E6 with linear transducer that had frequency of 7.5-12 MHz). The patient lies in the supine position with their hands on their heads. Both breasts and axillary regions are examined by the ultrasound for each patient. The suspicious breast findings were assessed by two expert radiologists and given BI-RADS 4. BI-RADS 4 are classified into BI-RADS 4A, 4B and 4C according to the descriptors of breast US BI-RADS lexicon 2013 that was mentioned in the introduction. The lesion characteristics include the mass, heterogeneous area, architectural distortion and peripheral ductal dilatation. The breast mass was assessed for its shape, margin, echogenicity and associated findings as skin thickening, nipple retraction, etc…

Fine needle aspiration technique and interpretation

Fine needle aspiration was performed for all patients under the ultrasound guide using 21-gauge needle. The site of the entry point on the skin was cleaned, then, the needle was inserted. The vacuum or negative pressure was generated in the needle and with in and out movement of the needle, the sample was taken. The material was placed on the slide then. The slide was stained with modified Papanicolaou stain. Then, the slides were reviewed on the microscope (Human Scope Premium 2006) by two cytopathologists.

The results of FNA cytology were classified into five categories from C1 to C5.

When the results of FNA are suspicious, malignant or equivocal, the biopsy was performed, then, the results of ultrasound and FNA were compared and analyzed.

Ethical consideration

The approval was taken from the scientific committee of the University of Baghdad

Written informed consent was taken from all included patients.

Statistical analysis

The collected data were analyzed statistically by the Statistical Package of Social Sciences software version 22. The Chi square and Fischer's exact tests were applied for analyzing the data as suitable. Two by two tables were used to acquire the validity findings of US-BIRADS in comparison to the final diagnosis. The level of significance (p value) was regarded statistically significant if it was 0.05 or less.

RESULTS

This study included 158 women presented with the mean age of (44.6 years) and range of 15-77 years; 27.8% of women were in the age group <40 years, 33.5% of them were in the age group 40-49 years, 19% of them were in the age group 50-59 years and 19.6% of them were in the age group of 60 years and more.

The ultrasound examination revealed that BI-RADS 4 A category was the most common category type which was found in (67.7%). The hypoechoic texture of the breast mass was the most common appearance found in about (52.5%). The right sided breast lesion (found in 50.6% of tumors) was more present than the left sided one. The upper outer quadrant was the most common site affected in breast (affected in 57%). Other ultrasound characteristics of breast lesions were illustrated in Table 1.

Table 1. Ultrasound characteristics of women with BI-RADS 4 breast lesion.

Variable		No.	%
US-BI-RADS			
4A		107	67.7
4B		28	17.7
4C		23	14.6
US description of breast lesion			
Mass	Hypoechoic	83	52.5
	Heterogeneous	38	24.1
	Isoechoic	11	7.0
	Complex	9	5.7
Peripheral duct dilation		8	5.1
Intraductal pathology		7	4.4
Complicated cyst		2	1.3
Side			
Right		80	50.6
Left		78	49.4
Site			
Upper outer quadrant		90	57.0
Lower outer quadrant		13	8.2
Upper inner quadrant		25	15.8
Lower inner quadrant		9	5.7
Retroareolar		21	13.3
Total		**158**	**100.0**

The fine needle aspiration has results for women with a shown breast lesion C1 (5.1%), C2 (7%), C3 (59.5%), C4 (11.4%) and C5 (17.1%). According to the FNA results, mild atypia was observed in (23.4%), moderate atypia in (15.2%), severe atypia in (5.1%), mild to moderate atypia in (18.4%), mammary carcinoma in (17.7%), intraductal papillary lesion was present in (3.8%), fibroadenoma in (7%), fibrocystic changes in (2.5%) and granulomatous mastitis in (7%). The final diagnosis revealed a benign breast lesion in 67.7% of women and a malignant breast lesion in 32.3% of women.

No significant differences were observed between women with a benign breast lesion and women with a malignant breast lesion regarding their ages (p=0.1). (Table 2)

Table 2. Distribution of women's age according to the final diagnosis.

Variable	Final diagnosis				P
	Benign		Malignant		
	No.	%	No.	%	
Age					0.1 NS
<40 years	33	30.8	11	21.6	
40-49 years	39	36.4	14	27.5	
50-59 years	19	17.8	11	21.6	
≥60 years	16	15.0	15	29.4	

There was a highly significant association between US-BIRADS 4 breast lesion and the final diagnosis (p<0.001); 41.2% of BI-RADS 4B women had a malignant breast lesion, while 37.3% of BIRADS 4C women had a malignant lesion. A highly significant association was observed between a hypoechoic breast mass and malignancy (p<0.001). (Table 3)

Table 3. Distribution of ultrasound characteristics according to the final diagnosis.

Variable	Final diagnosis				P
	Benign		Malignant		
	No.	%	No.	%	
US-BIRADS					**<0.001** S
4A	96	89.7	11	21.6	
4B	7	6.5	21	41.2	
4C	4	3.7	19	37.3	
US-lesion description					**<0.001** S
Hypoechoic	43	40.2	40	78.4	
Heterogeneous hypoechoic mass	32	29.9	6	11.8	
Isoechoic	10	9.3	1	2.0	
Complex mass	5	4.7	4	7.8	
Peripheral duct dilation	8	7.5	0	-	
Intraductal pathology	7	6.5	0	-	
Complicated cyst	2	1.9	0	-	
Side					0.2 NS
Right	51	47.7	29	56.9	
Left	56	52.3	22	43.1	
					0.3 NS
Site					
Upper outer quadrant	62	57.9	28	54.9	
Lower outer quadrant	6	5.6	7	13.7	
Upper inner quadrant	16	15.0	9	17.6	
Lower inner quadrant	6	5.6	3	5.9	
Retroareolar	17	15.9	4	7.8	

S=Significant, NS=Not significant.

There was a highly significant association between C4 and C5 FNA results of women with a breast lesion and malignancy (p<0.001). A highly significant association was observed between mammary carcinoma by the FNA results and the final diagnosis of breast cancer by biopsy (p<0.001). (Table 6)

Table 4. Distribution of cytology results according to thefinal diagnosis

Variable	Finaldiagnosis				P
	Benign		Malignant		
	No.	%	No.	%	
FNA results					**<0.001** S
C1	8	7.5	0	-	
C2	10	9.3	1	2.0	
C3	89	83.2	5	9.8	
C4	0	-	18	35.3	
C5	0	-	27	52.9	
FNA results					**<0.001** S
Mild atypia	37	34.6	0	-	
Moderate atypia	12	11.2	12	23.5	
Severe atypia	0	-	8	15.7	
Mild to moderate atypia	26	24.3	3	5.9	
Mammary carcinoma	0	-	28	54.9	
Intraductal lesion	6	5.6	0	-	
Fibroadenoma	11	10.3	0	-	
Fibrocystic changes	4	3.7	0	-	
Granulomatous mastitis	11	10.3	0	-	

S=Significant, NS=Not significant.

The validity findings of BI-RADS 4 breast lesions (4A, 4B, 4C) in regard to the final diagnosis were demostrted in Table 5.

Table 5. The validity findings of US-BIRADS 4 subdivisions in comparison to the final diagnosis.

BIRADS	Sensitivity	Specificity	+ve PV	-ve PV	Accuracy
BIRADS 4A	21.6%	10.3%	10.3%	21.6%	13.9%
BIRADS 4B	41.2%	93.5%	75%	76.9%	76.5%
BIRADS 4C	37.3%	96.3%	82.6%	76.3%	77.2%

There was a highly significant association between BI-RADS 4 breast lesion and the FNA results (p<0.001); 51.9% of BI-RADS 4C women had C5 FNA results, while 100% of BI-RADS 4A women had C1 FNA results. (Table 6).

Table 6. Distribution of the ultrasound BI-RADS 4 categories according to the FNA results categories.

Variable	FNA results										P
	C1		C2		C3		C4		C5		
	No.	%	No.	%	No.	%	No.	%	No.	%	
US-BIRADS											**<0.001** S
4A	8	100.0	9	81.8	82	87.2	6	33.3	2	7.4	
4B	0	-	2	18.2	8	8.5	7	38.9	11	40.7	
4C	0	-	0	-	4	4.3	5	27.8	14	51.9	

S=Significant.

Figure 1. 45-year old female. On her breast ultrasound, there is a lobulated hypoechoic mass given BIRADS 4A. FNA was done and it shows a benign finding (C2).

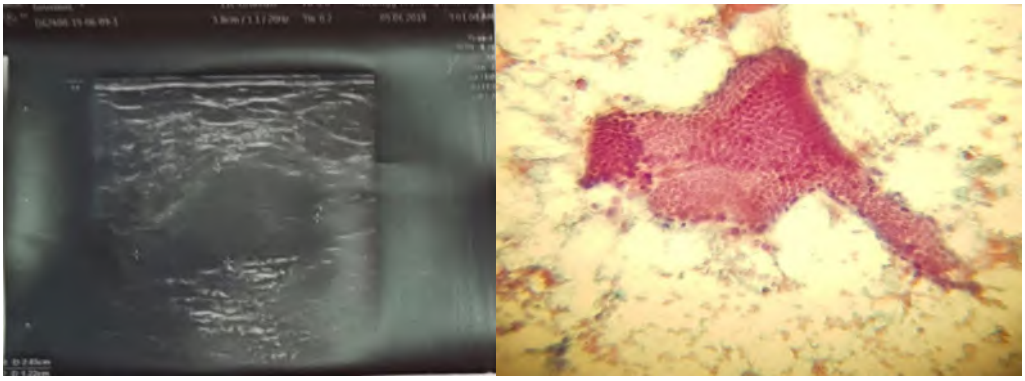


Figure 2. 50-year old female on regular follow up. Her breast ultrasound showed an oval shaped hypoechoic mass with an indistinct margin given BI-RADS 4 A. FNA was done from the lesion and revealed mild atypia (C3).

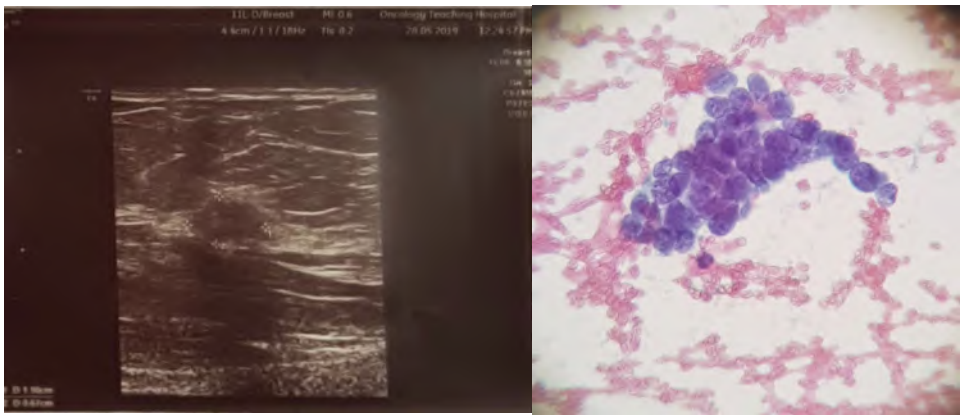


Figure 3. 31-year old female with a family history of breast cancer.
Her breast ultrasound showed a small ill-defined hypoechoic mass with an indistinct margin measuring (6x4mm) and given BI-RADS 4B. FNA was done and revealed suspicious findings (C4) and then proved to be malignant by a subsequent biopsy.

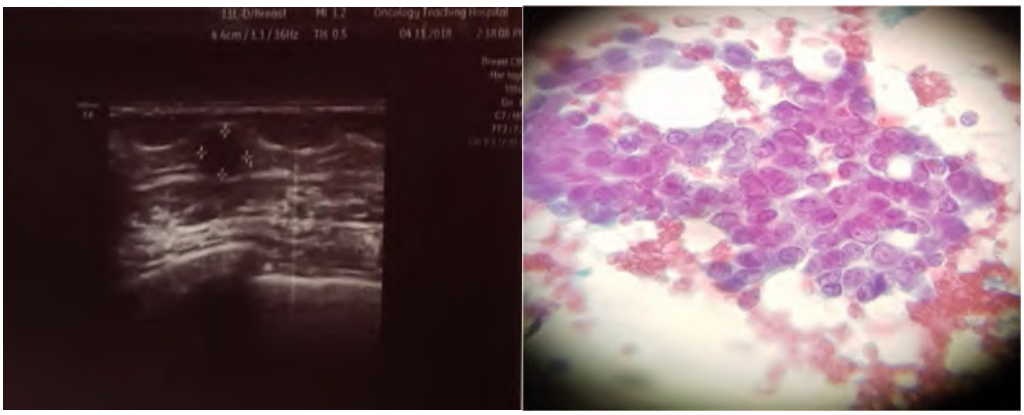
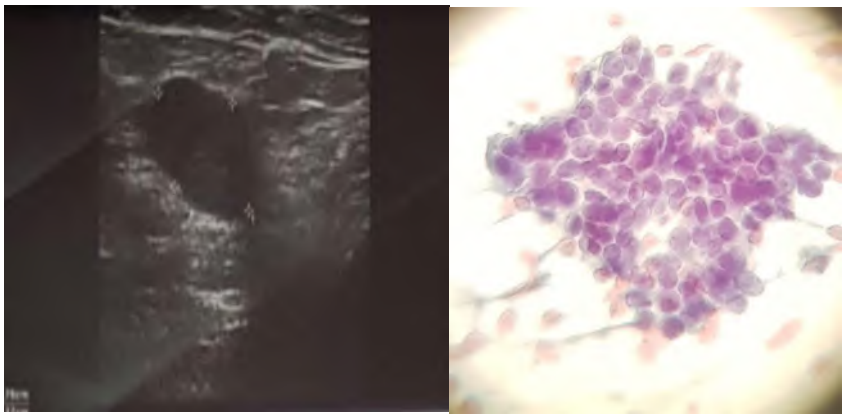


Figure 4. The breast ultrasound of 57year old female showed an ill-defined hypoechoic mass in the UUQ of the right breast and given BRADS 4B.
FNA of this lesion revealed malignant cells and proved to be malignant by a subsequent biopsy.



DISCUSSION

Breast carcinoma is the most common type of females' malignancies all over the world (19,20). Breast cancers represented 25% of all cancers and the main mortality cause for women. An early diagnosis of breast cancer is very important in the treatment that varies from lumpectomy to mastectomy. This precise diagnosis is mainly essential in the prevention of overtreatment or undertreatment with a poor prognosis (21).

The present study revealed a highly significant association between BI-RADS 4 of women with a breast lesion and the FNA results ($p<0.001$); 51.9% of BI-RADS 4 C women had C5 FNA results, while 100% of BI-RADS 4A women had C1 FNA results. This finding is similar to the results of Khaleel et al., a retrospective study in Iraq, which found a significant correlation between the ultrasound and the fine needle aspiration cytology findings in BI-RADS 4 breast lesions (22). The fine needle aspiration was commonly introduced for the assessment of palpable breast masses and it is done under the ultrasound guidance that made it as a novel diagnostic tool of a breast mass (23). Recently, the ultrasound guided-FNA is the main molecular diagnostic technique for breast cancer (24), while the morphological diagnosis of breast tumors was more valid by the core needle biopsy (25). The ultrasound imaging enables clinicians to recognize the nature and extent of a breast mass especially lesions with difficult localization by mammography and also the ultrasound is regarded as a complement imaging tool for mammography (26) . Our study found higher validity findings of the ultrasound BI-RADS 4C for the diagnosis of breast cancer as compared to BI-RADS 4A and 4B (the accuracy was 77% as compared to 13.9% and 76.5%, respectively). These findings are in agreement with the results of Filho et al study in Brazil which reported higher validity records of the ultrasound BI-RADS 4C in the diagnosis of breast carcinoma (27). Raza et al study in the USA documented that

these subcategories helped in providing more informative internal audit, improving the radiologic-pathologic correlation and improving the image-directed research (28). Additionally, Mustafa study in Iraq showed high agreement with the likelihood of malignancy after the application of BI-RADS terms in the category 4 and 5 (29).

In the current study, a benign breast lesion was finally detected in 67.7% of women and a malignant lesion in 32.3% of them. This malignancy proportion is lower than the results of Abedalarahman et al study in Iraq which found that 87.3% of suspicious benign lesions were a malignant breast mass as detected by histopathology (30). However, another Iraqi study carried out by Mustafa et al revealed that only 1.1% of women with suspicious lesions had a malignant breast mass (31). The ultrasound BI-RADS 4A was common BI-RADS in the present study with a highly significant association between BI-RADS 4C and breast malignancy. This finding is consistent with the results of Park et al study in South Korea which reported a significant concordance between BI-RADS 4C and a malignant breast mass in women with the negative mammography findings (32). Our study showed that a highly significant association was observed between a hypoechoic mass of women with a breast mass and malignancy ($p<0.001$). Similarly, Gokhale study in India reported that a hypoechoic mass is linked commonly with breast malignancy (33). Inconsistently, Kim et al study in South Korea stated than not all hypoechoic masses indicate breast malignancy, some benign breast lesions are characterized by hypoechoic lesions (34).

The fine needle aspiration found that a common breast lesion was C3 (59.5%) with a highly significant association between C4 and C5 FNA results of a breast lesion and malignancy ($p<0.001$). These findings are in agreement with the results of Arul et al study in India which found an obvious difference between FNA C3 and C4 results regarding benign and malignant breast lesions (35). In spite of these findings, the discrimination between FNA C3 and C4 is not easy due to the lack of specific diagnostic criteria (16). The present study revealed that the FNA results were commonly as mild atypia (23.4%), mild to moderate atypia (18.4%) and mammary carcinoma (17.7%), with a highly significant association between mammary carcinoma by FNA and the final diagnosis by biopsy ($p<0.001$). This finding coincides with the results of Mendoza et al study in China (36).

CONCLUSION

A clear relationship was observed between BI-RADS 4 subcategories and the fine needle aspiration cytology subgroups. BI-RADS 4-B is helpful in the discrimination between benign and malignant breast lesions; furthermore BI-RADS 4C has more acceptable validity in the diagnosis of breast malignancy.

Therefore, BI-RADS subcategories are encouraged to be included and mentioned in the ultrasound report for more accurate estimation of the lesion nature.

CONFLICT OF INTERESTS

Authors declare no conflict of interest.

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CHARACTERISTICS, CHEMICAL ANALYSIS AND BIOLOGICAL ACTIVITIES OF METHANOL EXTRACTS OF LICHENS *PLEUROSTICTA ACETABULUM* AND *CLADONIA SUBULATA*

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ABSTRACT

The aim of this study is to investigate the chemical composition of methanol extracts of the lichens Pleurosticta acetabulum and Cladonia subulata and their antioxidant, and anticancer activities. The phytochemical analysis of the extracts of lichens was determined by HPLC-UV method. The predominant phenolic compounds in these extracts were norstictic acid and salazinic acids in P. acetabulum, while hypoprotocetraric acid and fumarprotocetraric acid were the major metabolites detected in C. subulata. Total phenolics and flavonoids in the extracts were determined spectrophotometrically, with the varied amount from 21.31 to 73.45 mg GA/g and from 8.48 to 15.42 mg RU/g, respectively. The lichen extracts showed comparable and strong antioxidant activity, exhibited higher DPPH and hydroxyl radical scavengings, inhibitory activity towards lipid peroxidation and reducing power. Cytotoxic effects of lichens were tested against HeLa S3 and LS174 cell lines using MTT method.The cytotoxic effects of P. acetabulum and C. subulata extracts toward two cancer cell lines were in the range from 39.17 to >200 μg/mL IC_{50} value. The present study showed that the tested extracts of lichens demonstrated important antioxidant and anticancer effects. That suggests that these lichens can be used as new sources of the natural antioxidants and anti-cancer compounds.

Keywords: *Antioxidant activity, chemical composition, cytotoxic activity, lichen, phenols.*

INTRODUCTION

Lichens are complex symbiotic associations between fungi and algae which are important constituents of many ecosystems (1). *Cladonia subulata* was first described by G.H. Weber and F. H. Wigg. in 1780. This species is characterized by a squamulose thallus. *C. subulata* grows on bare soil over earth banks, rarely on wood, mainly in cool temperate regions. *Pleurosticta acetabulum* lichen was first described by J.A. Elix and H.T Lumbsch in 1988. *P. acetabulum* is characterized by a foliose, rosette-shaped thallus. This type of lichen belongs to thermophilic species of organisms and is found on the bark of deciduous trees in well-lit habitats. It can also be found on old tombstones and walls. Both lichen species are widespread on all continents (2-3).

To date, many lichens have proved to be a source of important secondary metabolites for pharmaceutical industries. Lichen extracts and lichen metabolites exhibit various biological activities such as antibiotic, antimycotic, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic properties. Therefore, lichens are natural antibiotics and potential drugs (4-5). Lichens synthesize a variety of organic compounds as primary and secondary metabolites. These compounds exist within the thalli and typically form crystals on the surface of the fungal hyphae. And since these compounds are poorly soluble in water, they can usually be isolated from the lichens by organic solvents (6). It was found that secondary metabolites of lichens exhibit a strong antioxidant activity due to the fact that they consist of phenolic groups that have the ability to scavenge toxic free radicals (7). Depsides and depsidones are the largest classes of secondary metabolites of lichens. Depsides, tridepsides, and tetradepsides consist of two, three, and four hydroxybenzoic acid residues linked by ester groups, while molecules depsidones have an additional ether bond between the aromatic rings. Depsidones in lichen are believed to arise by oxidative cyclization of depsides. It has been found that depsidones are more efficient antioxidants than depsides (8-9). So far more than 1000 primary and secondary metabolites, including phenolic compounds, dibenzofurans, depsides, depsidones, depsones, lactones, quinones and pulvinic acid derivatives, characteristic of lichens have been identified and some of them are isolated (10).

The aim of the present study was to identify the secondary metabolites of *Cladonia subulata and Pleurosticta acetabulum* from Serbia using HPLC-UV analysis and to investigate *in vitro* antioxidant and anticancer activities of the methanol extract from these lichens. Also, the aim of the study is to compare the influence of solvents of different polarities on the extraction of individual components from the samples with our earlier studies (11-12).

MATERIALS AND METHODS

Collection and identification of lichens sample

Lichens were collected at the site of the eastern slope of the mountain Kopaonik on the territory of the Republic of Serbia during May 2013. The specimens of the types of lichen: *Cladonia subulata* and *Pleurosticta acetabulum* were determined at the Department of Biology and Ecology, the Faculty of Natural Sciences and Mathematics, the University of Kragujevac using the relevant key and monographs (13). The samples were deposited under the following voucher numbers: 1010 (*Cladonia subulata*) and 109 (*Pleurosticta acetabulum*). The demonstration samples are preserved in facilities of the Department of Pharmacy, the Faculty of Medical Sciences, Kragujevac. The specimen of each species has been retained in our laboratory for future reference.

Preparation of the lichen extracts

The dried material (thallus) of the selected types of lichens is crushed to a coarse powder (2-6 mm), using a mill. Thereafter, a separate extraction (4 hours) was performed with methanol using the Soxhlet apparatus. For the extraction, 100 g of the crushed thallus of the tested lichen species and 300 mL of methanol were used. After the extraction, the resulting liquid extracts were filtered through the filter paper (Whatman, No.1). Evaporation of the solvents used for the extraction was performed under the reduced pressure on the rotary vacuum evaporator (IKA). In this way, dry extracts were obtained, which were stored in dark glass bottles and used for further testing.

High-performance liquid chromatography (HPLC) analysis

HPLC with UV detection was used to expand and identify individual constituents of the extracts. The analyses were performed on the Agilent 1200 Series using the C18 column (ZORBAX Eclipse XDB-C18; 25cm×4.6mm; 5 μm). Separate dot detection was performed using a Diode Array Detector (DAD) detector at 280, 330 and 350 nm, and the absorption spectra of the components are recorded in the range of 200 to 400 nm. Dissolved solubilized samples were filtered through using a pore size of 0.45 μm. Chromatographic separation was carried out using acetonitrile-water-phosphoric acid solvent system (90: 10: 0.1, v/v/v). The mobile phase flow rate was 1 mL/min. The column was thermostated at the temperature of 300 ^{0}C. Identification of individual constituents of the extracts was made by comparing the retention times (t_R) and UV spectra of constituents with standards (λ =200-400 nm). Used standards were obtained from the following sources: hypoprotocetraric acid is isolated from *Cladonia pyxidata*, fumarprotocetraric acid is isolated from *Hypogymnia physodes*, salazinic acid isolated from the *Lobaria pulmonaria*, norstictic acid from the lichen *Ramalina farinacea*, protocetraric acid is isolated from *Toninia Candida,* and evernic acid is isolated from the lichen *Evernia* prunastri (11-12).

Determination of total phenolics

Determination of total phenolics content was performed using the Folin-Ciocalteu method (14). The lichen extract was diluted to the concentration of 1 mg/mL, and aliquots of 0.5 mL were mixed with 2.5 mL of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 mL of $NaHCO_3$ (7.5%). The resulting mixture was left for 15 min at the 45 °C, after which absorbance was measured at 765 nm on spectrophotometer against the blank sample. All spectrophotometric analyses were performed on a Cary 300 UV-VIS Spectrophotometer from Agilent Technologies. The content of the total phenolics was calculated using the equation obtained from a standard gallic acid calibration curve (y = 0.007 x total polyphenols [mg GA/g of dry extracts] + 0.483, R^2 = 0.994) and the results were expressed in mg equivalents of gallic acid per g of the dry extract (mg GA/g).The values are presented as means of triplicate analyses.

Determination of total flavonoid content

The content of total flavonoids in the extracts was determined by the Markham spectrophotometric method (15). The reaction mixture was prepared by mixing a certain volume of the extract (2 mL) of the concentration 1 mg/mL with 2 mL of 2% methanol solution of aluminum (III) chloride. The absorbance of the samples was measured at 415 nm on the spectrophotometer compared to the blank test. Methanol in the place of extract was used as the blank. The total flavonoid content was determined using the equation obtained from a standard rutin calibration curve (y = 0.0296 x total flavonoid [mg RU/g of dry extracts] + 0.0204, R^2 = 0.9992) and the results were expressed in mg equivalents of rutin per g of the dry extract (mg RU/g). The values are presented as means of triplicate analyses.

Antioxidant activity

Determination of total antioxidant capacity

The total antioxidant activity of the lichen extracts was determined using the phosphomolybdenum method (16). This test is based on the reduction of Mo (VI)-Mo (V) by the antioxidant compounds and subsequent formation of a green phosphate/Mo (V) complex at acid pH. 0.3 mL of the sample extract was combined with 3 mL of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes with the reaction solution were incubated at 95 °C for 90 min. After that, the absorbance of the solution was measured at 695 nm using spectrophotometer versus the blank after cooling to room temperature. Methanol in the place of extract was used as the blank. Ascorbic acid (AA) was used as the standard. The total antioxidant capacity was determined as milligrams of ascorbic acid per gram of the dry extract (mg AA/g extract).

Determination of DPPH free radical scavenging activity

The free radical scavenging activity of the extracts was measured using the stable radical DPPH (1,1-diphenyl-2-picryl-hydrazil) according to the modification method from Kumarasamy (17). 8 mg DPPH was dissolved in 100 mL methanol to obtain the concentration of 80 µg/mL. Then, serial dilutions were carried out with the stock solution (1 mg/mL) of the extract. The resulting solutions (2 mL each) were mixed with DPPH (2 mL) and allowed to stand for 30 min for any reaction to occur, and the absorbance was measured at 517 nm. Ascorbic acid (AA) and butylated hydroxytoluene (BHT) dissolved in methanol were used as reference standards to make the stock solution with the same concentration (1 mg/mL). The control sample was prepared containing the same volume without the test compounds or reference antioxidants. Methanol 95% was used as the blank. The inhibition DPPH free radical scavenging activity (%) of the lichen extract was calculated using the following equation:

The inhibition capacity of DPPH radical (%) = Ac-As / Ac × 100,

where the Ac-absorbance of the control solution (negative control), As is the absorbance of the sample solution or standard. The IC_{50} value (µg/mL), defined as the concentration of extract needed to reduce the DPPH concentration of the radical by 50%, was obtained from the linear regression equation.

Determination of hydroxyl radical scavenging activity

In order to determine the ability of extracts to neutralize generated OH radical, the method described by Smirnoff & Cumbes with certain modifications was applied (18). The reaction mixture (3 mL) contains 1.0 mL of 1.5 mM FeSO4, 0.7 mL of 6 mM hydrogen peroxide, 0.3 mL of 20 mM and 1 mL of the sample solution. The absorbance was measured at 562 nm. Ascorbic acid and BHT were used as reference standards. The percentage of inhibition is calculated according to the equation:

The inhibition capacity of OH radicals (%) =Ac-As/Ac × 100,

where the Ac-absorbance of the control solution (negative control), As is the absorbance of the sample solution or standard. The IC_{50} value (µg/mL), defined as the concentration of extract needed to reduce the OH concentration of the radical by 50%, was obtained from the linear regression equation.

Determination of inhibition of lipid peroxidation

The antioxidant activity was determined by the thiocyanate method (19). The reaction mixture is made with 0.2 mL of extract samples (serial dilution from 1000 to 16.125 µg/mL), 0.2 mL linoleic emulsion (25 mg/mL in 99% ethanol) and 0.4 mL phosphate puffer (50 mM, pH = 7.4). The mixture is then incubated in the dark for 72 h at the temperature of 40^0C . An aliquot of the reaction mixture of 0.1 mL is

taken and 3 mL of ethanol (70%) and 0.1 mL of ammonium thiocyanate (30%) are added. Exactly 3 minutes after adding 0.1 mL of iron III chlorides (20 mM in 3.5% hydrochloric acid), the absorbance of the red-colored mixture is measured at 500 nm. Ascorbic acid and BHT were used as reference standards. The percentage of inhibition of lipid peroxidation is calculated according to the equation:

The inhibition of lipid peroxidation (%) =Ac-As/Ac × 100,

where the Ac-absorbance of the control solution (negative control), As is the absorbance of the sample solution or standard. The IC_{50} value (μg/mL), defined as the concentration of the extract that inhibits lipid peroxidation for 50%, was obtained from the linear regression equation.

Reduction capacity

The reducing capacity or reducing power was first described by Oyaizu (20). One milliliter of samples was mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide (1%). Then 2.5 mL of trichloroacetic acid was added to the mixture and the mixture was spinning at 3000 rpm for 10 minutes. Take 2.5 mL of the upper layer (supernatant), add 2.5 mL of distilled water and 0.5 mL of iron three chlorides. The absorbance of the solution was measured at 700 nm on the spectrophotometer compared to the blank test. Ascorbic acid was used as a positive control. An increase in the absorption of the solution shows how much reducing power is increased.

Cytotoxic activity

Cell line

The human cervix adenocarcinoma HeLa S3 cells (ATCC CCL-2.2) and human colon adenocarcinoma LS174 cells (ATCC CL-188) were purchased from the American Type Culture Collection (Manassas, VA, United States). The cell line was cultured in a nutrient medium RPMI-1640, (pH 7.2) with thermally inactivated (56 ^{0}C, 30 min.) fetal bovine serum (10 mL/100 mL), L-glutamine (3 mmol/L), streptomycin (100 mg/mL), penicillin (100 lU/mL), and HEPES (25 mM). The cell cultures were cultured in an incubator in the atmosphere saturated with water vapor, in the presence of 5% CO2, at the temperature of 37 ^{0}C.

Experimental design

The tested solution of extracts was made in DMSO (stock concentration= 100 mg/mL). The applied diluted solutions were the concentrations 200, 75, 25, 10 μg/mL. Cells for the cytotoxic activity (20000 cells/well) and antiproliferative activity (5000 cells/well) in 100 μL of a nutrient medium were seeded in 96-sterile plates and incubated for 24 hours under the atmosphere of saturated aqueous vapor at 37 ^{0}C and with 5% CO2. The cells were incubated with the test extracts of lichen, as well as the controls for the next 24 hours for the cytotoxic activity and 72 h for the antiproliferative activity, followed by MTT test. As a negative control, the cells that grow only in the culture medium are used, while positive controls were cis-DDP (cis-diammindihloroplatinum).

MTT test

The MTT standard in vitro test for viability and cell proliferation (21) was used. After the incubation of the cells with the extracts, the cells were washed with 100 μl of PBS (phosphate buffer solution) and MTT (20 μL) was added. The MTT reduction (absorption) measurement was performed spectrophotometrically at the wavelength of 540 nm on a multichannel spectrophotometer (Multiskan Ascent No354, Thermo Labsystems, Finland). The results are presented as the intensity of MTT reduction relative to the negative control. The absorbance of the control was taken 100% and in relation to it, the percentage values of the extracts were calculated relative to the control according to the formula:

% viability / proliferation of extracts = absorbance values of treated cells with extract or positive control / negative absorbance value X 100

The antitumor activity is expressed as the IC_{50} value. The IC_{50} value is defined as a concentration that inhibits cell survival by 50% or inhibits cell growth. The results are presented as the arithmetic mean of the tetraplicates for each concentration of the standard deviation.

Statistical analysis

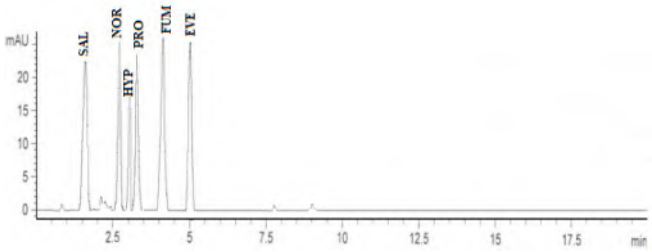
All measurements are repeated three times, and the results are displayed as the mean ± standard error (mean ± SD). The statistical analyses were performed using Microsoft Excel and SPSS software package, version 20 (IBM SPSS Statistics 20 Inc. Chicago, Illinois 60606-6307 U.S.A.). One-way ANOVA was used to determine differences between the mean measurement values, with statistical significance of $p < 0.05$.

RESULTS

The HPLC chromatogram for standards and the methanol extracts of the species *C. subulata* and *P. acetabulum* recorded at 254 *nm* are represented in Figures 1 and 2. The results of the HPLC analysis of the methanol extract of the *Pleurosticta acetabulum* indicate presence of four metabolites: salazinic acid (at the retention time t_R=1.56±0.20), norstictic acid (t_R=2.70±0.10), protocetraric acid (t_R=3.24±0.20) and evernic acid (t_R=5.08±0.10). The dominant peak in the chromatograms originates from depsidone compound, norstictic acid. The UV spectrum of norstictic acid has 3 absorption maxima (212, 239, 320 nm). Besides these signals in HPLC chromatogram of the methanol extract of *P. acetabulum*, medium to low-intensity signals were identified, originating from: salazinic acid, protocetraric acid and evernic acid. The results of the HPLC analysis of the methanol extracts of the *Cladonia subulata* indicate the presence of two metabolites: hypoprotocetraric acid (t_R= 3.10 ± 0.20 min) and fumaroprotocetraric acid (t_R= 4.14 ± 0.10 min). The

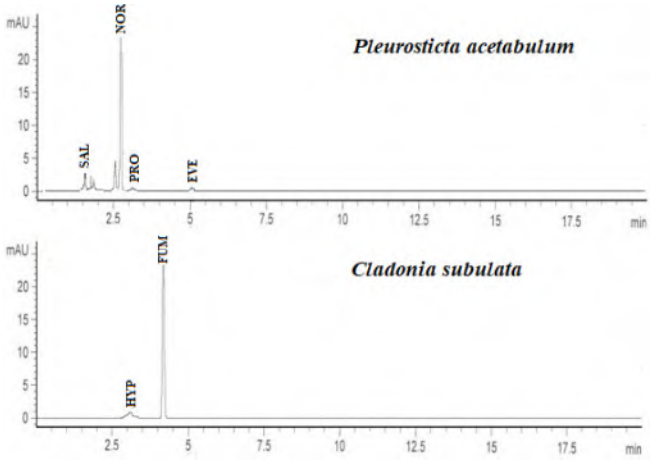
dominant peak in the chromatograms originates from depsidone compound, fumaroprotocetraric acid. The UV spectrum of fumaroprotocetraric acid has 3 absorption maxima (212, 240, 318 nm). Besides fumaroprotocetraric acid, the tested extracts contain depsidone hypoprotocetraric acid, whose signal intensity was significantly smaller than the peak from fumaroprotocetraric acid.

Figure 1. HPLC chromatogram of mixed standards used for identification of the lichen compounds at 254 nm



(SAL-Salazinic acid; NOR-Norstictic acid; HYP-Hypoprotocetraric acid; PRO-Protocetraric acid; FUM- Fumarprotocetraric acid; EVE- Evernic acid)

Figure 2. HPLC chromatograms of the methanol extracts of P. acetabulum and C. subulata at 254 nm.



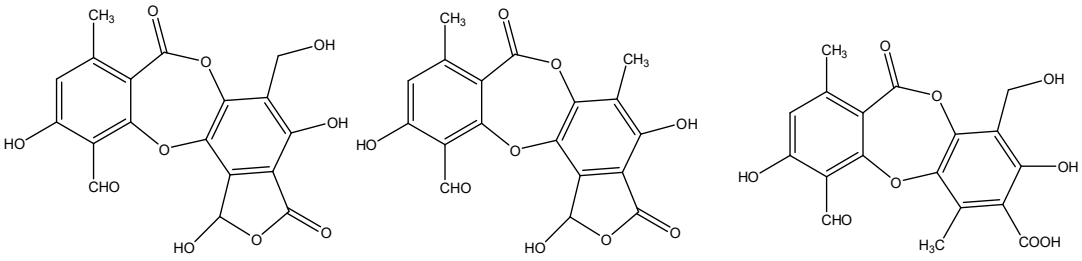
(SAL-Salazinic acid; NOR-Norstictic acid; HYP-Hypoprotocetraric acid; PRO-Protocetraric acid; FUM- Fumarprotocetraric acid; EVE- Evernic acid)

Table 1. Retention time of the examined lichen substances and their absorbance maxima (nm).

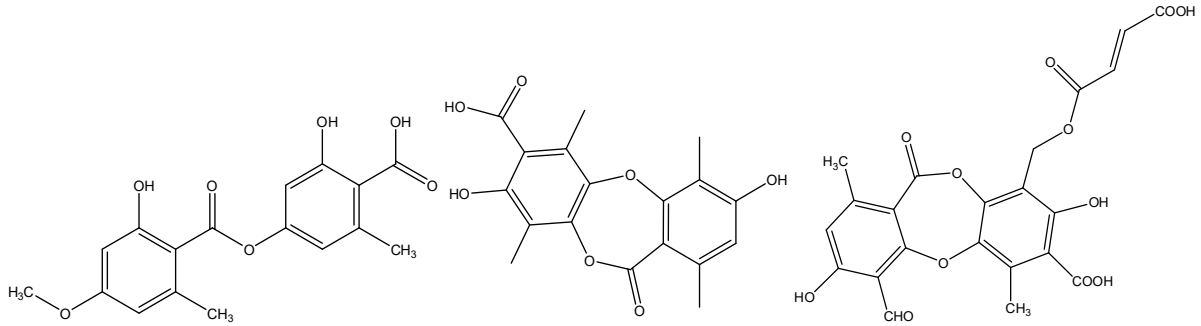
Lichen	Compound	Class substances	Retention time (t_R±SD)* (min)	Absorbance maxima (nm)
Pleurosticta acetabulum	Salazinic acid	Depsidone	1.56±0.20	212, 238, 310
	Norstictic acid	Depsidone	2.70±0.10	212, 239, 320
	Protocetraric acid	Depsidone	3.24±0.20	212, 242, 320
	Evernic acid	Depside	5.08±0.10	213, 270, 305
Cladonia subulata	Hypoprotocetraric acid	Depsidone	3.10±0.20	216, 258, 320
	Fumarprotocetraric acid	Depsidone	4.14±0.10	212, 240, 318

The structures of the detected compounds are shown in Figure 3.

Figure 3. Chemicals structures of the identified compounds



Salazinic acid Norstictic acid Protocetraric acid



Evernic acid Hypoprotocetraric acid Fumarprotocetraric acid

Table 2 shows the results of determination of total phenols, total flavonoids and the antioxidant capacity of the examined lichen extracts. The total phenolic contents expressed as gallic acid equivalents of the methanol extracts amounted to 73.45 mg GA/g for *P. acetabulum* and 21.31 mg GA/g for *C. subulata* , respectively. The results for the total antioxidant capacity showed that the methanol extracts of *P. acetabulum* with the antioxidant capacity of 74.29 mg AA/g possess a greater antioxidant activity than the methanol extract of *C. subulata.*

Table 2. Polyphenolic content (phenolic and flavonoids) and total antioxidant capacity of the methanol extracts of *Cladonia subulata* and *Pleurosticta acetabulum*

Lichen	Phenolics content (mg GA/g)	Flavonoids content (mg RU/g)	Antioxidant capacity (mg AA/g)
P. acetabulum	73.45±0.82	15.42±0.55	74.29±1.36
C. subulata	21.31±1.19	8.48± 0.57	25.36±0.94

*Values are expressed as mean ± SD of triplicate measurements;
GA - gallic acid equivalents; RU - rutin equivalents;
AA- ascorbic acid equivalents;

Table 3. Antioxidant activity of the examined *C. subulata* and *P. acetabulum* methanol extracts and control.

Lichen/control	IC_{50} (µg/mL)		
	DPPH scavenging activity	OH radical scavenging activity	Inhibition lipid peroxidation
P. acetabulum	48.52± 0.77	163.83± 0.95	74.30±1.48
C. subulata	296.75±0.61	595.35±7.78	151.96±2,79
Ascorbic acid	6.05±0.34	150.55±2.31	> 1000
BHT	15.61±1.26	33.92±0.79	1.00±0.23

*Values are expressed as mean ± SD of triplicate measurements;
AA- ascorbic acid equivalents; BHT- Butylhydroxytoluene

The assessment of the antioxidant activity showed that the tested extracts were able to scavenge DPPH radical (Table 3). The results of the hydroxyl radical scavenging activity of the tested extracts showed that the methanol extract of *P. acetabulum* had the highest activity (163.83 μg/mL), while the methanol extract of *C. subulata* showed the lowest activity (595.35 μg/mL). The results of the inhibitory activity towards lipid peroxidation (Table 3) demonstrated that all examined extracts of *P acetabulum* and *C. subulata* exhibited a significant inhibitory activity. The IC_{50} value of the methanol extract was 74.30 μg/mL and for *C. subulata* 151.96 μg/mL. The results of the reducing power of the tested lichen extracts are shown in Table 4. Measured values of absorbances varied in the range from 0.011 to 0.25. Higher absorbance indicates higher reducing power. The extract of *P. acetabulum* showed higher reducing power than *C. subulata* in all examined concentrations.

Table 4. Reducing power of the methanol extracts of lichens *Cladonia subulata* and *Pleurosticta acetabulum* and ascorbic acid

	Absorbance (700 nm)				
Lichen	**1000 µg/mL**	**500 µg/mL**	**250 µg/mL**	**125 µg/mL**	**62.5 µg/mL**
P. acetabulum	0.25±0.011	0.123±0.003	0.063±0.003	0.035±0.006	0.018±0.002
C. subulata	0.093±0.007	0.045±0.003	0.031±0.002	0.026±0.005	0.011±0.002
Ascorbic acid	2.113±0.032	1.654±0.021	0.0957±0.008	0.0478±0.004	0.0247±0.004

*Values are expressed as mean ± SD of triplicate measurements;

Table 5. Cytotoxic activity of the extracts on the HeLa S3 and LS174 cells line (after 24 h and 72 h incubation)

Lichen	***IC₅₀ (µg/mL)***			
	HeLa S3		LS174	
	24 h	**72 h**	**24 h**	**72 h**
P. acetabulum	64.30±2,89	39.17±5.54	66.09±1.61	>200
C. subulata	>200	>200	>200	>200
Cis-DDP	2.16 ± 0.67	0.78 ± 0.21	5.89 ± 0.92	2.48 ± 0.32

*Values are expressed as mean ± SD of triplicate measurement

Table 5 shows the IC_{50} values of cytotoxic/cytostatic activity of the tested extracts on HeLa and LS174 cells, determined by the MTT assay. The highest cytotoxic/cytostatic activity was shown by the methanol extract of *P. acetabulum* against HeLa S3 cells (IC_{50}= 64.30 µg/mL after 24 h of incubation; IC_{50}=39.17 µg/mL after 72 h incubation) and LS174 (IC_{50}= 66.09 µg/mL), while the methanol extract of *C. subulata* had no significant cytotoxic activity according to the cell lines either after 24 h or after 72 h of incubation.

DISCUSSION

The identification of secondary metabolites in the methanol extracts of *P. acetabulum* and *C. subulata* and their antioxidant and anticancer potentials were presented in this study. In the investigated extracts, depsidones and depsides were present as the most abundant substances classes. Comparing the methanol lichen extracts with our earlier study of the acetone extracts of *P. acetabulum* and *C. subulata,* the presence of the same metabolites was identified, but signal intensities and areas below the absorption maximum of certain secondary metabolites differed, which is consistent with the ability of the solvent (acetone and methanol) to dissolve more or less of these metabolites, by the principle of "dissolve similarly" (11-12). Such data indicate the influence of solvents of different polarity on the extraction of individual components from the samples (22).

The results for total phenolics and flavonoids content showed that the methanol extract of lichens had a higher total phenolic content than the acetone extract which we examined in our earlier research (11). Theobtained results that the methanol extracts contain the highest content of phenol and flavonoids are in accordance with the literature data where it has been shown that the phenol compounds are more soluble in the polar solvents (23).

Until now, many researchers have investigated the antioxidant properties of many lichens extracts and some of them showed a very good antioxidant activity (24-26). If we compare the same lichens species but different extraction solvent, the methanol extract had a greater antioxidant capacity than the acetone extract. There is a positive correlation between total phenols and the results of the antioxidative activity testing. These results agree with the literature (27-28). Based on the IC_{50} values, it is evident that the methanol extract of *P. acetabulum* exhibits the highest antiradical activity against DPPH· (48.52 μg/mL) compared to the acetone extract of *P. acetabulum* and acetone and methanol extracts of *C. subulata* but still significantly less than commercially used butylhydroxytoluene and ascorbic acid (11-12). Also, the methanol extracts of *P. acetabulum* and C. *subulata* showed a stronger antioxidant activity than the extract of *Toninia candida* and *Usnea barbata* (29), and many other lichen species (8, 27). The results of the antioxidant activity (OH radical scavenging activity, inhibition lipid peroxidation and reducing power) suggest that the methanol extracts of *P. acetabulum* and *C. subulata* were free radical scavengers, acting possibly as primary antioxidants. The tested extracts show a strong antioxidant activity against different oxidative systems. The strong antioxidant activity is the result of a high total phenolic content of the tested extracts (25). Phenolic compounds express the antioxidant effects mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (30). Some metabolites of lichens in

their structure contain phenolic groups which are considered to be a key element for the antioxidative efficiency (31). All identified secondary metabolites possess two phenolic groups in their molecules. Norstictic acid, evernic acid, salazinic acid, fumarprotocetraric acid and protocetraric acid, isolated from different lichen species are relatively strong antioxidant agents (8, 32-34). Nevertheless, it should be taken into consideration that individual phenolics may have distinct antioxidant activities, there may be antagonistic or synergistic interactions between phenolics and other compounds (35).

The methanol extracts of lichens showed a weaker cytotoxic effect according to the tested cell lines compared to the acetone extracts in our previous studies (11-12). There are previous reports on examination of the cytotoxic activity of many lichens (36-37), but in this study, the cytotoxic activity of the methanol extracts of *P. acetabulum* and *C. subulata* was explored for the first time. Relatively few lichen substances have been screened in detail for a biological activity and therapeutic potential, principally due to difficulties in obtaining them in quantities and purities sufficient for structural elucidation and pharmacological testing (38). So far, a limited number of studies have been published where the mechanism of action against cancer cell lines was explored (39). The molecular mechanism of cell death by the lichen compounds includes cell cycle arrest, apoptosis, necrosis, and inhibition of angiogenesis (40). There are several studies about the antitumor activity of depsides and depsidones including some of the identified lichen metabolites in the examined extracts (8, 32-34). Generally, depsidones showed a stronger cytotoxic activity than depsides. The strong biological activity of some depsidones may be due to the strong hydrogen bond between the aldehyde group at C3 and the hydroxyl group at C4. Depsidones, salazinic acid, norstictic acid, and fumarprotocetraric acid, were evaluated for their cytotoxic activity towards hepatocytes from a rat and lymphocytes from rat spleens (41-42). The research has shown that salazinic acid, stictic acid and fumarprotocetraric acid showed apoptosis of hepatocytes in a dose-dependent manner with stictic acid showing the strongest apoptotic activity. Also, in the work of Pejin and associates, it has been shown that the results suggest a moderate anticancer activity towards malignant HT-29 of stictic acid (43). This may indicate that norstictic acid can be considered as a promising lead compound for the design of novel human colon adenocarcinoma drugs.

CONCLUSION

This is the first research dealing with the chemical composition, antioxidant and cytotoxic activity of the methanol extracts of these lichens. The extracts of these lichens showed significant antioxidant and anticancer activities in vitro. The present study provides the data supporting the use of *P. acetabulum* and *C. subulata* extracts as natural antioxidant agents and confirms that these lichens represent a significant source of phenolic compounds. There is a need to continue to investigate the research in the field of phytochemistry and biological activity of lichens with particular reference to the compound of lichens that gives promising results. The attention should also be paid to identifying the specific mechanisms of action and extensive clinical studies to use promising drug therapies based on the lichens substances.

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CONFLICT OF INTEREST

None.

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ASSESSMENT OF SEXUAL BEHAVIOR AND HABITS OF MEDICAL AND NON-MEDICAL STUDENTS

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ABSTRACT

The aim of this study is the assessment of sexual behavior and habits of medical and non-medical students in Belgrade. The research is designed as cross-sectional study conducted in the period from November until January 2016/2017. An anonymous and standardized questionnaire from the "National Health Survey" research protocol was used and variables of interest were added. A total of 1268 randomly selected students participated, from higher education institutions in Belgrade (College for Health Studies "Milutin Milankovic, Medical College of applied sciences in Zemun, Belgrade, Business Academy and Faculty of Security Studies in Belgrade). Results: Respondents from both groups almost had sexual relations with the opposite sex, although 2.2% of students of medical profession and 1.7% of non-medical profession had sexual relations with the same sex. Condoms are most often contraceptive measures used in both groups, (>40%). The most common reason for not using condom in both groups were partner's trust or reducing pleasure during intercourse ($p<0.005$ and $p<0.851$ respectively). Students of non-medical professions comparing to students of medical professions, on the second year of study (7.3% vs 5.5%), and on the third year of study (14.1% vs 2.4%) stated that the reason for not using a condom is the high cost. Conclusions: According to the obtained results, students of medical professions were prone to risky sexual behaviour, despite greater knowledge about reproductive health and risky sexual behaviors. There is a significant need for an evaluation of educational programs about sexual and reproductive health of students from all orientations.

Keywords: *Students, sexual behavior, habits.*

INTRODUCTION

Youth is period of life during which beside biological and psychological maturation, the process of including an individual in the social community also takes place. During studies, society has some expectations from young people, expectations that they will develop skills and abilities which will help them to take significant roles in every part of society (1). Disharmony between biological and psychosocial maturity opens a possibility for risky behaviors which can disturb psychophysical and reproductive health of the adolescent population. Risky sexual behavior of students implies first sexual intercourse at a young age, more frequent partner change, not using contraceptive methods, inconsistent condom use for protection of STI, etc. As a consequence of ignorance, incomplete or inaccurate information, as well as taking reproductive health for granted, there are unwanted pregnancies (usually ending in abortion) or STIs which can have long term consequences on psycho-physical health (2).

Results of the research of health of the population of Serbia in 2013, shows that 33.1 % of young people in Serbia already had sexual intercourse, which is 4.1 % more than showed in the 2006 research. Although an increasing number of young people in the Republic of Serbia follow modern tendencies in sexual behavior, a small number use effective methods of contraception (3).

Young people in the age group of 15-24 years represent 25% of the sexually active population, and over 60% of all newly acquired STIs worldwide are registered in this age group (4). Studies show that a great number of young people do not have elementary knowledge about anatomy and physiology of reproduction, and that their knowledge about contraception and STI is poor and filled with prejudice (5, 6). One of the important reasons for this kind of behavior is that education of young people about reproductive health for now is not regulated by the system, and because of that the source of knowledge and information for young people are their peers, media as well as the internet. Research in relation to sexual behavior of students shows that students are a high-risk population for acquiring and transmitting STIs (7). Risky sexual behavior of students implies more frequent partner change, entering sexual relations under the effect of psychoactive substances, inconsistent condom use, etc. (8, 9). Considering that young people and students are a population that is especially at risk, the attention in the research is mostly focused on their knowledge on risks, significance of preserving reproductive health, use of contraceptive measures, and general knowledge on STIs (10). It is considered that a higher level of knowledge on sexual health provides many benefits to these categories. Promoting knowledge in relation to STIs, as well as developing awareness of all complications and consequences of infections that could occur certainly affects the reduction of risky behavior(10).

THE AIM OF THE PAPER

The aim of the paper is an assessment of sexual behavior and habits of medical and non-medical students.

PATIENTS AND METHODS

Research is based on the cross-sectional study and the study population consisted of 1268 students from four higher education institutions of the University of Belgrade of medical and non-medical professions. For the study we used an anonymous and standardized questionnaire from the “National Health Survey” research protocol where variables of interest were added (11).

The sampling method is a stratified (medical / non-medical profession, year of study) random sample. In the research were included the following higher educational institutions of Belgrade University: Medical College of applied sciences in Zemun, Belgrade, College for Health Studies “Milutin Milankovic”, Business Academy and Faculty of Security Studies in Belgrade. Respondents were students of medical and non-medical educational profiles.

Prior to the start of the research, the Deans of the selected faculties gave written approval for conducting anonymous surveys. The ethical standards of the research are harmonized with the international (Helsinki Declaration) and specific legislation of our country. In order to respect the privacy of the subject of research and the confidentiality of information, all necessary steps have been taken in accordance with the Law on Personal Data Protection ("Official Gazette of the Republic of Serbia", No. 97/08, 104/09), the Law on Official Statistics (“Official Gazette RS”, No. 104/09) and the European Parliament Directive on the protection of personal data with regard to personal data (Directive 95/46 / EC). For conducting the research, the consent of the Ethics Committee of the Faculty of Medical Sciences was obtained.

Statistical analysis

The complete statistical analysis of data was performed using the statistical software package, PASW Statistics 18® [SPSS (Hong Kong) Ltd., Hong Kong]. All variables were presented as the frequency of certain categories. Differences between categorical variables were tested by Chi-square test. The normality of the data was assessed using the Kolmogorov-Smirnov test. The relationship between the variables was tested by Spearman’s coefficient correlation. All the analyses were estimated at $p < 0.05$ level of statistical significance.

RESULTS

Our results show that in answers to the question about having sexual relations there is a significant difference between years of study for students of medical profession $p < 0.001$ (Table 1.). The largest number of students who have had sexual relations were on the 3rd year of study (97.3%), and the smallest number were on the first year (79.3%). On the other hand, if we compared the same year of study between students of medical and non-medical profession, results show that on the all the 3 years of study there are more respondents of medical profession who have had sexual

relations (First year 79.3% *vs.* 76.1%; Second year 90.9% *vs*. 74.3%; Third year 97.3% *vs*. 82.0%).

Respondents from both groups almost as a rule had sexual relations with people of opposite sex, but there are 2.2% of students of medical profession and 1.7% of students of non-medical profession who had sexual relations with people of the same sex.

Regarding the type of sexual intercourse that respondents had, our results show that students of both medical and non-medical professions more often had all 3 type of sexual intercourse (vaginal, anal and oral) in the third and second year of studies compared to the first year of studies (First year *vs*. First year: p =0.555; Second year *vs*. Second year: p <0.001; Third year *vs*. Third year: p <0.001). When we compare students of medical and non-medical profession, results show that oral sex is by 47.5% and anal sex is by 17.2% more often practiced by students of non-medical profession and students of medical profession more often practice vaginal sex 97.3% (Table 1.)

If the frequency of sexual relations is compared, a significant difference can also be observed between students of medical profession relative to the year of study (p <0.001). With a higher year of study there are less students who never had sexual intercourse (First year 20.7%, Second year 9.1%; Third year 2.7%), and the number of students who had sexual intercourse often increases (First year 40.1%, Second year 44.6%; Third year 55.9%). If we compare students of medical and non-medicals professions we find significant differences on the second year of study (9.1% *vs.* 25%; p <0.001), and on the third year of study (2.7% *vs.*18%; p <0.001).

A significant difference in the use of condoms during vaginal sexual intercourse was found in students of medical professions (p <0.001). Comparing the year of study, with the increase of the year of studying also increased the number of students who never use condoms during vaginal intercourses and decreases the number of those who always use condoms. Comparing students of medical and non-medical professions of the same year of study, results shows that on the second year of study (16.6% *vs.* 10.1%), and on the third year of the study (24.3% *vs*. 11.5%) there are more students of medical professions who participate in unprotected sexual intercourses. Regarding the condom use during anal intercourse, 88.3% students of medical professions and 64.8% students of non-medical professions on the third year of study in both groups never had that type of intercourse (Table 2). The results show that students stated that the most common reason for not using condom in both groups were that they trust their partner or that the condom reduces pleasure felt during intercourse (students of medical professions p <0.005; students of non-medical professions p <0.851). Students of non-medical professions comparing to students of medical professions, on the second year of study (7.3% *vs*. 5.5%), and on the third year of study (14.1% *vs*. 2.4%) stated that the reason for not using a condom is the high cost of condoms (Table 2.).

There are no significant differences about using female condoms. Students of medical professions most often used it in the second year of studies, 4.2 % of them. Regarding the use of contraceptive measures, condoms are most often used in both groups, by over 40% of subjects. Students of medical professions more commonly use birth control pills (14.7% *vs*. 4.0%), and coitus interruptus (pull-out method) (27.9% *vs*. 9.8%) than students of non-medical professions (Table 2).

Table 1. Sexual habits of students

Variables	Medical profession No 645			Non-medical profession No 623		
Did you ever have sexual relations?	**I year**	**II year**	**III year**	**I year**	**II year**	**III year**
yes	180 (79.3)	279 (90.9)	108 (97.3)	175 (76.1)	205 (74.3)	100 (82.0)
no	47 (20.7)	28 (9.1)	3 (2.7)	55 (23.9)	71 (25.7)	22 (18.0)
p value	<0.001*			0.246*		
With whom you have sexual relations:						
With persons of the opposite sex	225 (99.1)	302 (98.4)	111 (100.0)	225 (97.8)	274 (99.3)	120 (98.4)
With persons of the same sex	2 (0.9)	4 (1.3)	-	3 (1.3)	1 (0.4)	-
With persons of both sex	-	1 (0.3)	-	2 (0.9)	1 (0.4)	2 (1.6)
p value	0.623*			0.343*		
Oral sexual intercourse						
yes	52 (22.9)	91 (29.6)	34 (30.6)	66 (28.7)	112 (40.6)	58 (47.5)
no	175 (77.1)	216 (70.4)	77 (69.4)	164 (71.3)	164 (59.4)	64 (52.5)

Variables	Medical profession No 645			Non-medical profession No 623		
p value	0.161*			0.001*		
Anal sexual intercourse						
yes	18 (7.9)	43 (14.0)	11 (9,9)	29 (12.6)	39 (14.1)	21 (17.2)
no	209 (92.1)	264 (86.0)	100 (90.1)	201 (87.4)	237 (85.9)	101 (82.8)
p value	<0.001*			0.241*		
Vaginal sexual intercourse						
yes	178 (78.4)	277 (90.2)	108 (97.3)	174 (75.7)	205 (74.3)	100 (82.0)
no	49 (2.6)	30 (9.8)	3 (2.7)	56 (24.3)	71 (25.7)	22 (18.0)
p value	<0.001*			<0.001*		
Practicing sexual relations:						
Every day	21 (9.3)	22 (7.2)	5 (4.5)	20 (8.7)	21 (7.6)	8 (6.6)
2-3 times a week	91 (40.1)	137 (44.6)	62 (55.9)	88 (38.3)	103 (37.3)	56 (45.9)
1-3 times a month	47 (20.7)	93 (30.3)	30 (27.0)	46 (20,0)	52 (18.8)	19 (15.6)
Once every three months	17 (7.5)	12 (3.9)	6 (5.4)	16 (7.0)	14 (5.1)	10 (8.2)
Once every six months	3 (1.3)	5 (1.6)	1 (0.9)	6 (2.6)	8 (2.9)	6 (4.9)
Once a year	1 (0.4)	10 (3.3)	4 (3.6)	3 (1.3)	9 (3.3)	1 (0.8)
Never	47 (20.7)	28 (9.1)	3 (2.7)	51 (22.2)	69 (25.0)	22 (18.0)
p value	<0.001*			0.504*		

Chi-square test, No - number.

Table 2. Condom use and risky sexual behavior of students

Variables	Medical profession No 645			Non-medical profession No 623		
Did you ever have sexual relations?	**I year**	**II year**	**III year**	**I year**	**II year**	**III year**
yes	180 (79.3)	279 (90.9)	108 (97.3)	175 (76.1)	205 (74.3)	100 (82.0)
no	47 (20.7)	28 (9.1)	3 (2.7)	55 (23.9)	71 (25.7)	22 (18.0)
p value	<0.001*			0.246*		
With whom you have sexual relations:						
With persons of the opposite sex	225 (99.1)	302 (98.4)	111 (100.0)	225 (97.8)	274 (99.3)	120 (98.4)
With persons of the same sex	2 (0.9)	4 (1.3)	-	3 (1.3)	1 (0.4)	-
With persons of both sex	-	1 (0.3)	-	2 (0.9)	1 (0.4)	2 (1.6)
p value	0.623*			0.343*		
Oral sexual intercourse						
yes	52 (22.9)	91 (29.6)	34 (30.6)	66 (28.7)	112 (40.6)	58 (47.5)
no	175 (77.1)	216 (70.4)	77 (69.4)	164 (71.3)	164 (59.4)	64 (52.5)
p value	0.161*			0.001*		

Variables	Medical profession No 645			Non-medical profession No 623		
Anal sexual intercourse						
yes	18 (7.9)	43 (14.0)	11 (9,9)	29 (12.6)	39 (14.1)	21 (17.2)
no	209 (92.1)	264 (86.0)	100 (90.1)	201 (87.4)	237 (85.9)	101 (82.8)
p value	<0.001*			0.241*		
Vaginal sexual intercourse						
yes	178 (78.4)	277 (90.2)	108 (97.3)	174 (75.7)	205 (74.3)	100 (82.0)
no	49 (2.6)	30 (9.8)	3 (2.7)	56 (24.3)	71 (25.7)	22 (18.0)
p value	<0.001*			<0.001*		
Practicing sexual relations:						
Every day	21 (9.3)	22 (7.2)	5 (4.5)	20 (8.7)	21 (7.6)	8 (6.6)
2-3 times a week	91 (40.1)	137 (44.6)	62 (55.9)	88 (38.3)	103 (37.3)	56 (45.9)
1-3 times a month	47 (20.7)	93 (30.3)	30 (27.0)	46 (20,0)	52 (18.8)	19 (15.6)
Once every three months	17 (7.5)	12 (3.9)	6 (5.4)	16 (7.0)	14 (5.1)	10 (8.2)
Once every six months	3 (1.3)	5 (1.6)	1 (0.9)	6 (2.6)	8 (2.9)	6 (4.9)
Once a year	1 (0.4)	10 (3.3)	4 (3.6)	3 (1.3)	9 (3.3)	1 (0.8)
Never	47 (20.7)	28 (9.1)	3 (2.7)	51 (22.2)	69 (25.0)	22 (18.0)
p value	<0.001*			0.504*		

Chi-square test, No - number.

DISCUSSION

Sexual behavior varies from one society to another depending on race, ethnicity, gender and socio-economic status. The average age of the first sexual intercourse varies in most countries. In highly developed countries more that 50% of adolescents had already had their first sexual intercourse at the age from 15 to 18 years old. Unfortunately, in many countries there is no comprehensive sex education program for this age group (12).

Students who had attended programs of sexual education more often use contraceptives. Some of them get information about sexual health and contraceptives from their friends, television and health workers. Comparing the knowledge and sexual practice among students of different areas of education, it has been noticed that students of some medical sciences show healthier behavior than other students (13).

In a study performed at the University in Palermo among students of medical professions most students, over 75.31% of them, already had some sexual experience (14). In our study, students of medical professions had significantly more sexual intercourses than students from Italy, while students of non-medical professions in our study had approximately the same percentage of sexual intercourse. In our study same as in study from Palermo, most students were from the first year of study and the least from the third (14).

Most subjects in our study were of heterosexual orientation, which is confirmed by the study from Italy where also the most subjects were of heterosexual orientation, and a lesser number of subjects were of homosexual orientation (p =0.213) and bisexual orientation (p =0.166) (14). As for the types of sexual intercourse that are practiced, students of the University from Brasil, just as students from our study, mostly practiced vaginal and oral sexual intercourses. (15).

Unlike the study from Spain where every other students had sexual intercourse 1-2 times per week, in our study the most common answer about sexual frequency is 2-3 times per week (13).

Concerning the condom use during sexual intercourses, students from our study as well as students from the studies from other Universities from Europe and from the world, show that, despite the level of knowledge about risky sexual behaviour, still only consider vaginal sexual intercourse as the most risky type of sexual intercourse during which they use condoms.

Research from the University of California among students of medical professions shows that condoms were used mostly during vaginal sexual intercourse 48.5%, then anal 26.0 %, and least in oral intercourse 6.2% (16). Research from Italy among students of medical professions shows that more than 92 % of students do not use condoms during oral sexual intercourse (17).

Students from the department of Kinesiology in Texas, during anal sexual intercourses use sometimes or rarely condoms, with a higher number of sexual partners condom use is declining, 1 partner 18.8%, 2-4 partners 5.4%, and over 5 partners 1.7% (18).

As in the above mentioned studies, in our study a high percentage of students almost never use a condom during oral intercourse, while during vaginal intercourse most adhere to measures of prevention and safe sex. Despite the risks of unprotected sex, students are replacing condom use with other forms of contraception.

The reasons of not using a condom, in first and last year of study from the research from University from Croatia, is using others types of contracepties, while our students from all years of study stated that they trust their partners (19).

Unlike the study done in Congo, where a large percentage of respondents, over 80% had experience with the use of female condoms, in our study we have the opposite situation where over 90% of students had never used a female condom (20).

A study from Spain shows that regarding the use of contraceptive measures the most commonly used was the hormone therapy used by a quarter of respondents, while 3.6% used emergency contraception, every fifth postcoital pill, and 2.6% of them had legal abortion (13). While our respondents, in addition to the condom, which is in the first place as the main type of contraception, also use hormonal pills and interrupted intercourse, while during oral and anal sexual intercourse, condoms are never used to a large extent.

CONCLUSION

This study describes sexual habits and risky sexual behavior among students of medical and non-medical professions from Universities in Serbia. Student of medical professions are prone to risky sexual behavior despite a certain level of knowledge about reproductive health and risky sexual behavior which they acquired during formal education. Based on the results of the study, there is a significant need to evaluate education related to student sexual health. Young people should receive adequate education from professionals, and not gather knowledge from the internet, friends and other non professional sources of information. There is a need to introduce sex education programs in high schools as well as universities. We also believe that it is necessary to introduce education for young people who do not attend universities and thus provide them with an adequate level of information.

Based on previous research as well as relevant data related to uterine cancer, which is extremely widespread on the territory of the Republic of Serbia, and is significantly related to the risky sexual behavior of young people, all of the above should be taken into consideration and preventive educational programs should be started intensively.

ETHICS APPROVAL

The ethical standards of the research are harmonized with the Helsinki Declaration. In order to respect the privacy of the subject of research and the confidentiality of information, all necessary steps have been taken in accordance with the Law on Personal Data Protection ("Official Gazette of the Republic of Serbia", No. 97/08, 104/09), the Law on Official Statistics (Official Gazette RS, No. 104/09) and the European Parliament Directive on the protection of personal data with regard to personal data (Directive 95/46 / EC). For conducting the research, the consent of the Ethics Committee of the Faculty of Medical Sciences was obtained.

FUNDING

None.

CONFLICT OF INTEREST

None.

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THE COMPLICATIONS OF CATARACT SURGERY IN PATIENTS WITH PSEUDOEXFOLIATION

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ABSTRACT

The senile cataract represents the blurring of the crystalline lens after the age of 65. It occurs due to metabolic changes in the crystalline lens which occur over the years. The only effective way to treat cataract is the surgical one. Pseudoexfoliation is an age related systemic disorder. PEX represents the accumulation fibrillar material in the extracellular matrix of the tissue. The most known ocular manifestation of the PEX are the collection at iris pupillary margin and anterior lens capsule. This accumulation is associated with many intraoperative and postoperative complications in patients scheduled for cataract surgery. The aim of the study was to investigate the prevalence of the surgical complications during phacoemulsfication in patients with PEX. The study included 91 patients scheduled for cataract surgery divided into two groups (PEX group 46, control group 45 patients). Poor intraoperative midryasis, zonular dehiscence, postoperative corneal edema, anterior chamber inflammation, elevated intraocular pressure and tear film instability had particularly higher rate of occurring in PEX group comparing to the control group ($p<0.001$). The highest mean value of intraocular pressure was observed in PEX group on the first postoperative day 25.6 ± 1.1 mmHg, while the best corrected visual acuity was measured in control group 0.71 ± 1.2 one month after phacoemulsification. Cataract surgery in patients with PEX carries great risk, but with adequate preoperative planning, the awareness of the potential complications, can provide safe and routine phacoemulification in these patients.

Keywords: *Cataract, pseudoexfoliation, complication, glaucoma.*

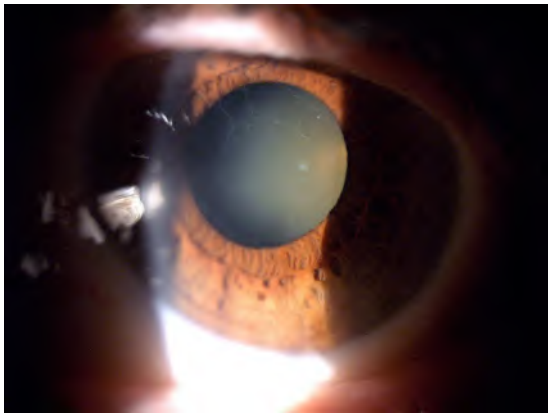
INTRODUCTION

The senile cataract represents the blurring of the crystalline lens after the age of 65. It occurs due to metabolic changes in the crystalline lens which occur over the years (1). The only effective way to the treat cataract is the surgical one (2). Through the centuries the surgical technique was improving. In the last few decades cataract surgery is based on usage of ultrasound energy to aspirate the cataract. This technique is known as phacoemulsification (3). Cataract still remains the worlds' leading cause of reversible blindness in the elderly population. Knowing that senile cataract begins to develop in every person above the 65 years old, explains why the phacoemulsification is the most common surgical procedure worldwide (4).

Pseudoexfoliation (PEX) is an age related systemic disorder. It affects many visceral organs and blood vessels. PEX represents the accumulation of fibrillar material in the extracellular matrix of the tissue (5). PEX shows a strong geographic presentation. It is a very common condition in Scandinavia, where its incidence is over 22%, while in Chine its rate is about 0.5% (6). In the eye, PEX is found in the conjunctiva, cornea, anterior chamber, iris, anterior lens capsule, ciliary body, zonules (Figure 1). This condition is described as a pseudoexfolation syndrome (7). The most known ocular manifestations of PEX are the accumulation at iris pupillary margin and anterior lens capsule. By blocking the trabecular meshwork PEX aggravates the aqueous humour outflow, which leads to the intraocular pressure (IOP) rise. That is followed by a characteristic visual field defects and optic nerve damage. This condition in known as pseudoexfolation glaucoma (XGF) (8). It is also reported that patients with PEX are in a higher risk to develop a senile nuclear cataract (5). The presence of PEX in conjunctival goblet cells, corneal endothelium, iris and zonule can have a huge effect at cataract surgery. The accumulation of PEX in these tissues is associated with many intraoperative and postoperative complications in patients scheduled for cataract surgery (9) (10) (11).

The aim of this study was to investigate the prevalence of the surgical complications during phacoemulsfication in patients with PEX.

Figure 1. PEX



PATIENTS AND METHODS

The study was prospective and carried out between January 2019 and January 2020 at the Clinic of Ophthalmology, Kragujevac Clinical Center, Kragujevac, Serbia. It included 91 patients scheduled for cataract surgery. For the research, in every patient only one eye was included in the study, so the total number of eyes were equal to the number of the patients (n=91). Participants were divided into two groups depending on presence of pseudoexfoliation. The first group included 46 patients with diagnosed pseudoexfoliation syndrome. The second group included 45 patients without presence of the pseudoexfoliation.

The most important criterion was the presence of the senile cataract. Patients with another type of cataract, such as presenile, traumatic, iatrogenic, metabolic, congenital, were excluded from the study. Visual acuity in every patient before the surgery had to be 0.2 by Snellen chart and lower. Patients with previous history of intraocular surgery, dry eye, glaucoma, uveitis, keratitis, laser treatment, conjunctival or corneal degeneration, were also excluded from the study. Those patients who underwent some other type of cataract surgery, such as extracapsular or intracapsular cataract extraction, also could not participate in the investigation. Patient with preoperative zonular dehiscence, lens subluxation, vitreous body loss were excluded. With the approval of the institutional Ethics Committee and according to the tenets of the Declaration of Helsinki, all the patients gave their written consent at the beginning of the study.

Before the surgery all the patients passed complete ophalmological examination (best corrected visual acuity, intraocular pressure measurement, detailed slit-lamp examination, retinal examination, ocular ultrasonography). Using the slit-lamp a presence of the PEX were carefully analysed. Patients who were suspected having a glaucoma passed additional retinal examination, visual field testing and central corneal thickness measurement. Dry eye tests were also performed. We used Schirmer test to measure the quantity of the tear film. The test strips were placed in the lateral part of the inferior conjunctival fornix. Values shorter than 5 mm, after 5 minutes, indicated a dry eye syndrome. The quality of the tear film was investigated by using tear breakup time test (TBUT). This test is the indicator of the lipid layer of the tear film. The test is based on usage of the cobalt blue light of the slit lamp biomicroscope and fluorescein strips. After the staining of the corneal surface with fluorescein, time until the first dry spots appear were counted. Values under 10 seconds were considered as pathological.

Five days before the phacoemulsification 0.3% solution of ofloxacin was administrated in every patient (sol Floxal®, Dr.Gerhard Mann, Chem.-Pharm. Fabric GmbH, Berlin, Germany). All the cataract surgeries were performed by one experienced surgeon. Phaco machine for all surgeries was Stellaris (Bausch & Lomb, Rochester, NY, USA). On the surgery day, maximal mydriasis was achieved applying topical phenylephrine hydrochloride ophthalmic solution 2.5%.

Tetracaine eye drops was the only anesthetic drug used during the surgery. Sterile ruler was used to measure the maximum achieved intraoperative mydriasis. Main corneal incision and paracentesis were made using 2.75mm and 1.5 mm wide surgical knifes. If it was necessary, due to better visualisation, anterior lens capsule was stained using trypan blue solution. A cohesive viscoelastic then fulfilled the anterior chamber. Continuous curvilinear capsulorhexis, hydrodisection and nucleus rotation followed. When the nucleus was completely separated from the lens capsule, the "stop and chop" and "divide and conquer" technique were used to crack the nucleus. The remaining lens cortex was aspirated using bimanual irrigation and aspiration. When the capsular bag was cleared, cohesive viscoelastic was again injected, and foldable monofocal artificial intraocular lens (IOL) was implanted in capsular bag with adequate injector. In cases where capsular bag was not intact, such as posterior capsule rupture, or zonular dehiscence, anterior chamber intraocular lens was implanted. Then viscoelastic was aspirated and intracameral solution of cefuroxime with 1 mg / 0.1 ml balanced salt solution was injected (sol. Nilacef®, Hemofarm A.D., Vršac, Serbia). The main corneal incision and paracentesis were hydrated by balanced salt solution using a blunt injection needle. Postoperatively, dexamethasone tobramycin eye drops (Tobradex®, Alcon-Couvreur NV, Puurs, Belgium) was administrated five times per day for one month.

Detailed opthalmological examination was repeated on the first postoperative day, and then three, six and twelve months after cataract surgery. Using a Snellen charts best corrected visual acuity was calculated in every patient during one year follow-up period.

IBM SPSS version 22.0 was used for the statistical analysis in the study. According to the normality of distribution, paired t-test, $\chi 2$, Person test, Mann-Whitney test, were utilized in analyzing associations between the continuous variables (BCVA, IOP, dry eye tests, patients' demographic characteristics). Categorical variables were expressed as frequencies. Values $p<0.05$ and $p<0.001$. were considered to be statistically significant.

RESULTS

The PEX group consisted of 24 female and 22 male patients. The mean age in this group was 75.6 ± 7.1 years (range 67 - 82 years). The control group included 23 female and 22 male participants. The mean age was 69.3 ± 4.5 (range 65 - 78 years). No statistical significance was noticed in sex distribution among groups (p=0.784). Statistically significant difference was recorded in patients' age. Patients from the first group were statistically significantly older compared to those from the control group (p=0.034).

Intraoperative complications in both groups are shown in Table 1. As it can be seen, statistically significantly difference among groups was observed in every intraoperative complication measured in the study. The highest statistically difference was recorded in pupil diameter and zonular dehiscence.

Table 1. Intraoperative complications in PEX group and control group

Intraoperative complications	**PEX group** number of patients (%)	**Control group** number of patients (%)	**p value**
Maximal midryasis (mm)	5.02 ± 0.6	6.72 ± 0.7	p<0.001*
Posterior capsule rupture	6 (13.04 %)	2 (4.44%)	p=0.031*
Vitreous body loss	3 (6.52%)	1 (2.22%)	p=0.038*
Zonular dehiscence	5 (10.86%)	2 (4.33%)	p=0.006*
Intraoperative miosis	10 (21.73%)	7 (15.56%)	P=0.017*

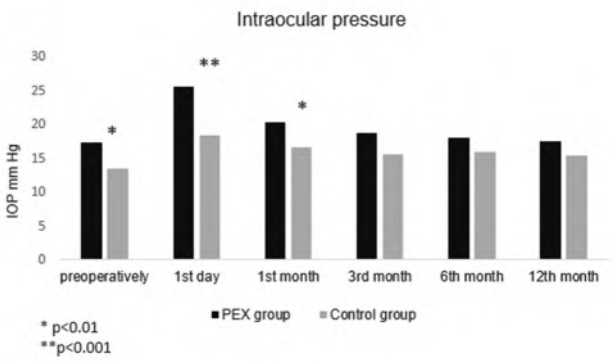
Table 2. Postoperative complications in PEX group and control group

Postoperative complications	**PEX group** number of patients (%)	**Control group** number of patients (%)	**p value**
Cystoid macular edema	3 (6.52%)	2 (4.44%)	p=0.065
Nucleus fragment dislocation	2 (4.34%)	1 (2.22%)	p=0.052
Posterior lens capsule opacification	0.09 ± 0.02	0.08 ± 0.025	p=0.065
Corneal edema	9 (19.56%)	5 (11.11%)	p=0.016*
Retinal detachment	0	1 (2.22%)	p=0.317
IOL decentration	2 (4.34%)	1 (2.22%)	p=0.057
Anterior chamber inflammation	10 (21.73%)	4 (15.55%)	p=0.021*

Postoperative complications were also more frequent in PEX group (Table 2). Especially high statistical significance was noticed in the incidence of postoperative corneal edema and anterior chamber inflammation. Other measured complications were not statistically significant. Possible postoperative complications, such as suprachoroidal hemorrhage, endophthalmitis were not noticed in any patient during the follow-up period.

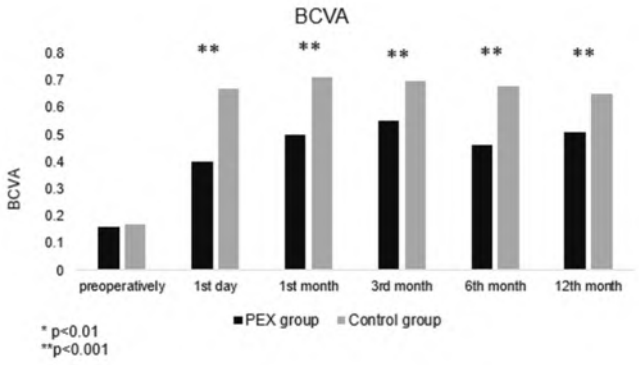
Intraocular pressure values were higher in PEX groups during all measurements. The first three visit showed statistically significantly difference among the groups, with the highest difference one month after phacoemulsification (Figure 2).

Figure 2. Intraocular pressure during the study



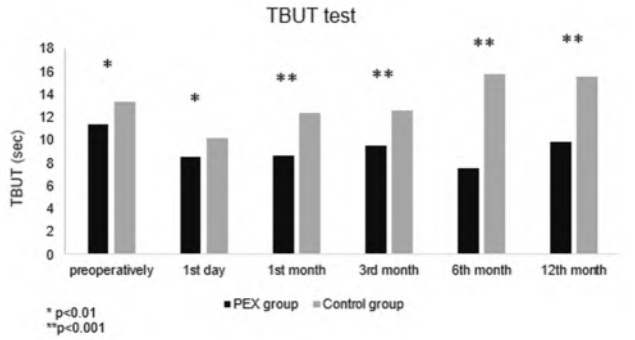
Best corrected visual acuity was similar at the beginning of the investigation (I group: 0.16 ± 0.02; II group: 0.17 ± 0.01; p=0.124). From that moment until the end of follow-up period high statistical significance maintained among the groups (Figure 3).

Figure 3. Best corrected visual acuity during the study



Tear break up time test seemed to be the test with the highest difference between the PEX and the control group. Statistical significance was present before the phacoemulsification and remained in that way for the next twelve months (Figure 4).

Figure 4. Tear break up time test during the study



Unlike to TBUT test, Schirmer test showed no statistical difference among the groups during the whole period of the study. Preoperatively Schirmer test values in both groups were above 10 mm (I group 22.45 ± 1.2 mm, II group=20.12 ± 0.8 mm, p=0.065). On the first postoperative day PEX group had mean value 26.12 ± 2.2 mm, while control group had 25.57 ± 2.1 mm, again without statistical significance, p=0.078. This trend continued during next four measurements, one month after the cataract surgery (I group: 20.12 ± 0.8 mm, II group: 21.56 ± 1.2 mm) three months (I group: 18.14 ± 2.4 mm, II group: 19.10 ± 1.1 mm), six months (I group: 17.47 ± 2.2 mm, II group: 18.78 ± 2.4 mm) and twelve months (I group: 20.32 ± 4.2 mm; II group: 19.68 ± 2.6 mm).

DISCUSSION

Cataract surgery is the most common surgical procedure worldwide. Still it is not completely without risk (12). The presence of PEX makes this risk even higher. Our results strongly indicated that the accumulation of this fibrillar material in ocular tissue was associated with higher incidence of all possible intraoperative complications. These results are in a correlation with earlier investigations (6) (10).

Particularly high statistical significance was noticed in pupil diameter, zonular dehiscence and intraoperative miosis among the groups. PEX is usually located in iris and ciliary zonule, causing the iris atrophy and zonular weakness (13). That fact requires extreme caution and precision during the phacoemulsification. Our study also revealed a higher rate of posterior capsule rupture in PEX group, which could be consequence of poor mydriasis and zonular instability during phacoemulsification.

Cataract surgery breaks down the hemato-ocular barrier releasing the proinflammatory mediators in humour aqueous (14). It is believed that inflammation in anterior chamber is responsible, as well as the remaining viscoelastic, for the intraocular pressure rise after phacoemulsification. Our results are similar with the previous studies (15) (16). In PEX patients there is a higher rate of inflammation due to presence of pseudoexfoliation in anterior chamber. Subsequently, by blocking the trabecular meshwork, PEX causes increased rise of IOP after the cataract surgery. IOP values were constantly increased in PEX group during the study with the highest

statistical significance one month after phacoemulsification ($p<0.001$).

Phacoemulsification technique is based on using ultrasound energy to crack the lens nucleus and aspirate it. This energy also disturbs the corneal endothelial cells (17). It is known that these cell are responsible in maintaining the cornea transparency by pumping the ions to anterior chamber. Lower number of endothelial cells leads to water influx and the development of corneal edema (18). Deposition of PEX material in corneal endothelial cells decreases their morphology and function, which could be the reason for the higher rate of postoperative corneal edema in PEX patients in our study. That is the explanation, including all other intraoperative and postoperative complications, for the statistically significantly lower postoperative BVCA in PEX group which is shown in Figure 3.

Dry eye tests indicated the presence of the dry eye after the surgery of cataract. Statistical difference in tear film stability were noticed even preoperatively among the groups. That could be explained by the presence of PEX material in conjunctival goblet cell which disturbs their function. Phacoemulsification can cause the development of dry eye by many ways: transection of corneal nerves, exposure to the microscopic light, by releasing proinflammatory mediators. During the first postoperative month we recorded pathological values of TBUT test in both groups, while the Schirmer test results were even compensatory increased. After that period patients from control group managed to increase their tear film stability. Dry eye remained present in PEX group during the end of follow-up period.

CONCLUSION

Patients with PEX had more frequent occurrence of the intraoperative and postoperative complications. Poor intraoperative midryasis and zonular dehiscence, postoperative corneal edema and anterior chamber inflammation had particularly higher rate of development comparing to the control group. Elevated IOP and tear film instability were worse in PEX group as well. All these factors can disrupt the final outcome of the surgery and patient's satisfaction. Phacoemulsification has improved cataract surgery in all aspects, but even this technique is not without risks. Our investigation revealed potential risks in patients with pseudoexfoliation syndrome scheduled for cataract surgery. Adequate preoperative planning, the awareness of the potential intraoperative and postoperative complications, can provide safe and routine phacoemulification in these patients.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the Declaration of Helsinki. The protocol of the study was approved by the Ethical committee from the Clinical Center Kragujevac. Each patient gave informed consent to participate in the study.

CONFLICT OF INTEREST

None.

ACKNOWLEDGEMENT

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PSYCHOMETRIC PROPERTIES OF THE QUALITY OF LIFE QUESTIONNAIRE - CERVICAL CANCER 24 (QLQ CX 24) TRANSLATION TO SERBIAN

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ABSTRACT

Cancer of the cervix has a progressive character and is one of the most significant public health problems in many countries. Our research aimed to translate EORTC QLQ CX 24 (European Organization for Research and Treatment of Cancer; Quality of life questionnaire-cervical cancer 24) from English to Serbian, to create essential cultural adaptations and to analyze psychometric properties of the translation in a model of female inpatients with cancer of the cervix. The QLQ CX 24 was translated and adapted according to internationally established guidelines, and then tested on a sample of 100 Serbian females with cancer of the cervix. The testing was repeated three times on the same patients. We calculated the internal consistency (Cronbach's alpha), criterion validity, convergent validity, and discriminative validity of the QLQ CX 24. We used factor analysis to discover the original construct. The Serbian translation of QLQ CX 24 showed good internal consistency, showed satisfactory reliability, and temporal stability. In the first, when was rated by the investigators Cronbach's alpha was 0.607, and one month later when the questionnaire also was rated by investigators Cronbach's alpha was 0.696. When the scale was rated by females themselves Cronbach's alpha was 0.802. Divergent as well as convergent validity tests had good results. The factorial analysis exposed six domains. The Serbian translation of QLQ CX 24 is a trustworthy and appropriate specific instrument for measuring the quality of life in females with cervical cancer.

Keywords: *Quality of life, questionnaire, cervical cancer, validation, females.*

INTRODUCTION

Cancer of the cervix has a progressive character and is one of the most significant public health problems in many countries (1). According to some studies, cervical cancer is the fourth commonest cancer in women with about 500,000 cases diagnosed with cervical cancer and about 270,000 women who die from this disease every year all over the world (1-3). Although the disease is now diagnosed more readily and the management of cervical cancer is advancing, both the disease itself and the treatment modalities carry significant morbidity with a negative impact on the quality of life (QOL) (1, 4). Surgery can produce certain functional disorders, radiotherapy may cause injury of vaginal mucosa and chemotherapy may have a variety of undesirable effects, like diarrhea, constipation, nausea, changes in weight, and hormonal imbalance (1).

Quality of life is an important health outcome of any disease beyond traditional ones, like morbidity and mortality. Measuring QOL is also helpful for the identification of patients' problems and making the most appropriate treatment plan for individual patients (1).Numerous prognostic factors for QOL in females with cervical cancer have been studied and published in the literature. As an example, some studies found that spiritual well-being, maladaptive coping, and reproductive concerns were separately affecting QOL, other studies imply that family, social, and intimate relationship played a vital role on perception of QOL. There is also stress that this disease causes to the relationship. In the study Khalil et al., 39 % of females who were married when diagnosed got separated afterwards. Seventy two percent of them attributed their divorce to the cervical cancer (4).

Several questionnaires like Female Sexual Function Index (FSFI), Vaginal Health Index, Urogenital Distress Inventory short form (UDI-6), International Consultation on Incontinence Questionnaire short form/female lower urinary tract symptoms (ICIQ-FLUTS SF) were developed to evaluate problems with the functioning of the female genitourinary system (5). The most frequently used questionnaire for measuring QLQ in females with cervical cancer is EORTC QLQ CX 24 (European Organization for Research and Treatment of Cancer; Quality of life questionnaire-cervical cancer 24) (6, 7). EORTC QLQ CX 24 is a scale developed specifically to deal with the quality of life amongst patients with cervical cancer, planned to be used as an attachment to the EORTC QLQ-C30 (European Organization for Research and Treatment of Cancer; Quality of life questionnaire- cancer 30) (8, 9). This scale contains 24 questions for measuring different aspects of quality of life that had been found significant by a team of professionals in females with cervical cancer. The items of the questionnaire are grouped into three groups (Symptom Experience, Body Image, Sexual /Vaginal Functioning), also with 5 single-items: sexual worry, sexual activity, and sexual enjoyment, lymphedema, peripheral neuropathy, menopausal symptoms. The quality of life of the females is inversely proportional to the marked right answer. The QLQ CX 24 usually appears to be appropriate for everyday use in clinical practice, but it was not translated and adapted to Serbian language and cultural surroundings until now.

Our research aimed to translate EORTC QLQ CX 24 from English to Serbian, to create essential cultural adaptations, and to analyze psychometric properties of the translation in a model of female inpatients with cancer of the cervix.

METHODS

All females gave written informed consent before the investigation began. The study was permitted by the Ethics Committee of Clinical Center Kragujevac, Serbia (No. 01/18-5291), and conducted according to principles laid down in the Declaration of Helsinki about experimentations on human subjects. We got permission for translation and cultural adaptation from the EORTC Study group on Quality of life for QLQ CX 24 and from author Bojana Dinic for Emotional Regulation Questionnaire (ERQ).

Patients

Final Serbian adaptation of the QLQ CX 24 was tested for reliability on female inpatients who were hospitalized for brachytherapy at the Oncology Clinic of Clinical Center Kragujevac, Serbia. The study was performed from September 2018 to October 2019. The patients fulfilling the inclusion criteria were those with cancer of the cervix, hospitalized, and educated. The exclusion criteria were: lactation, pregnancy, mood disorders, cognitive impairment (previously confirmed in a patient's medical documentation), mental retardation, and incomplete patient's files. The participation of the patients was successive nature, i.e. all patients who were at the Oncology Clinic on the day of study (and fulfilled inclusion and exclusion criteria) were offered the questionnaire. During the initial meet, the questionnaires were completed in two ways: at first, by the researcher who were questioning the patients, and second, by the females themselves one week later, at their homes. The females again finished the questionnaires by the researcher one month afterward (10).

Translation and cultural adaptation of the original QLQ CX 24 questionnaire

According to the guiding principle of the International Society for Pharmacoeconomics and Outcomes Research (ISPOR), translation and cultural adaptation of the QLQ CX 24 were finished (11). The 24-item QLQ CX 24 was originally designed to consist of five multi-item scales on clinically different dimensions (sexual functioning, body image, and gastrointestinal, urologic, and vaginal symptoms) and several single-item measures. The original questionnaire has the following subscales: Symptom Experience, Body Image, Sexual/Vaginal Functioning, and 5 single-item scales: Lymphedema, Peripheral Neuropathy, Menopausal Symptoms, Sexual Worry, Sexual Activity, and Sexual Enjoyment. In the beginning, the original scale was primarily translated from English to Serbian by two independent native Serbian language speakers, an expert translator, and a bilingual

university professor. They translated the questionnaire separately from each other, and then they gathered and the translations were synchronized to one Serbian version. The synchronized Serbian version was then translated reverse to English by a native English language speaker, who was not responsive to the original English version of the QLQ CX 24. Researchers then compared the back-translation to English was with the original English version, and the final Serbian version of the QLQ CX 24 was agreed upon at the new meeting of investigators. Finally finishing the translation of QLQ CX 24 to Serbian was then tested on 10 female inpatients with cervical cancer at the Oncology Clinic, Clinical Center Kragujevac, Serbia. Following the pilot testing, the minor changes of the wording were made, and then the final Serbian version of the QLQ CX 24 was copied and organized for testing. Also, with a specially designed questionnaire, we collected demographic data about patients (10, 12,13).

Serbian version of QLQ CX 24, the Serbian version of the QLQ C30 Questionnaire, and the Serbian version of the Emotion Regulation Questionnaire were finished by the females themselves and by the researchers after interviewing the females. Chronological stability of the translated QLQ CX 24 was tested by the repeated achievement of the questionnaire 30 days after the first one.

Statistics

Kolmogorov–Smirnov test was used to test if answers to the questionnaires were normally distributed, in the case that the answers were not distributed normally, nonparametric tests were used. The consistency of the Serbian version of QLQ CX 24 was checked by measuring the inner stability through the result of the Cronbach's alpha. Through investigation of the correlation of the individual items, convergent validity was assessed with the total score of the QLQ C30 and ERQ instruments. For all tests of criterion and convergent validity, we calculated Spearman's level correlations. When the answers were not normally dispersed factor structure of the Serbian translation of QLQ CX24 was examined by the method of principal axis factoring (12, 13). According to the condition that eigenvalues had to be superior to 1, new factors were extracted. The factors we extracted were then compared with their respective QLQ CX24 items and therefore named (14).

All calculations in this study were performed by SPSS software, version 18. The stage of importance was set to $p < 0.05$.

RESULTS

The original version of the QLQ CX 24 was tested on the sample of 100 female inpatients: mean age 51.55±12.63 years, smokers being 33% of the patients (mean number of cigarettes 5.40±8.92, mean years of smoking 6.77±10.98), and alcohol drinkers (5 % of the patients)(Table 1).

Completing the questionnaires

The usualtime taken of finishing all the questionnaires of the study was less than 45 minutes, and the duration of completing QLQ CX 24 was less than 20 minutes. Only 20 patients answered all questions in the three questionnaires. Most of the patients (about 80%) answered the first 19 items of the QLQ CX 24. Therefore only answers to the first 19 questions of the QLQ CX 24 were entered to the statistical calculations of reliability and validity.

Consistency and factor analysis

We got results about mean values, variance, skewness and kurtosis of the distribution of responses to each of the questions, after testing the QLQ CX 24 questions (Table 2). When the scale was rated by researchers, Cronbach's alpha was 0.607. We separated the questionnaire by split-half method (Spearman-Brown), and the coefficient for the questionnaire as a whole was calculated by the formula Spearman-Brown, and its significance was 0.579. Reliability appears to be solid with the Cronbach's alpha of 0.802 after the questionnaire was rated by the females themselves (a week following the ranking by the researchers).

The measure of sampling adequacy (Kaiser-Meyer-Olkin) was 0.642 and Bartlett's test of sphericity was important ($p<0.001$). By orthogonal rotation six factors were extracted, illuminating in total 70.7 % of the variance. The eigenvalue of the first factor was 5.37 (28.28 % of variance), of the second 2.59 (13.64 % of the variance), of the third 1.84 (9.69 % of the variance), of the forth 1.44 (7.61% of the variance), of the fifth 1.20 (6.35 % of the variance) and the sixth 1.04 (5.44 % of the variance). The rotated part matrix is exposed in Table 3. The cut-off point for transmission of the question to a factor was loading superior to one. The items 2 and 5 belong to the first factor, which reflects problems with both defecation and urination. The items 15, 16, 17 belong to the factor 2 and reflect the self-perception of physical appearance. Items 6 and 7 belong to the third factors and describe problems with urination. Factor 4 includes items 4, 8, 9, 12 and 13 and describes the feeling of being ill in general. The items 10 and 14 belong to the fifth factor and describe sensations of autonomic functions. The items 1, 11,18 and 19 belong to factor 6 and describe problems related to sexual activity.

Validity and chronological stability

Through non-parametric correlation between scores of the QLQ CX 24 (when it was rated by researchers and by females themselves) and scores of the QLQ-C30 (when it was rated by researchers and by females themselves), divergent criterion validity was tested. A score of the QLQ CX24 was calculated as the sum of individual items ratings for each of the patients. Through non-parametric correlation between scores of the QLQ CX 24 (when it was rated by researchers and by females themselves) and scores of the ERQ (when it was rated by researchers and by females themselves), convergent criterion validity was tested. According to the non-normal distribution of some of the scores non-parametric

correlation was used. Spearman's correlation coefficients are shown in multi-trait, multi-method matrix (Table 4). The Table 4 shows how items of the translated scale are grouped in six domains (factors) that reflect facets of the quality of life of patients with cervical cancer. Titles of the domains were given in an attempt to verbally describe aspect of quality of life that items within the domains are referring to. It is obvious from the Table 3 that main aspects of quality of life in patients suffering from cervical cancer relate to functional sphere (defecation and urination), loss of sexual function and distorted perception of physical appearance.

The QLQ CX 24 showed pleasing chronological stability: when rating (by explorer) was repeated on the same females one month later the correlation between the scores (Spearman's coefficients) was 0.831 ($p = 0.000$). After the repeated rating Cronbach's alpha was 0.696. The correlation coefficient of interclass was 0.607 ±0.483 on the initial rating, and 0.693±0.599 on repeated rating.

DISSCUSION

This study presents data from the translation and confirmation of the EORTC QLQ-CX 24. It is the first investigation to achieve a psychometric confirmation of the EORTC QLQ-CX 24 in a Balkan country, Serbia. The most important explanation why this study is significant is the fact that the morbidity and survival rate of cervical cancer is rising, which will guide to an increase in the need for tools able to review QOL of such patients. Moreover, the results show that the translation of the EORTC QLQ-CX24 to Serbian is cross-culturally suitable in countries with Serbian-speaking populations.

The Serbian translation of the QLQ CX 24 showed solid reliability when was rated by the researcher as well as by females themselves. Our results showed that measures of chronological stability, correlations, and internal consistency with convergent or divergent scales were ahead of the values in the literature considered as sufficient to establish validity and reliability (15). The values from our study were moreover very similar to those obtained after testing the reliability and validity of the QLQ-C30 and QLQ CX 24 (8, 9). Factor analysis revealed six factors (Problems with defecation and urination; Self-perception of physical appearance; Problems with urination; Feeling of being ill in general; Sensations of autonomics function; Problems related to sexual activity).

The mean age of our patients (51.6 years) was similar to the patients included invalidation of QLQ CX 24 (9). In our sample 33 % of females were smokers; almost all meta-analyses and multi-center investigations spot that smoking is a significant co-factor for cervical squamous cancer and maybe also for cervical adenocarcinoma (16). When original QLQ CX 24 was developed values of Cronbach's alpha varied from 0.72 to 0.87 which is similar to our results with Cronbach's α between 0.6 and 0.8 depending on if it was rated by investigators or by patients themselves (9). If we compare our results with results from validation in other countries, we will notice a similar outcome (17-21).

Our factor analysis revealed six factors (Problems with defecation and urination; Self-perception of physical appearance; Problems with urination; Feeling of being ill in general; Sensations of autonomics function; Problems related to sexual activity) so there are differences between original QLQ CX 24 and validated scale in our country. This could be explained with a different population structure in Serbia in comparison with the country where the original questionnaire was developed. We have received solid results regarding the validation of the questionnaire. The scale evidenced good item convergence ($r=0.1$-0.7), similar to the study in China (21) and Etiopia (20). Our study has several limitations. The sample size was relatively small (100), and the patients were enrolled successively, during one year, but they were very compliant with the study protocol, enabling testing of temporal stability and evaluation of testing mode effect. Uni-centricity is another limitation, which may have introduced the investigator's bias. The last limitation is that patients did not answer all questions. Most of the patients answered only the first 19 items of the QLQ CX 24. Therefore, only answers to the first 19 questions of the QLQ CX 24 were entered into the statistical calculations of reliability and validity. There could be many reasons why we got such a result. First, some of the patients had not sexual activity. Second, females felt shame to answer such questions. It should be noted that the questionnaire originated in developed countries and that Serbia is a developing country.

In conclusion, the QLQ CX 24 is a trustworthy and suitable specific instrument for measuring QOL in patients with cervical cancer, but patients need to fill the questionnaire by themselves. This translation supplements the range of instruments that can be used in clinical practice to measure the quality of life in such patients.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was permitted by the Ethics Committee of Clinical Center Kragujevac, Serbia (No.01/18-5291), and conducted according to principles laid down in the Declaration of Helsinki about experimentations on human subjects. Each patient gave informed consent to participate in the study.

CONFLICT OF INTEREST

None.

ACKNOWLEDGMENT

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Table 1. Characteristics of the study sample

Variable	Mean ± standard deviation*/median (range)** (n = 100)
Age (years)	51.55±12.63
Smokers	33 (33%)
Number of cigarettes	5-40
Years of smoking	1-45
Alcohol consumption	5 (5%)
Type of tumor	
Carcinoma planocellulare	71(71%)
Adenocarcinoma	29(29%)
Charlson Comorbidity Index	1-4
Chemotherapy	71(71%)
Radiotherapy	89(89%)
Surgery	30(30%)
*Data were normally distributed; **Data were not normally distributed.	

Table 2. Mean values, standard deviation, skewness and kurtosis of responses to items of QLQ CX 24

	Number of patients	Mean	Std. Deviation	Skewness	Kurtosis
Have you had cramps in your abdomen?	100	2.390	1.332	1.104	1.254
Have you had difficulty in controlling your bowels?	100	1.980	0.994	0.543	-0.924
Have you had blood in your stools (motions)?	100	1.410	0.697	1.421	0.565
Did you pass water/urine frequently?	100	2.310	0.981	-0.008	-1.129
Have you had pain or a burning feeling when passing water/urinating?	100	1.860	0.804	0.380	-1.011
Have you had leaking of urine?	100	1.840	2.966	6.463	42.677
Have you had difficulty emptying your bladder?	100	1.660	2.170	8.492	79.682
Have you had swelling in one or both legs?	100	1.730	0.885	1.007	0.109
Have you had pain in your lower back?	100	2.090	1.518	4.086	22.518
Have you had tingling or numbness in your hands or feet?	100	1.740	1.235	4.475	31.124
Have you had irritation or soreness in your vagina or vulva?	100	1.570	0.685	0.991	0.564
Have you had discharge from your vagina?	100	1.870	0.895	0.865	0.054

	Number of patients	Mean	Std. Deviation	Skewness	Kurtosis
Have you had abnormal bleeding from your vagina?	100	1.750	0.845	0.915	0.095
Have you had hot flushes and/or sweats?	100	2.390	1.469	4.011	22.427
Have you felt physically less attractive as a result of your disease or treatment?	100	1.650	0.936	1.136	-0.014
Have you felt less feminine as a result of your disease or treatment?	100	1.720	0.943	1.033	-0.112
Have you felt dissatisfied with your body?	100	1.760	1.016	1.031	-0.264
Have you worried that sex would be painful?	99	1.798	1.049	0.903	-0.641
Have you been sexually active?	96	1.645	0.994	1.291	0.308
Has your vagina felt dry during sexual activity?	21	2.523	0.980	-0.249	-0.833
Has your vagina felt short?	20	2.700	1.080	-0.439	-0.974
Has your vagina felt tight?	20	2.500	1.100	-0.132	-1.259
Have you had pain during sexual intercourse or other sexual activity?	20	2.800	1.005	-0.594	-0.490
Was sexual activity enjoyable for you?	20	2.100	0.911	0.713	0.154

Table 3. The rotated component matrix of QLQ CX 24 with factor loading per each item.
Items within the shaded cells belong to the corresponding factor.
Number of patients who responded: 100.
The cut off point for assigning an item to a factor was loading greater than one.

	FACTORS					
	Problems with defecation and urination	**Self-perception of physical appearance**	**Problems with urination**	**Felling of being ill in general**	**Sensations of autonomic functions**	**Problems related to sexual activity**
Have you had cramps in your abdomen?	0.074	0.091	0.129	-0.005	-0.061	0.379
Have you had difficulty in controlling your bowels?	0.497	-0.019	-0.022	-0.052	0.315	-0.091
Have you had blood in your stools (motions)?	-0.064	0.040	1.000	-0.077	-0.076	0.186
Did you pass water/urine frequently?	0.167	-0.152	-0.018	0.704	0.173	0.004

	FACTORS					
	Problems with defecation and urination	**Self-perception of physical appearance**	**Problems with urination**	**Felling of being ill in general**	**Sensations of autonomic functions**	**Problems related to sexual activity**
Have you had pain or a burning feeling when passing water/urinating?	0.982	0.170	0.010	0.061	-0.159	-0.015
Have you had leaking of urine?	-0.009	0.015	0.262	0.046	0.050	-0.093
Have you had difficulty emptying your bladder?	0.330	-0.034	0.534	-0.066	0.043	0.145
Have you had swelling in one or both legs?	0.046	-0.186	0.247	0.553	0.162	-0.204
Have you had pain in your lower back?	0.021	0.143	0.229	0.442	0.237	-0.235
Have you had tingling or numbness in your hands or feet?	-0.047	0.243	0.089	0.261	0.678	-0.100
Have you had irritation or soreness in your vagina or vulva?	0.182	-0.013	0.208	0.260	0.027	0.441
Have you had discharge from your vagina?	-0.200	0.141	-0.134	0.853	-0.159	0.331
Have you had abnormal bleeding from your vagina?	0.137	0.406	0.255	0.409	-0.133	0.031
Have you had hot flushes and/or sweats?	0.142	-0.011	0.053	-0.005	0.704	0.515
Have you felt physically less attractive as a result of your disease or treatment?	0.049	0.614	0.087	-0.194	0.266	0.087
Have you felt less feminine as a result of your disease or treatment?	0.035	1.030	-0.021	0.010	-0.019	-0.093
Have you felt dissatisfied with your body?	0.156	0.636	-0.010	0.064	0.001	0.320
Have you worried that sex would be painful?	-0.078	0.272	0.077	-0.029	-0.006	0.444
Have you been sexually active?	-0.176	0.039	-0.125	0.034	0.113	0.689

Table 4. Multi-trait, multi-method correlation matrix (non-parametric Spearman's coefficients).
Number of patients who responded: 100

	QLQ C30 rated by investigator	ERQ rated by investigator	QLQ C30 rated by a patient	ERQ rated by a patient	QLQ CX 24 rated by investigator	QLQ CX 24 rated by a patient
QLQ C30 rated by investigator	1	Rho=0.007 p=0.948	Rho=0.738 P=0.000	Rho=0.091 P=0.369	Rho=0.347 p=0.001	Rho=0.196 p=0.058
ERQ rated by investigator	Rho=0.007 p=0.948	1	Rho=-0.043 p=0.668	Rho=0.915 p=0.000	Rho=-0.151 p=0.142	Rho=-0.124 p=0.232
QLQ C30 rated by a patient	Rho=0.738 p=0.000	Rho=-0.043 p=0.668	1	Rho=-0.102 p=0.310	Rho=0.227 p=0.026	Rho=0.284 p=0.006
ERQ rated by a patient	Rho=0.091 p=0.369	Rho=0.915 p=0.000	Rho=-0.102 p=0.310	1	Rho=-0.088 p=0.396	Rho=-0.212 p=0.040
QLQ CX 24 rated by investigator	Rho=0.347 p=0.001	Rho=-0.151 p=0.142	Rho=0.227 p=0.026	Rho=-0.088 p=0.396	1	Rho=0.774 p=0.000
QLQ CX 24 rated by a patient	Rho=0.196 p=0.058	Rho=-0.124 p=0.232	Rho=0.284 p=0.006	Rho=-0.212 p=0.040	Rho=0.774 p=0.000	1

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EFFECTS OF ANTIPSYCHOTICS ON BONE MINERAL DENSITY IN SCHIZOPHRENIA

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ABSTRACT

Previous researches have shown that patients with schizophrenia who were using antipsychotics in their treatment developed changes in Bone Mineral Density and body composition, leading to osteoporosis and modifications in weight, skeletal muscle mass index and percent of fat tissue. Results of many studies suggested that the use of antipsychotic causes hyperprolactinemia and consequently lower bone mineral density values were observed. Further, antipsychotics increase food intake and have possible effects on metabolism, causing changes in weight, glucose level and lipid status, all of which can be a risk for developing metabolic syndrome and result in changing of bone mineral density. Antipsychotics change cytokine profiles in patients with schizophrenia and on the other hand the influence of T cells, B cells and inflammatory cytokines on osteoclasts and on osteoblasts was also established. In assessing the effects of antipsychotic on bone metabolism very important is to consider the duration of the treatment and clinical course of the disease, but undeniable effect is careless life style and inadequate physical activity that patients with schizophrenia have. Our attempt is to give an overview of the newest findings in this field, regarding the direct effects of antipsychotics on the bone metabolism, but also through prolactine elevation, metabolic and immune changes. Better understanding of the underlying mechanisms of schizophrenia and changes in bone mineral density could improve our clinical practice: affect to choice of the individually most appropriate antipsychotic, point to the need to monitor possible immunmetabolic changes during the treatment and improvement of the life quality of this vulnerable population.

Keywords: *Bone mineral density, osteoporosis, antipsychotics, schizophrenia.*

INTRODUCTION

Osteoporosis is a chronic, progressive, metabolic disease of the bone system, primarily characterized by lossof the bone mass (1). Bone is constantly remodeled, osteoblasts produce and secrete matrix proteins and transport minerals into the matrix and so they synthesize bone, and the osteoclasts break down the tissues (2). The dysfunction between bone resorption and bone formation lies in the basics of osteoporosis. Reduction of the bone mass and damage to the bone tissue microarchitecture increases the risk of bone fractures and their consequences, which significantly impairs patients' quality oflife (1, 2). Dual-Energy X-ray Absorptiometry (DEXA) is considered to be the gold standard for the diagnosis of osteoporosis. Measuring sites are the lumbar spine and hip. The diagnosis of osteoporosis is based on the values ofthe T score expressed in Standard Deviation (SD). T scorerepresents the deviation value, obtained from the meanof Bone Mineral Density (BMD) of young healthy persons (between 20 and 40 years of age). A normal finding is the T-score above or equal to -1 SD, osteopenia corresponds to T scores between -1 and -2.5 SD, and osteoporosis is less than or equal to -2.5 SD (3).

The causes of osteoporosis are numerous, such as endocrine, nutritional and connective tissue disorders, bone marrow disease etc. (4). Also, use of some drugs can lead toosteoporosis, one of which is antipsychotic usage. Antipsychotics are the first line treatment choice in schizophrenia,as well as in other forms of psychosis (5). Schizophreniais a group of psychotic mental disorders, with a specific constellation of psychopathological phenomena, which in a chronic course could lead to the disorganization of the patient's personality (6).

One of the most explored possible effects on bone metabolism is hyperprolactinaemia, which is due to antipsychotics blockade of the tuberoinfundibular system (7). Increased food intake and weight gain are consequencesof the blockade of the effect of dopamine in the medullaryperiventricular pathway while patients are being treated with antipsychotics (7). Metabolic disorders, elevated blood sugar levels, lipid disorders, especially insulin changes are possible consequences of the application of antipsychotic therapy that could lead to changes in bone metabolism and osteoporosis (7). Also, it was already accentuatedthe influence of the immune system of T cells, B cells and cytokines, both on osteoclasts and on osteoblasts (8, 9).

It has recently been found that there is an association of stressful events in early age and long-term impact on inflammatory response and potential vulnerability to develop specific behavioral and metabolic changes in later life(10-12). It is clear now that in patients with schizophrenia there are metabolic changes before the antipsychotic application (13-15), but also after its use (16, 17). The detailed data and analysis of the possible effects of antipsychotic treatment on bone mineral density in patients with schizophrenia is still lacking.

ANTIPSYCHOTICS' DIFFERENT RECEPTOR PROFILE AND BONE MINERAL DENSITY

Clinical guidelines suggest that patients with schizophrenia should be treated either with conventional or first-generation antipsychotics (chlorpromazine, levomepromazine, promazine, flufenazine, haloperidol and others) or with atypical or second-generation antipsychotics (clozapine, risperidone, olanzapine, quetiapine, ziprasidone, sertindole, quetiapine, aripiprazole, paliperidone and others) (6). The first-generation antipsychotics are characterized by a strong affinity and blockage of primarily dopaminergic, but also noradrenergic and cholinergic receptors (6). Atypical antipsychotics act polyvalent, their neurotransmitter activity is balanced at several neurotransmitter systems, but a central effect is on the serotonergic and dopaminergic receptors (6).

Although treatment with antipsychotic drugs hasdesirable effects in wide range of symptoms, they havealso shown their side-effects. Researches carried out so far suggest that the patients with schizophrenia have a higher incidence of osteoporosis due to antipsychotic treatment (18).

As it was previously mentioned, antipsychotics inhibit or block dopamine receptors in the brain (7, 19). There arefive types of dopamine receptors in the central nervous system. Almost all antipsychotic drugs block dopamine D2-receprots in the brain, but recent new antipsychotics are acting more on other receptors and do show significant clinical differences from other antipsychotics (19). Atypical antipsychotics have a higher affinity for serotonin 5HT2A compared to D2 receptors. For example, clozapine acts onserotonin 5-HT2 receptors, dopamine D4-receptors, noradrenalin alpha 1 receptors and histamine H1 receptors more strongly than it does on dopamine D2 and D1 receptors (7, 19, 20).

As the block of the dopamine D2 receptors occurs it comes to the release of prolactin and later on consequently,hyperprolactinemia (21). Clinical researches showed that high levels of prolactin can in many ways cause reduction of BMD. One way may be through hormonal side effects caused by high levels of prolactin: reduced secretion of gonadal hormone, reduced sex hormone levels, affecting the synthesis of 25-OH-vitamin D and the absorption of calciumin the intestine (21, 22). Also direct impact of prolactin onosteoblasts should be considered, as study showed reducednumber of these cells, and thus reduced bone proliferation and consequently low bone mineral density (21, 22).

Depending on the strength of the action and which receptors will occupy more, the effects of antipsychotics will vary, and will not have the same impact on inducing hyperprolactinemia (23). According to their effect on prolactin levels antipsychotic can be categorized as prolactinsparing or prolactin-raising (24). Prolactin-raising are conventional antipsychotics and some atypical antipsychotics: risperidone, amisulpride and paliperidone. Prolactin sparing are other

atypical antipsychotics: aripiprazole, clozapine, olanzapine, quetiapine, ziprasidone. This division is particularly useful in order to reduce adverse side effects, especially now referring to BMD in men and women.

In patients with schizophrenia before treatment with antipsychotics it should be also considered if the level of prolactin is already high and what it was caused by. The usual normal prolactin levels in peripheral blood are below530 mIU/L (25 ng/ml) in women and 424 mIU/L (20 ng/ ml) in men (24). If there is a need the following precautions can be taken; reducing the dose of the antipsychotic or switching to a prolactin-sparing agent (23, 24).

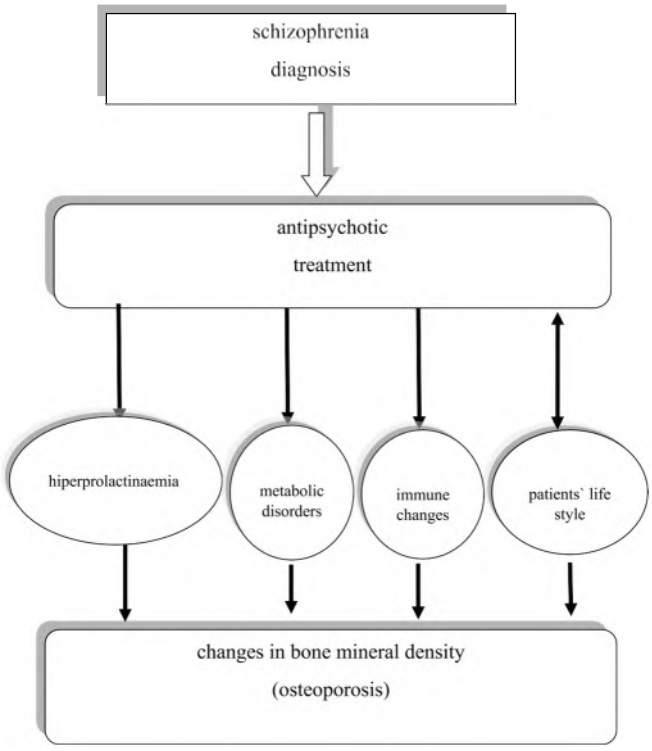

Figure 1. After diagnosis of schizophrenia and initiation of therapy withantipsychotics, possible mechanisms that lead to changes in bone mineral density

Possible side effects after the use of antipsychotics in the treatment of schizophrenia are the development ofhyperprolactinaemia, then metabolic disorders, followed by elevated blood sugar levels, lipid disorders, especiallyinsulin changes are possible consequences of the application of antipsychotic therapy which by itself could lead to changes in bone metabolism and osteoporosis. Also, influence of the immune system of T cells, B cells and cytokines, both on osteoclasts and on osteoblasts, after the usage of antipsychotics. And last but not the least, patients' life style could have an impact and contribute to the changes in bone mineral density leading to osteoporosis.

Hyperprolactinaemia is not the only undesirable effect of the use of antipsychotics. Other effects that can make possible changes on BMD include obesity, changes in the level oflipid status and systemic glucose levels, and their expressionvary with the use of different antipsychotics also dependingon the occupation of determined receptors (25, 26).

Studies showed that for lipid status disorder risk is high with usage of clozapine, olanzapine, and quetiapine, lower with risperidone and amisulpride, and the smallest risk to cause unhealthy cholesterol is after treatment with ziprasidone and aripiprazole (27, 28). Diabetes is expected to follow weight gain, and so it usually appears as a consequenceof the use of antipsychotics that cause obesity, except for olanzapine and clozapine which may directly induce glucose dysregulation without weight gain (27, 28). It should be noted that difference in BMD values in the application of antipsychotics from different groups, is basically due to affinity for receptors.

Studies so far have mostly paid attention on the effect of hyperprolactinaemia based on the effects on the hypothalamo-pituitary-gonadal axis resulting in impaired secretion of the luteinizing hormone and follicle-stimulating hormone, which consecutively lead to lower gonadal hormone secretion and has been suggested as a leading mechanism of bone loss in schizophrenia (21, 29).

Osteoporosis can occur in both men and women (30, 31). More women get osteoporosis than men, as estrogen deficiency following menopause or surgical removal of the ovaries is correlated with a rapid reduction in bone mineral density, while in men, a decrease in testosterone levelshas a comparable, but not so pronounced effect (31, 32). Estrogen is the major hormonal regulator of bone metabolism both in women and men. Estrogen effects on the key cells involved in bone metabolism: osteocytes, osteoblasts,osteoclasts, and T-cells. Its role are inhibition of bone remodeling, decreasing bone resorption, and maintaining ofbone formation (32). After the usege of antipsychotics current evidence show that postmenopausal women and oldermen were more vulnerable to hyperprolactinemia-related low BMD than premenopausal women and younger men (21). Because of this knowledge, it is also important to consider selecting appropriate antipsychotics in therapy, preferring prolactin-sparing if needed.

ANTIPSYCHOTIC-INDUCED HYPERPROLACTINAEMIA AND DECREASE BONE MINERAL DENSITY

Cores of the tuberoinfudibular part of the hypothalamus synthesize dopamine, which by the portal hypophyseal circulation is then transported into the pituitary glandand inhibits the secretion of prolactin. Antipsychotics, as dopamin D2-receptors antagonists, bind to D2 receptors of lactotroph cell in the pituitary and lead to release of prolactin. After some time it comes to hiperprolactinemia andthe effect on bone metabolism in the form of bone densityreduction occurs as consequence (33,34).

The study that compared bone mineral density values and prolactin level (PRL) and their correlations before and12-

monhs after treatment with conventional and atypical antipsychotics in patients with shizophrenia showed negative correlation between BMD values and PRL after treatment with conventional antipsychotics (35). Lower BMD values were found in patients who were on treatmentwith conventional antipsychotics than ones treated with atypical antipsychotics. Also PRL level was higher after treatment, and significantly after use of conventional antipsychotics comparing those after atypical antipsychotics treatment (35).

The study that was based on relationship of secondgeneration antipsychotics and risk of osteoporosis in patients with schizophrenia found out that PRL levels were significantly higher in patients treated with antipsychotics compared to healthy control subjects (36). It was noted that Tartrate-Resistant Acid Phosphatase 5b (TRACP-5b) levels significantly correlated with PRL in the female patients. TRACP-5b is an enzyme derived from the osteoclast that can be quantified in serum samples and is very useful boneresorption marker. The results also showed that hyperprolactinaemia lead to suppression of sex hormones, which probably caused changes in bone metabolism and lead to osteoporosis (36).

Researchers have also tried to determine if there is a change in bone biomarkers in patients with schizophrenia who were switched or have add-on therapy of atypical antipsychotic aripiprazol (37). Prolactin, estrogen and testosterone, urinary N-Terminal telopeptide (NTX) and serum Bone-Specific Alkaline Phosphatase (BSAP) levels were measured in certain intervals. Approximately 90% of the organic matrix of bone is type I collagen, a helical protein that is cross-linked at the N- and C-terminal ends of the molecule. The amino acid sequences and orientation of thecross-linked alpha 2 N-telopeptide of type 1 collagen makeit a specific marker of human bone resorption. N-terminaltelopeptide (NTx) molecules are mobilized from bone by osteoclasts and subsequently excreted in the urine. Elevated levels of NTx indicate increased bone resorption. Measuring BSAP provides a general index of bone formation and a specific index of total osteoblast activity. It was showed that NTX concentration was significantly reduced in group which was treated only with aripiprazol, but not changed much where aripiprazol was added. On the other hand, BSAP levels were significantly reduced in both groups of patients, as well as PRL. So it was concluded thataripiprazol shaped better bone metabolism profile (37).

De Hert et al. (2016) presented that raised PRL do affect and lead to increased risk of the occurrence of osteoporosis, and also showed the difference in the values of PRL after using the first generation of antipsychotics and after using the second generation antipsychotics, which proved to have more favorable profile (38). Other study also confirmed these findings and indicated that antipsychotics caused hyperprolactinemia made effect on bone mineral density in the form of reduction and finally osteoporosis (39).

Tseng at al. (2015) in their study made comparisons in changes of BMD after treatment with prolactin-raising and prolactin-sparing antipsychotics (40). They revealed moredecreased, worse BMD values in patients with shizophrenia who were on treatment with prolactin raising antipsychotics. Further studies revealed the same, increased prolactin levels, lower bone mineral density in patients with schizophrenia treated with antipsychotics, and as a consequence osteoporosis (41, 42).

EFFECTS OF ANTIPSYCHOTICS-INDUCED METABOLIC DISORDERS ON BONE

MINERAL DENSITY

So far only one study has pointed out the relationship and impact of antipsychotics on metabolic disorders and then consequently on the bone metabolism in the patients with schizophrenia (43). This study tried to determine whether there was a link between patients treated with antipsychotics for a longer period and modification in bone mineral density, due to changes in the values of glycemia, lipids, insulin, weight gain, and metabolic syndrome. Therewere two groups of patients, one with drug-naive first episode patients and second who were at least 6 months treated with antipsyhotics and normal controls. Results showedthat PRL levels were higher in both patients' group thanin healthy controls. Bone turnover markers that were usedin this study were osteocalcin and Serum Beta-Crosslaps (β-CroosLaps). Osteocalcin is produced by osteoblasts, essential to bone mineralization and it is often used as a marker for the bone formation process for osteoporosis. During bone resorption proteases degrade collagen and β-CroosLaps is released into the circulation. It is a specific marker for the degradation of Type I collagen. Most of the organic matrix of bone consists of Type I collagen. Increased levels of β-CroosLaps are found in serum in statesof increased bone resorption.

Bone turnover marker, β-CroosLaps was higher in patients who were on antipsychotics long-term use than in subjects from other two groups, but in osteocalcin there was no difference. Total cholesterol and triglyceride measured levels were higher in group with patients who were on antipsychotics long-term use than in group with drugnaive first episode patients. Levels of high density lipoprotein cholesterol showed no difference between two groupsof patients. Glucose levels were higher in both groups of patients and were in correlation with bone turnover markers, osteocalcin and β-CroosLaps. In group of patients whowere on long-term use of antipsychotics, serum insulin level was very high and correlated positively with osteocalcin levels. Increased secretion of bone turnover marker osteocalcin may be due to the action of insulin as it stimulates the mitogen in osteoblasts. The study of Zang et al. (2016) (43) certainly gave and uncovered possible mechanism between use of antipsychotics and osteoporosis, mostly through abnormalities in lipid metabolism, glucoseand insulin changes.

ANTIPSYCHOTICS-INDUCED IMMUNE CHANGES AND BONE MINERAL DENSITY

To our knowledge, there are previously conducted studies that have explored or indicated a direct relationshipbetween the use of antipsychotics, changes in the immunesystem, and consequent disorders in bone metabolism.The influence and alteration of the immune system on theoccurrence of osteoporosis is distinctly known (44-47), as it is also established that the effect of antipsychotics can lead to changes in the immune system (50). However, no research has been carried out on this subject so far, to explore the possible link between immune system alterationsand osteoporosis in patients with schizophrenia.

Osteoblast can be induced to increase secretion of cytokines, which acting on the osteoclasts and then stimulatebone resorption (44). One review study indicated on the roleof regulatory T cells in bone remodelling proces. Throughcellular and molecular mechanism, by secreting Transforming Growth Factor-beta (TGF-β), interleukin (IL)-10, as well as IL-4 cytokines, regulatory T cells participated in bone remodeling and could lead to inflammatory bone lossor osteoporosis (44). Other research study has shown that association in correlated expression of Th1 cytokines (IL-12 and Interferon-γ (IFN-γ)) and bone-resorbing cytokines (Tumor Necrosis Factor-α (TNF-α)) have a significant rolein the bone resorption caused by inflammation (45). As forB cells, it is assumed that the occurrence of osteoporosis is due to the changed axis RANK/RANKL/OPG, whose B cellsare active regulators during inflammation (45). Azizieh et al.(2017) (46) pointed out that higher levels of proresorptive cytokines (TNF-α, IL-6, IL-12 and IL-17) and lower levels of the antiresorptive cytokines (IL-4, IL-10, and IL-23) wereproduced in women with low BMD compared to women with normal BMD. It has been shown that during inflammatory diseases there could be a relationship between changesin BMD and C-reactive protein serum values (47). In a largepopulation study group sample results showed that CRP concentration was inversely and independently associatedwith BMD (47).

Now we know that changes in the immune system occur in schizophrenia even before the treatment with antipsychotics itself (48, 49). Afterwards the effect of antipsychotics on changes and alteration in the immune system is demonstrated, too. Recent study monitored and analyzed changes in the serum levels of type-1 (TNF-α, IFN-γ), type-2 (IL-4, IL-10), type-17 (IL-17) and regulatory cytokines (TGF-β, IL-27, IL-6) after use of antipsychotic drug inpatients with schizophrenia (50). Divided in three groups patients with First Episode Psychosis (FEP) that were unmedicated, patients with Schizophrenia in relapse (SC in relapse) treated with antipsychotics and healthy control group were studied subjects and cytokine measurements were done on day 0 and day 30 of the treatment. After antipsychotic treatment in FEP group lower levels of IL-4, IL-6and IL-27 were found. In SC relapse group, after therapy, serum levels of IL-6 and IL-4 were decreased. Serum levelsof IL-17 were lower in FEP group, but without significant difference between two measurements. TGF-β serum levels were increased in both patients group, but after treatment it came up even more in FEP group.

After all, we can expect that immune changes that happened in patients with schizophrenia both before and afterthe use of antipsychotics have effect on bone mineral density. By all means, we are unable to contend with certainty,but the assumption that those changes would lead to a reduction in bone mineral density and eventually osteoporosis. Therefore, it is necessary to pay attention to these possible impacts in the forthcoming researches.

SCHIZOPHRENIA PATIENTS' CHARACTERISTICS AND LIFE STYLE AS CON-FOUNDING FACTORS OF DECREASE

BONE MINERAL DENSITY

Greater prevalence of osteoporosis in patients with schizophrenia was established considering risk factors, thesociodemographic and clinical characteristics of these patients and the type of antipsychotics they used in the treatment (51). Study showed significantly lower BMD values in patients with schizophrenia compared to healthy control group. They also showed correlation between BMD and age, where older age and longer use of antipsychotics and duration of schizophrenia were in correlation with significant reduction in bone mineral density (51). Gender difference was found in this study, showing that women had worse changes in BMD than men. They revealed that patients treated with clozapin had better results in terms of bone density compared to other patients treated with other antipsychotics. By no means, this study indicated that it should be considered that the treatment of patients with poorer clinical characteristics and lifestyle should initially be treated with an antipsychotic that is less risky, possibly from the group of prolactin-sparing antipsychotics.

Patients with schizophrenia do not have adequate physical activity (52). Besides, because of the use of antipsychotics, the intake of food is not desirable, it is enhanced and leads to obesity (53). As risk factors in this group of patients,they may lead to the onset of somatic complications and increased mortality (54, 55). Of course, it is also possible thatmetabolic changes exist even before the disease and beforethe use of antipsychotics in these patients. Therefore, it is very important that as factors that can be changed and affected to, we should take into account both physical activity of these patients and nutrition, including even diet.

Curcic et al (2017) (56) revealed that physical activity significantly improved the aerobic capacity of those with schizophrenia and improved some psychiatric aspects of functioning. Recommended physical activity may also be of helpful in preventing co-morbidity in these patients.

CONCLUSION

Antipsychotics are very useful tool and necessity in the treatment of schizophrenia. But, antipsychotics in the treatment of schizophrenia could lead to osteoporosis and the risk of fractures. Exhaustive literature search revealed that several possible mechanisms could be responsible for this process, but definitely more work and research is needed to enlighten this issue and consider which mechanism is dominant. The direct and indirect mechanisms of antipsychotics that could cause osteoporosis should be defined and it is also important to determine which antipsychotic has the least unwanted effect on bone metabolism. So far the second generation of antipsychotics showed lower impact on bone metabolism. Also, the least attention has been directed towards the immune changes caused by antipsychotic treatment that could lead to osteoporosis in patients with schizophrenia.

It is necessary to raise awareness of the consequences and impact on the quality of life and work, taking care of themselves, aiming not only to achieve the remission of the disease, but to prevent the comorbidity of osteoporosis and fractures, as part of the treatment of the underlying disease (57). The existence of comorbidity in patients with schizophrenia should also be considered. Disturbances in several organic systems exist and should be taken into account the relationship between those disorders in the central nervous system (CNS) and those that are not in the CNS system (58). All disorders that are in cardiometabolic system, immune system, endocrine system, or disorders in the CNS system itself, such as brain structural, or changes in neurophysiological or neurochemical parameters, can contribute mortality and morbidity and so this should not be ignored during the illness itself.

So, it is important even during the illness to be aware that there are factors that we can act to, correct them, change patients' habits and in this way reverse the course of disorder and also changes in bone metabolism in patients with schizophrenia during their treatment with antipsychotics (59, 60). Ultimately, it is important to develop and implement preventive measures and multidisciplinary approach for the purpose of better bone health in these patients in terms of nutrition, physical activity and general lifestyle (60).

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CONFLICT OF INTEREST

None.

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ILEOCECAL INTUSSUSCEPTION OF THE ADULT INDUCED BY THE GASTROINTESTINAL STROMAL TUMOR OF THE ILEOCECAL VALVE - A CASE REPORT

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ABSTRACT

Adult intussusception is a rare entity which is distinct frompaediatric cases in incidence, aetiology, and management. It rep-resents 5% of all intussusceptions and is the cause of 1% of allintestinal obstructions, 0,08% of all abdominal surgeries and 0,003-0,02% of all hospital admissions. Ileocolic intussusceptionin adults is a unique variant in which nearly 100% of cases havea malignant lead point. In our report, we described a case of apatient with ileocecal intussusception caused by a rare type of thegastrointestinal tumor. The female patient was admitted to hospi-tal for occasional pain in the lower right quadrant of the abdomenfollowed by abdominal discomfort and appearance of blood in thestool. The result of CT scan of the abdomen and pelvis showed atumor mass and intussusception at the ileocecal junction, whichwas confirmed peroperatively. Open right hemicolectomy wasperformed adhering to oncological principles. The final patho-logic diagnosis indicated the gastrointestinal stromal tumor of theileocecal valve. The diagnosis of intussusception in adults is deli-cate, and timely surgical treatment can be vital. Patients with thepalpable abdominal mass, digestive tract obstruction, gastroin-testinal bleeding, or lead point computed tomography must un-dergo a surgical examination. Given a high risk of malignancy,primary surgical resection using oncologic principles presentsthe best option for the treatment of ileocecal intussusception inadults.

Keywords: *Intussusception in adults, ileocecal valve, GIST tumors.*

INTRODUCTION

Intussusception in adults is a very rare occurrence. It represents 5% of all intussusceptions and is the cause of 1% of all intestinal obstructions, 0,08% of all abdominal surgeries and 0,003-0,02% of all hospital admissions (1). The overall incidence of intussusception in adulthood has been estimated to be around 2-3 cases/1,000,000 population/year (2). Most often, in 66% of cases, we are talking about intussusception of the small intestine into the small intestine, while ileocecal forms and intussusceptions at different levels of the colon occur in 34% of cases (3). In 60% of cases, intussusception of the small intestine in adults is caused by benign lesions. The rest are caused by malignancy (30%) or are idiopathic (10%). However, most colonic intussusceptions are caused by malignancy (60%) (1).

In our report, we described a case of a patient with ileocecal intussusception caused by a rare type of the gastrointestinal tumor.

CASE REPORT

The female patient, 79 years old, was admitted to hospital for occasional pain in the lower right quadrant of the abdomen followed by abdominal discomfort and appearance of blood in the stool. The patient reported intermittent bloating, and weight loss of 15 kg in the last 6 months., The patient also reported occasional nosebleeds and occasional coughing up of blood. We found out from the patient's past medical history that she underwent cardiac surgery 25 years ago - triple coronary bypass surgery and a few years later, resection of the aortic arch aneurysm. In addition to the cardiovascular disease, the patient suffers from hypertension, chronic obstructive pulmonary disease and diabetes. Due to the diseases mentioned above, the patient receives an extensive therapy, including oral anticoagulants. She denied any tobacco, alcohol or illicit drug use.

The examination included her vital signs: the temperature of 36.5 degrees Celsius, blood pressure of 160/100 mm Hg, and oxygen saturation of 93% on room air. The patient had irregular heartbeat (arrhythmia absoluta). The physical examination revealed presence of the palpable mass and mild pain in the lower right quadrant of the abdomen. There was no tenderness of palpation. The rectal examination revealed presence of the raspberry-colored mucus.

The basic laboratory blood tests were performed. During the examination, there were signs of anaemia: RBC $3.16x10^9$/L (reference range 3.86 - 5.08); HGB 83g/L (reference range 110 - 157); HCT 0,259 (reference range 0.356 - 0.470. Prothrombin time (PT) and international normalized ratio (INR) were raised to 52.6 (reference range: 9.1-12.1) and 4.760 (reference range for patients with the anticoagulant therapy: 2.0-4.0), respectively. Partial thromboplastin time (PTT) was 64.0 sec (reference range: 25.0-35.0). The blood tests for inflammatory markers were negative. The analysis of tumor markers for colorectal cancer was performed. The results were in the reference range: CEA 1.7ng/mL (reference range: 0.0 - 5.0); AFP 7.11 IU/ml (reference range: 0.00 - 7.40). The urine analysis was negative for the urinary tract infection. The gynecological examination was unremarkable. The plain abdominal X-ray and abdominal ultrasound showed normal findings.

CT-scan confirms extensive ileocecal intussusception extending up to the transverse colon. A nodular structure was observed near the distally involved small intestine, which may suggest a tumor and would explain intussusception in the patient at this age. Edema of the bowel wall and free fluid in the lumen were observed (Figure 1).

Surgical intervention is indicated. Intraoperative findings indicate intussusception about 20cm of the terminal ileum in the lumen of the cecum (Figure 2A). Ileocecal valve cannot be identified. In the lumen of the cecum, there is a palpable tumor mass about 8 cm in diameter (Figure 2B). The appendix does not participate in intussusception. No signs of bowel ischemia were observed, there was no free fluid in the abdomen. Open right hemicolectomy was performed adhering to oncological principles (Figure 3). Re-establishing of the intestinal continuity was done with side- to- side ileocolic anastomosis. The patient tolerated the procedure well with minimal blood loss and no complications.

The final pathologic diagnosis indicated the gastrointestinal stromal tumor of the ileocecal valve. The tumor size was 76 mm in diameter; the base of the tumor was on the ileocecal valve, 26 mm in diameter (Figure 4). The tumor did not grow into the intestinal wall. In the surrounding pericolic adipose tissue, 18 lymph nodes with no metastatic deposits were found and analyzed. Immunophenotype profile: CD117++, CD34++, alpha-smooth muscle actin (α-SMA)+, desmin+, S-100+. GIST diagnosis was confirmed on the routine HE-stained sections (×100) of the tumor tissue (Figure 5A) and immunohistochemical analysis (×100). Tumor cells were diffuse positive for CD117 (Figure 5B). GIST diagnosis was confirmed on (e-h) immunohistochemical analysis (×100). Tumor cells were focally positive for desmin, α-SMA, CD34 (Figure 5C); S-100 protein and Ki-67 (×200) proliferation index was very low (Figure 5D).

The patient was discharged thirteen days after the operation.

Figure 1. Computed Tomography abdomen and pelvis with oral contrast.

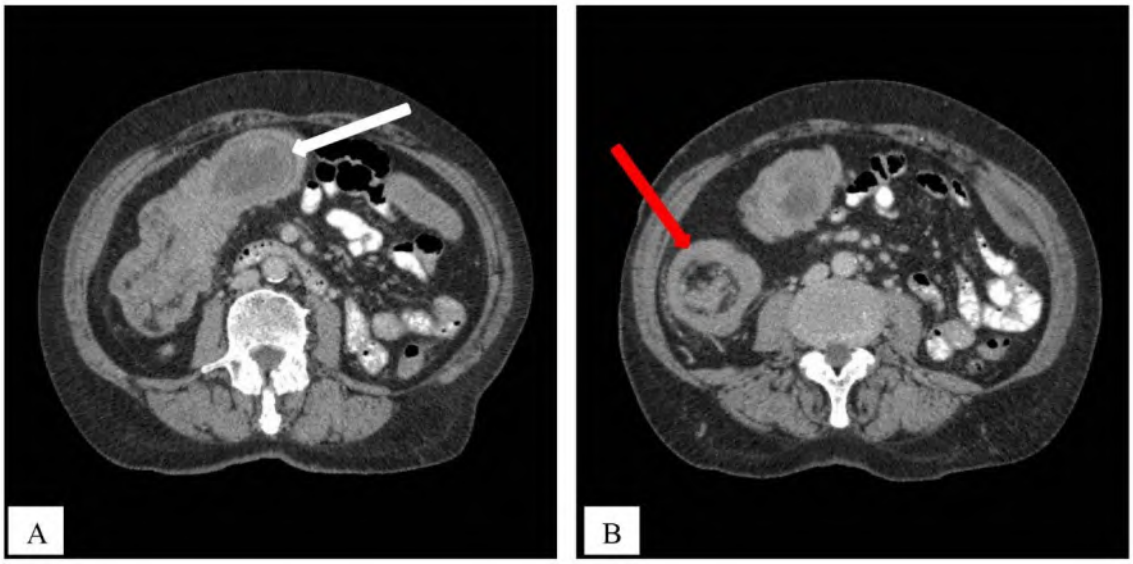
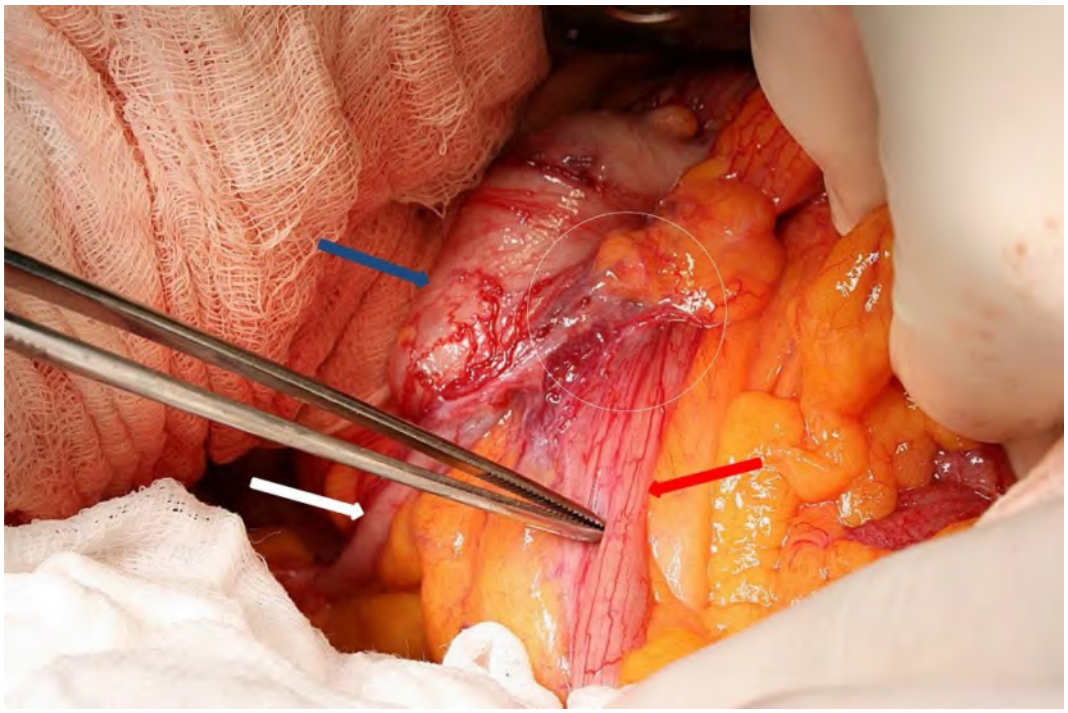
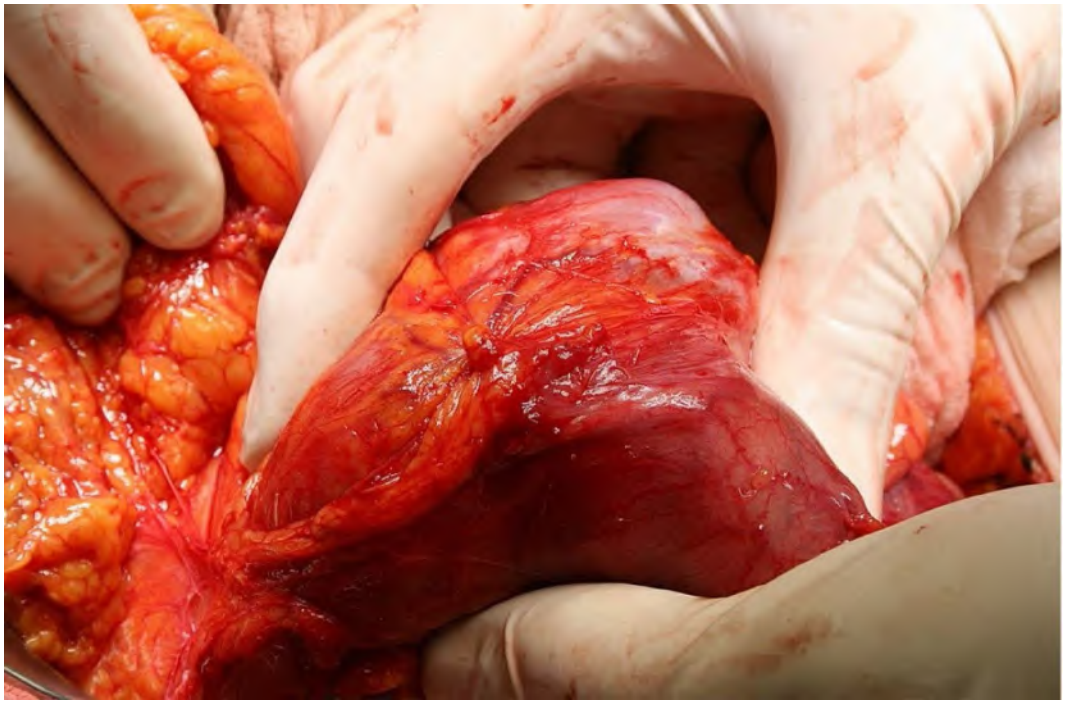


Figure 2A. Surgical specimen.



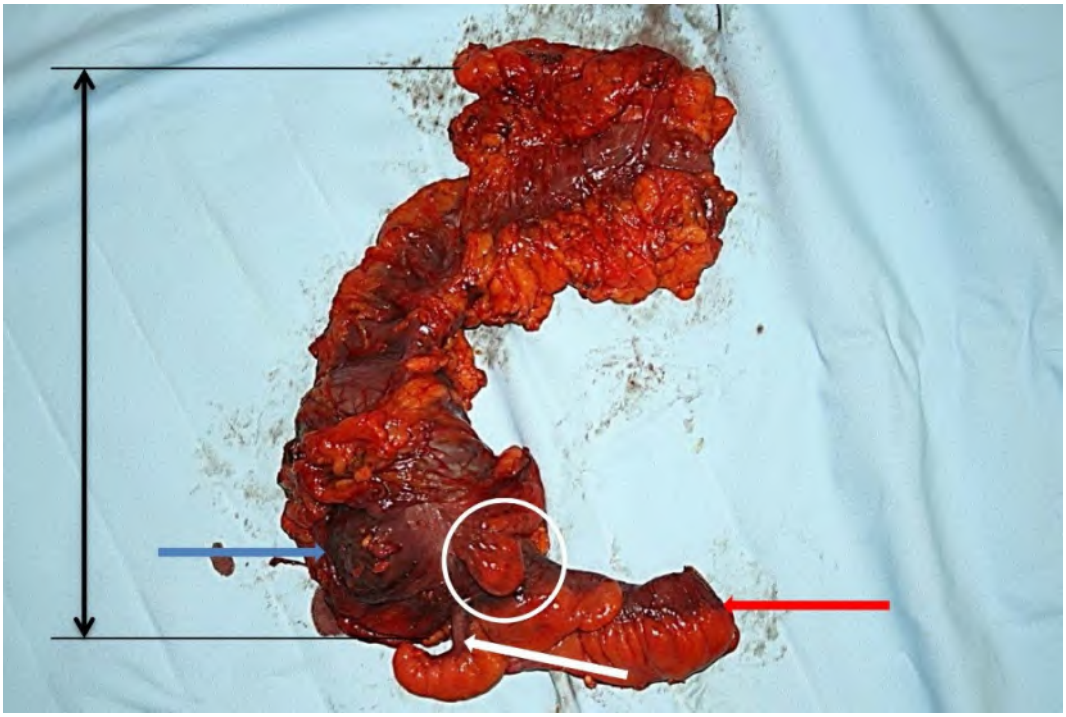
The terminal ileum (red arrow) telescoping (white circle) into the cecum (blue arrow).
The appendix is seen in a normal position (white arrow).

Figure 2B. Surgical specimen. Externally visible tumor which represents a lead point.



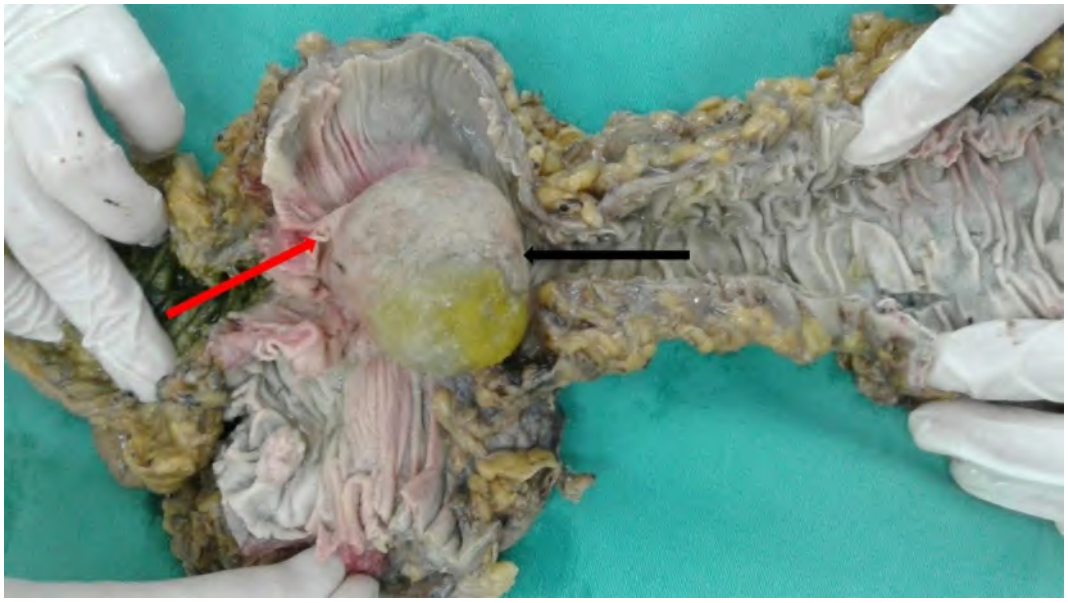
Note that the bowel is healthy, and mesentery has no enlarged lymph nodes.

Figure 3. Surgical specimen.



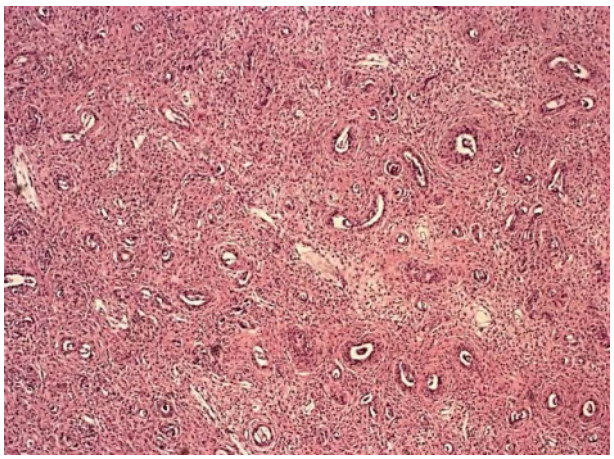
Resected segment showing intussusception(the terminal ileum- red arrow;
the site of intussusception- white circle; the cecum- blue arrow; the right colon- black arrow;
the appendix is seen in a normal position- white arrow)

Figure 4. Macroscopic appearance.



The opened fragment of the right colon showed a tumoural mass
76mm in diameter (black arrow) protruding into the lumen of the cecum.
The base of the tumor was onthe ileocecal valve, 26 mm in diameter (red arrow).

Figure 5A. Microscopic appearance.



HE-stained sections (×100) of the tumor tissue

Figure 5B. Microscopic appearance. Tumor cells were diffuse positive for CD117.

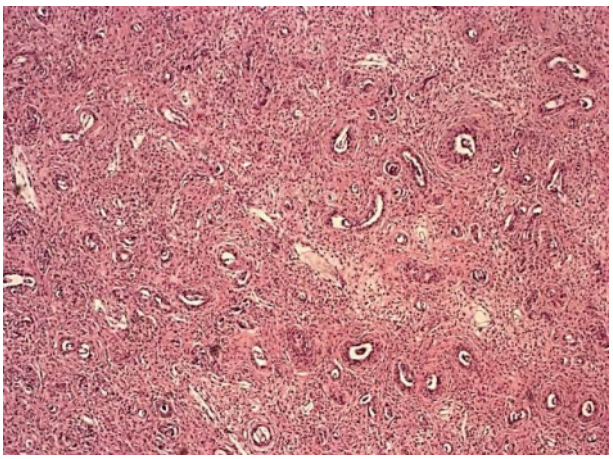
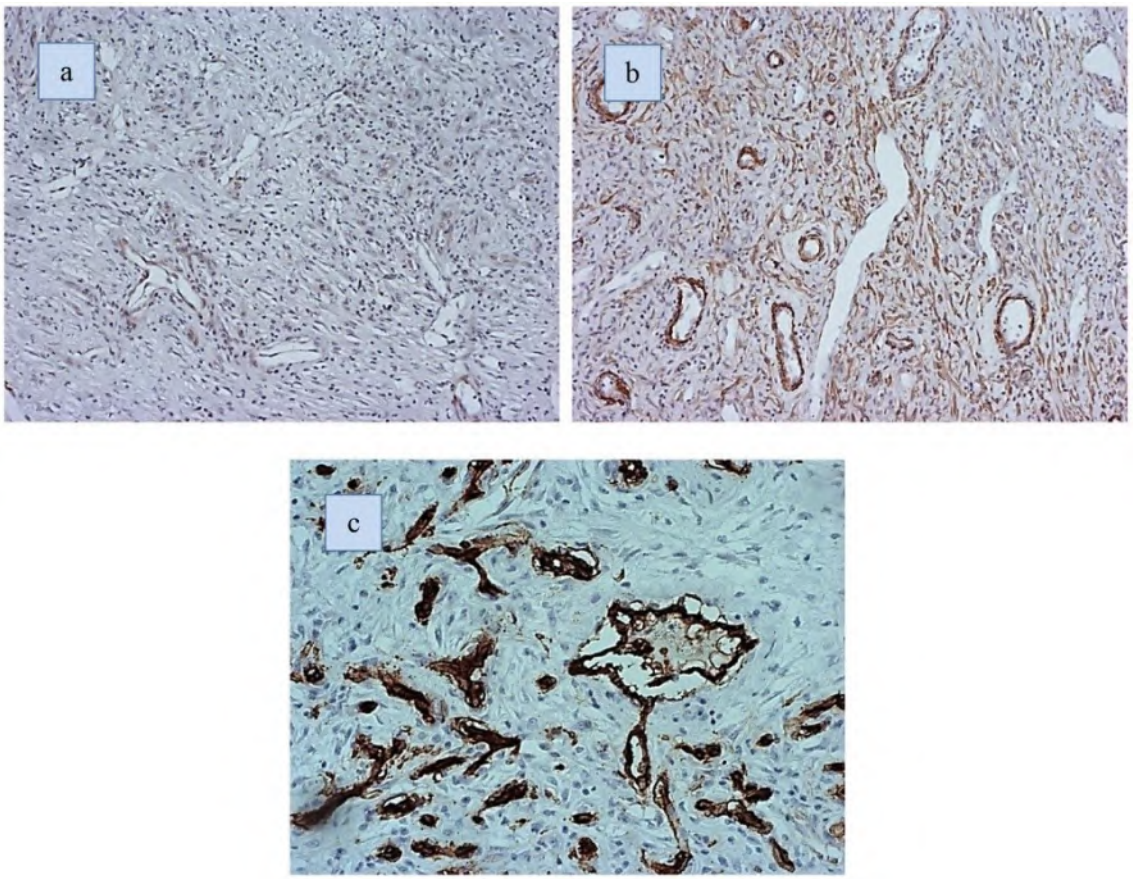
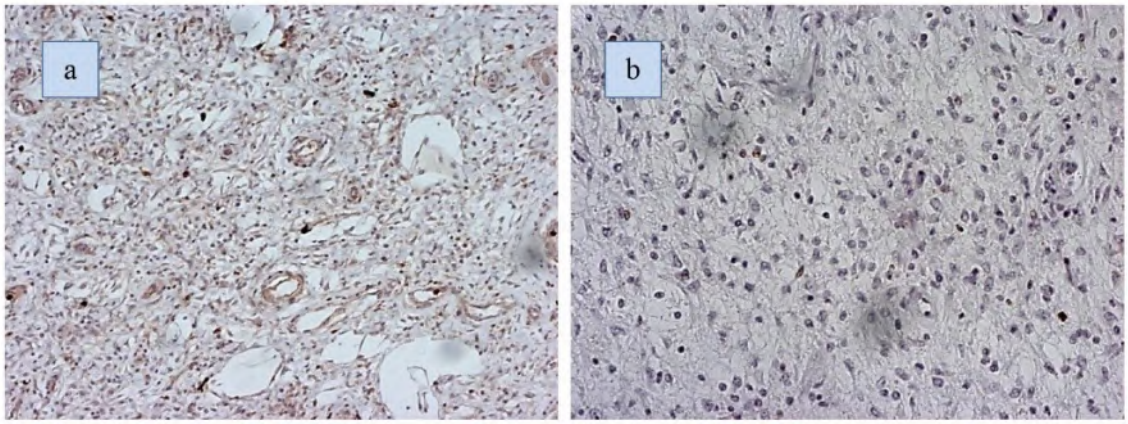


Figure 5C. Microscopic appearance.



Tumor cells were focally positive for desmin (a), α-SMA (b) and CD34 (c)

Figure 5D. Microscopic appearance. S-100 protein (a) and Ki-67 (b) (×200) proliferation index were very low



DISCUSSION

Intussusception is defined as the telescoping of the proximal part of intestine together with the mesentery into the adjacent segment, leading to impaired peristalsis, obstruction and possible disorders of intestinal vascularization (4).

Intussusception is usually classified either according to the etiology (benign lesion, malignant lesion, or idiopathic) or by the location (entero-enteric, ileo-colic, ileo-cecal, colocolic or appendiceal) (5, 6).

Intussusception was first described by Dr. Paul Barbette in 1674 and first treated by Sir Jonathan Hutchinson in 1871. (7). Before 1871, intussusception was classified as a serious life-threatening condition that had a high mortality rate (7).

Intussusception in adults is a very rare occurrence. It represents 5% of all intussusceptions and is the cause of 1% of all intestinal obstructions, 0,08% of all abdominal surgeries and 0,003-0,02% of all hospital admissions (1). Some studies report the cases of intussusception in adults in patients younger than 13 and older than 90 years, with the highest incidence occurring in the 30-50 age group. The ratio of men to women was approximately 2:1 (8). In the case we presented, the patient was at older age compared to the average age of patients with intussusception in adults.

In direct contrast to pediatric etiologies, adult intussusception is associated with an identifiable cause in 90% of symptomatic cases, with an idiopathic cause in 10% of cases (9). Benign or malignant neoplasms cause two-thirds of cases of intussusception in adults; the remaining cases are caused by infections, postoperative adhesions, Crohn granulomas, intestinal ulcers (Yersinia), and congenital abnormalities such as Meckel diverticulum. In our case, the patient's past medical history was negative for gastrointestinal diseases. Also, our patient had no previous abdominal surgery or abdominal trauma. Of the cases caused by neoplasms, 50% of them are malignant (10). Malignant lesions include primary tumors such as carcinoids, adenocarcinoma, malignant polyps, GISTs, leiomyosarcomas, lymphoma and metastatic tumors, most commonly melanoma (11). The most common malignant cause of colonic intussusception is primary colonic adenocarcinoma and the most common benign cause is colonic lipoma (10). Ileocolic intussusception in adults is a unique variant in which nearly 100% of cases have a malignant lead point, namely, cecal adenocarcinoma involving the ileocecal valve (12). In the case of our patient, there are two significant characteristics. First, the lead point of ileocecal intussusception is a rare type of the gastrointestinal tumor - GIST. And second, the ileocecal valve is an extremely rare primary localization of GISTs and gastrointestinal tumors in general (13-20).

In the extensive literature reviewed, no similar cases have been described. These facts may be a starting point for further research.

In adults, the clinical presentation of intussusception is otherwise nonspecific, rarely presenting with the classic triad of acute abdominal pain, palpable mass, and bloody stool (21, 22, 8**).** Instead, it presents with symptoms of the small or large bowel obstruction. The most common presenting symp- tom is abdominal pain, (22, 23) with associated symptoms consistent with partial obstruction: nausea, vomiting, obsti- pation, gastrointestinal bleeding, change in bowel habits, constipation, or bloating (8, 24). Wang et al. (12) found abdominal cramping pain in nearly 80% of patients as a leading symptom; the palpable abdominal mass, however, was found in less than 9%. The symptoms are usually acute, lasting for days to weeks, but they can rarely be chronic, and lasting for months to years (12).

Our patient did not have the acute abdominal pain, but rather occasional chronic pain in the lower right quadrant of the abdomen that lasted for months. The symptoms of bowel obstruction were mild in the form of abdominal discomfort and occasional bloating. Such a clinical picture can be explained by the fact that the onset and duration of clinical symptoms of intussusception are significantly longer in the colon than in the disease of the small intestine, 62.5 to 35.7%, respectively (12). The symptoms in our patientwere not specific for intussusception. Intermittent abdominal pain and bloating along with occasional rectal bleeding are symptoms associated with a long list of differential diagnoses. However, the physical examination that revealed presence of the palpable mass in the lower right quadrant of the abdomen helps in narrowing down and focusing attention to a subset of possible etiologies. Diagnostic procedures were performed.

A number of different radiologic methods have been described as useful in the diagnosis of intussusception: CT scan, barium studies, abdominal ultrasound, plain film, angiography, and radionucleotide studies (25).The plain abdominal X-rays are typically the first diagnostic tool and show signs of the intestinal obstruction, and may provide information regarding the site of obstruction. However, some authors cite a series of cases of intussusception in adults in which the plain x-rays of the abdomen were not relevant for a diagnosis, (26) which is confirmed in the case of our patient. The result of the plain abdominal X-ray was negative. Contrast studies can help to identify the site and cause of intussusception, particularly in more chronic cases. In the past, colon intussusception was diagnosed with a contrast enema showing a crab claw-like shadow, but the accuracy of preoperative diagnosis was only 20-25 % (27). In the case of our patient, a contrast study was not done for technical reasons. Ultrasound is well established as the first-line imaging modality for diagnosing intussusception in children (28). Features on the ultrasound include a typical ‘target lesion’ or ‘pseudo-kidney’ appearance on longitudinal imaging (28). The ‘target lesion’ demonstrates concentric layers of different echogenicities, which correspond to the oedematous bowel wall and central invaginated mesenteric fat (29). In children, the ultrasound may be 98-100% sensitive and 88-

89% specific in diagnosing intussusception (28). Ultrasonography as a diagnostic test of intussusception in adults requires an experienced examiner. The limitations include obesity and bowel gases that can trap typical findings, and information about mesenteric vasculature, location, and surrounding interior is not clearly defined (30). In the case of our patient, the abdominal ultrasound examination was not relevant to the diagnosis. Despite the extensive experience of the diagnostician, the typical 'target lesion' or 'pseudo-kidney' was not observed. Flexible endoscopy including colonoscopy and small bowel enteroscopy may be a useful diagnostic tool in patients with subacute or chronic intermittent bowel obstruction (31). It permits the confirmation of intussusception, location and biopsy to aid with the diagnosis and plan of surgery (32). Small lesions can be snared endoscopically if the surrounding bowel appears normal without signs of inflammation or ischemia, however, lesions larger than 2 cm with a wide base should not be excised due to the increased risk of perforation of the bowel (30). Colonoscopy is most useful for adult intussusception involving the colon, terminal ileum and cecum, (25) however, due to the age and multiple morbidity of our patient, colonoscopy was not performed.

A contrasted CT scan of the abdomen and pelvis is the most sensitive and specific radiological investigation for intussusception and is the modality of choice in adults (33). The reported diagnostic accuracy of CT in adult intussusception varies but may reach 100% (34). The characteristic features include a soft tissue mass, target or sausage shaped, enveloped with an eccentrically located area of low density. The findings of a bowel within bowel configuration with or without mesenteric fat and mesenteric vessels are pathognomonic for intussusception (35). CT scan of the abdomen and pelvis of our patient showed a target lesion in the right lower quadrant with obstruction of contrast and pericolon fat accumulation, indicating intussusception at the ileocecal junction. (Fig. 1) CT scan also provides other critical information such as the length and diameter of intussusception, three dimensional views of the bowel and surrounding viscera, possible lead point, type and location of intussusception, mesenteric vasculature, possibility of strangulation, and likelihood of partial or complete bowel obstruction (36). CT in our patient demonstrates a swirling mass containing fat in the region of the ascending colon/cecum. Intussusception of the ileum into the cecum is detected which extends through to the mid-transverse colon. At the apex, a tumor is suspected. The bowel wall is thickened (the intussuscipiens), but no signs of the vascular compromise have been observed. Perihepatic, mesenteric, right colic gutter and pelvic free fluid. No free gas (Fig. 1).

Before the advent of diagnostic modalities, immediate laparotomy and bowel resection without reduction were the standard of care and advocated by most surgeons (37, 38). The current controversy remains on the extent of surgical resection vs reduction of intussusception. The initial favor to resect en-block theintussuscepted bowel segment was based on the theoretical risks of venous embolization of tumor cells on the bowel manipulation as well as on the risks of perforating the ischemic, friable, edematous bowel which may lead to seeding of tumor cells and microorganisms into the peritoneal cavity (39). The laparoscopic approach offers both diagnostic and therapeutic options for intussusception in adults. Certain small bowel intussusception especially in younger patients may have a benign, physiological cause and laparoscopy with reduction may be an acceptable strategy. However, caution should be exercised when using laparoscopy in patients with a severe bowel obstruction where visualization may be poor, and the bowel manipulation may further risk perforation and increase surgery morbidity (40). Of two equally well-grounded opinions, we are closer to the opinion of authors who have the view that all colonic intussusceptions should be resected en-bloc without reduction, as most of these could harbor a pathological etiology and may not respond to the conservative management (25, 41- 43).

CONCLUSION

Adult intussusception is a rare entity which is distinct from paediatric cases in incidence, aetiology, and management. Ileo-colic intussusception is often caused by the lead point pathology which can be a submucous lipoma but it may be a malignant lesion. Patients with the palpable abdominal mass, digestive tract obstruction, gastrointestinal bleeding, or lead point computed tomography must undergo a surgical examination. With advances in laparoscopic surgical techniques and outcomes, an experienced surgeon can approach this disease laparoscopically. Given a high risk of malignancy, reduction is most often prohibited and primary surgical resection using oncologic principles presents the best option for the treatment of ileocecal intussusception in adults.

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.

FUNDING

No funding was received from any sources.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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SUCCESSFUL TREATMENT OF MASSIVE PULMONARY EMBOLISM WITH RESCUE FIBRINOLYSIS IN YOUNG PATIENT WITH HOMOCYSTINEMIA - CASE REPORT

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ABSTRACT

Pulmonary embolism (PE) is the most frequently missed diagnosis in the urgent clinical department with serious consequences. Patients with unprovoked PE have increased risk of recurrent PE. Approximately 5 to 8% of PE patients have inherited thrombophilias. A solated homocystinemia is a rare cause of unprovoked acute pulmonary embolism. Timely diagnosis and proper treatment can prevent complications, costs and mortality and provide patient better quality of life. We are presenting a 42-year-old woman was admitted to our emergency department with the first episode of severe dyspnea and chest pain. She had no history of previous cardiovascular or respiratory disease and no history of previous pulmonary embolism (PE) or deep vein thrombosis (DVT). Urgent echocardiography showed indirect signs of pulmonary embolism which was confirmed by the pulmonary artery CT angiography performed one day after the patient's admission. After two days of heparin infusion, she developed a hemodynamic instability with cardiogenic shock and was treated successfully with fibrinolysis. After the clinical stabilization, she was put on the rivaroxaban therapy, which was recommended for additional six months. The thrombophilia profile was done two weeks after stopping the therapy with rivaroxaban. The thrombophilia panel came back positive for high levels of homocysteine (67 µmol/L), with other thrombophilia results within normal limits. The patient was stable during the follow-up period. Pulmonary embolism should be always suspected in younger patients with acute severe dyspnea even without provocable risk factors. High suspicion level and fast diagnosis are lifesaving. In younger patients presented with unprovoked pulmonary embolism, clinicians should consider inherited prothrombotic factors and homocystinemia as a potential cause. Rescue fibrinolysis is a lifesaving therapy in hemodynamic worsening in intermediate high-risk PE patients. A longer anticoagulation therapy should be considered in these cases with novel oral anticoagulants that are recommended as safer and superior therapy.

Keywords: *Pulmonary embolism, homocystinemia, thrombophilia.*

INTRODUCTION

Pulmonary embolism (PE) is the third vascular cause of death. PE mortality rates exceed 10% at 30 days and 16% at 3 months. 5% of PE patients are presented with a hemodynamic instability and cardiogenic shock which is related tothe high intrahospital mortality (1). Homocysteine is a sulfhydryl amino acid formed from demethylation of dietary methionine. The studies examining the relationship between an elevated plasma homocysteine concentration, have appreciated the relationship between homocysteinemia and venous thrombosis. The elevated level of homocysteine is a risk factor for both arterial and venous thromboembolism (2). The overweight and obese individuals have 2-3-fold increased risk for deep vein thrombosis (DVT) and PE (3). The scientific data also show potential impact of body fat distribution and cardiometabolic abnormalities associated with central obesity with the risk of arterial and venous thrombosis. Venous stasis as a consequence of obesity, may be of more importance for venous thromboembolism (4).

CASE REPORT

A 42-year-old woman was admitted to our emergency department with the first episode of severe dyspnea and chest pain. She had no history of previous cardiovascular or respiratory disease, no history of PE or DVT. The symptoms started at home one hour before the arrival to our clinic, not provoked by any physical effort. The patient denied any provocable PE risk factors (no history of injury, no surgical treatment, bed rest over 72h, no cancer history, no signs of DVT or previous PE/DVT). She also denied recent respiratory infection or longer flights. Additionally, we have obtained an information on the contraceptive use five years before this event. She had an increased body weight, with BMI 28kg/m^2, without other CV risk factors. Physical examination showed tachycardia, tachypnea with a respiratory rate 16/min, without abnormal pulmonary findings and no heart murmurs. ECG showed sinus tachycardia with HR 130 bpm, RBBB, and S1Q3T3 sign. Blood pressure was 125/90mmHg. No signs of DVT were found at the clinical examination. The Wells score for PE probability was <4 (PE unlikely).

Echocardiography was performed immediately after the emergency department examination in order to evaluate the cause of patient's symptoms. The examination showed an increased right ventricle (RV) size, increased RV to LV ration >1, reduced RV function (TAPSE 15, TDI S' 8), presence of McConnell's sign, severe tricuspid regurgitation with dilated non-collapsible v. cava -21mm, and signs of pulmonary hypertension (SPAP 54mmHg). The LV function was normal, with the left ventricular ejection fraction 65%, and no wall motion abnormalities. There were no thrombus formations weened in the RV cavities or pulmonary artery. Due to the clinical presentation and echocardiography findings of the RV dysfunction and signs of pulmonary hypertension, the patient was admitted to our intensive care unit for suspected PE and the anticoagulation treatment with unfractionated heparin was started.

Laboratory results showed an increased leucocyte level of 11 x10^9/l, Hgb 145 g/l, hematocrit values 49%, platelet counts 285x 10^9/l, glomerular filtration rate (GFR) - 98 ml/min/1.73m^2. Serum electrolytes, renal and liver function tests were within a normal range. hs-Troponin I (ABBOT Essay) was elevated – 182 ng/l (referent values for women 0-34.2 ng/l), D-dimer levels were increased- 9.835 ng/ml (cut off value <500 ng/ml). Hemostasis findings 4 hours after heparin infusion showed a good therapy response with a prolonged prothrombin time and activated partial thromboplastin time monitoring (56 sec), INR 1.8.

Doppler ultrasonography of the lower extremities performed on the second hospital day, showed no signs of DVT.

CT angiography was performed one day after the patient's hospital admission in order to definitely confirm PE. The CT result showed massive pulmonary embolism with riding thrombus over the pulmonary trunk, extending to the right and left pulmonary artery with central filling defects. A large partial-lumen occluding filling defect was noted in the left main pulmonary artery, which was extending further into the hilar branch, occluding the lumen completely. Another larger non-complete lumen occluding filling defect was noted in the right main pulmonary artery. These filling defects were extending into the segmental and sub-segmental branches of the lateral segment of the right middle and bilateral lower lobe. The pulmonary trunk was dilated to -33 mm. There was no evidence of mediastinal pathology. The evaluated sPESI score was >1, which indicated a high 30-day death risk (10.9%). Based on the hemodynamic profile, echocardiography findings of the RV dysfunction, sPESI score and elevated troponin levels, the patient was initially assessed at an intermediate high risk for early mortality.

Treatment and complication

Due to the high probability of PE based on the echocardiography findings, the intravenous heparin therapy was started (7.500 IE iv bolus, with a continued infusion of 30.000 IE/24h). The patient tolerated the treatment well, with a significant dyspnea reduction, the heart rate decreased to 80-90 bpm after three hours, the respiratory rate was 11/min and O_2 saturation 90% with an oxygen mask. Two days after the admission and Heparin infusion, the patient developed again severe dyspnea, with a hemodynamic instability with BP reduction to 85/55mmHg, tachycardia with HR 142 bmp, cold and wet periphery, reduction of 0_2 at room temperature to 81%. Gas analyses showed respiratory acidosis with pH 7,32, increased pCO_2 53kPa, HCO3 25mEq/L, lactate 2,1 mmol/l. Due to the shock development, the patient was given rescue fibrinolysis with Actilisae 100mg infusion for two hours. The patient hemodynamically stabilized after the first hour of Actilisae infusion, with BP normalization to 110/70mmHg, HR 100 bpm, 0_2 89% on room air. The gas analyses normalized after 4 hours. The patient received heparin infusion for three days after fibrinolysis and continued the management with rivaroxaban with recommended doses of 15 mg 2x1 for 21

days than 20mg 1x1 for further six months due to the episode of unprovoked PE. The patient was discharged after 10 days, clinically stable, without dyspnea and decreased hs-Troponin values to 45 ng/l.Control echocardiography before the discharge showed normalization of the RV function, reduction of tricuspid regurgitation and no signs of pulmonary hypertension.

Follow-up

The patient came to the first control after one month. She was asymptomatic, clinically stable and physically active. She did not report side effects from the rivaroxaban therapy. D-dimer values normalized (325 mg/l). The second control was performed after three months with no echocardiographic signs of the right ventricular dysfunction or pulmonary hypertension. The thrombophilia profile was done two weeks after stopping the therapy with rivaroxaban after six months (factor V Leiden mutation, prothrombin gene mutation, protein C, protein S and AT III activity or deficiency, anti-beta 2 glycoprotein, anticardiolipin antibodies, and serum homocysteine levels). The thrombophilia panel came back positive for high levels of homocysteine (67 μmol/L), with other thrombophilia results within normal limits. Vitamin B12, folate, and vitamin B6 levels were normal.

Figure 1. Admission ECG showed sinus tachycardia with 130 bpm, RBBB and S1, Q3, T3 pattern

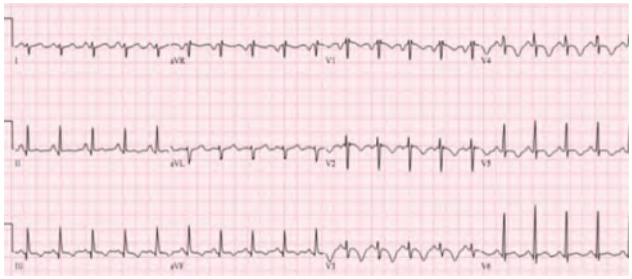
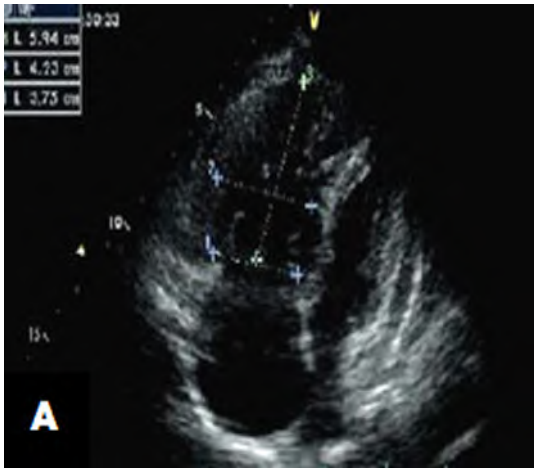
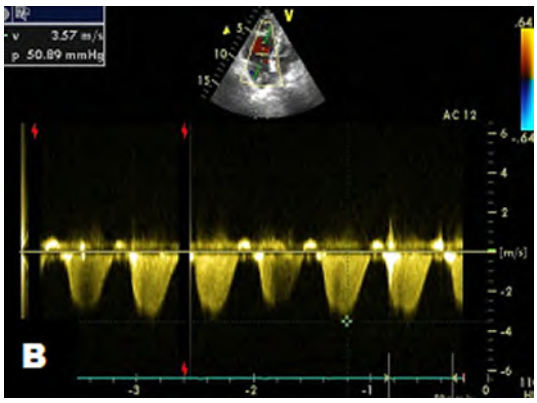


Figure 2.
A. Apical 4 chamber view showing increased right heart cavities with RV:LV ratio >1;



B. Color doppler showing severe tricuspid regurgitation



C. Short axis view showing D shaped left ventricle

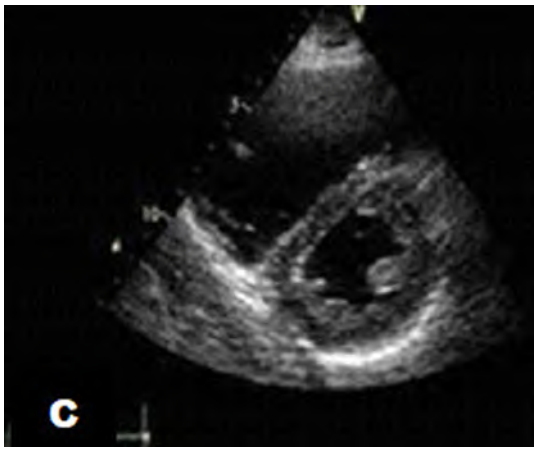
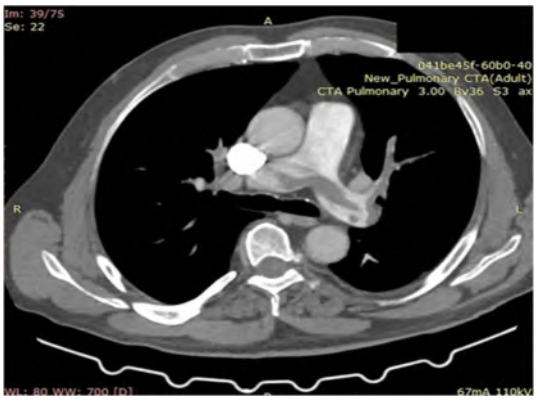


Figure 3. CT angiography of pulmonary artery showing a large partial-lumen occluding filling defect in the left main pulmonary artery, extending further into the hilar branch, occluding the lumen completely. Another larger non-complete lumen occluding filling defect was noted in the right main pulmonary artery.



DISCUSSION

We present the case of a successful treatment of intermediate high-risk PE patient complicated by cardiogenic shock. Although it was the case of massive PE, initially, the underlying etiology was unknown. The final discharge diagnosis of unprovoked VTE was made. The thrombophilia panel testing after discontinuation of the rivaroxaban six-month therapy showed increased blood homocysteine levels. Our patient has an increased body weight, which is a risk for PE. High clinical suspicion is very important for the diagnosis of acute PE in young patients, especially in the absence of history suggestive of DVT or additional provocable risk factors, since this condition is not uncommon and potentially fatal (5). Homocysteine is a sulfhydryl amino acid formed by demethylation of dietary methionin. Homocystinemia is a rare cause of acute pulmonary embolism, which increases a risk of repetitive PE episodes (6). The major acquired factors leading to homocysteinemia are nutritional deficiencies of folate, vitamin B12, and/or vitamin B6, folate antagonist administration (metotrexate, phenytoin), vitamin B6 antagonists (estrogen, theophylline), disturbed renal function, as well as hypothyroidism (6). It is determined by genetic mutations and/or acquired disruption in the homocysteine metabolism pathways. The most frequent genetic causes of homocysteinemia are defects in the gene encoding for enzymes of homocysteine metabolism (the gene encoding for cystatione beta synthase and defects of the gene encoding for methylenetetrahydrofolate reductase e – MTHFR – the most common being MTHFR C677T polymorphism) (7). Homocysteine is known to induce venous thromboembolism (VTE) by multiple mechanisms, including a toxic effect on the clotting cascade, injury to the vascular endothelium and antagonism of the synthesis and function of nitric oxide (8). Any young patient presented with unprovoked PE should have thrombophilia and homocysteine level evaluated,and should have close monitoring for PE, as early recognition of the problem can prevent this serious disease. The normal blood levels of homocysteine range from 5-15 μmol/L (9). Individuals with severe homocysteinemia have the homocysteine concentrations in the range of 50 to500 ftmol/L(10). The classification of homocysteinemia is as follows: 1. moderate risk, 15 to 30 μmol/L; 2. intermediate risk, 30 to 100 μmol/L; 3. severe risk, >100 μmol/L. Our patient had abnormally high homocysteine levels with normal vitamin B12 and folate levels and no abnormalities of other thrombophilia tests. It is necessary to monitor the patient with homocysteinemia carefully due to the high risk of recurrence of thromboembolic events. A case-control study by Falcon et al. found that homocysteinemia was a risk factor for thrombosis in people younger than 40 years (11). Vitamins B6 and B9 or B12 supplements, while they lower the homocysteine level, do not change the risk of heart disease, stroke, or death (12).

Obesity has been consistently reported as a moderate risk factor for venous thrombosis. According to the literature and the MEGA study, the relative risk of venous thrombosis associated with obesity was higher in women than in men (13). The weight gain is associated with an increased risk for cardiovascular disease, diabetes and hypertension, but also with an increased risk for VTE, particularly among obese individuals. The prothrombotic changes in individuals with obesity may contribute to the VTE risk. Several studies have shown that chronic inflammation, assessed by CRP, is associated with obesity. The inflammation stimulates synthesis of factors involved in the coagulation cascade (14,15). The plasma levels of PAI-1, CRP and factor VIII are elevated in obesity, and high levels of these factors have been associated with an increased VTE risk in several studies particularly in women (16).

The patients with PE and cardiogenic shock have the intrahospital mortality rate between 30-60%. Rescue fibrinolysis is a lifesaving treatment in hemodynamically unstable patients (17,18). It also saved the life of our patient, who had favorable clinical and one-year outcome. The latest European Society of Cardiology PE guidelines indicate at least three-month anticoagulation therapy for patients with unprovoked PE. We decided to follow up our patient yearly and more closely due to an increased risk for repeated PE.

Based on the modified Wells score, our patient was assessed at the admission as low PE probability and managed appropriately. However, this case highlights how the Wells score fails to take into account the risk factors that are commonly known to increase the risk of VTE and change prognosis. In this case, obesity and previous use of oral contraceptive pills were the major risk factors, suggesting that obesity when combined with the oral contraceptive use, ends up with a significant increase of PE risk. The ThromboEmbolism Risk (ESTHER) study by Canonico *et al.* also indicated that obesity alone or oral estrogen use by itself each increased the risk of VTE by 4.0- and 5.6-fold, respectively; however, OR approached more than 20-fold when these two risk factors were combined among women who were obese and were taking oral estrogen (19). Obesity and insulin resistance have been reported to increase the risk of VTE in a BMI-dependent manner (20). Rather than the current narrow criteria, a wider range of prothrombotic factors should be considered within the scoring system. Obesity is a known risk factor for VTE, and the data from the Nurses' Health Study found that, among the most obese subjects (body mass index >35), there was a 6-fold increase in the risk when compared to the normal-weight subjects (21). This same study showed that hypertension and cigarette smoking were also associated with an increased risk of idiopathic PE (22,23). Any scoring system that is used to determine the probability of VTE should include more of the relevant risk factors. Also,the Wells score does not take into account the different gradients in severity of VTE risk factor, instead equating all the included risk factors as similarly influential to the overall score.

CONCLUSION

We described a successful treatment of massive pulmonary embolism complicated by cardiogenic shock in young overweight woman with homocystinemia. Pulmonary embolism should be always suspected in younger patients with

acute severe dyspnea even without provocable risk factors. High suspicion level and fast diagnosis are lifesaving. In younger patients presented with unprovoked pulmonary embolism, clinicians should consider inherited prothrombotic factors as a potential cause. Further research is needed to explain the interaction between the genetic markers of thrombophilia, nutritional factors, comorbidities affecting homocysteine metabolism and acquired risk factors for pulmonary embolism. Rescue fibrinolysis is a lifesaving treatment in intermediate risk patients with hemodynamic deterioration.

The patient informed consent form was obtained for the preparation and publication of this case report.

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.

FUNDING

None.

CONFLICT OF INTEREST

None.

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

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

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

RESEARCH AND PUBLICATION ETHICS

Research Involving Human Subjects

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

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

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

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