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RUTHENIUM(II) COMPLEXES AS POTENTIAL APOPTOSIS INDUCERS IN CANCER THERAPY

CASE REPORT

FE ANALYSIS OF THE SYMPTOMATIC NAVICULAR - A CASE REPORT

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ORIGINAL SCIENTIFIC ARTICLE

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IMMUNOLOGICAL ASPECTS OF DEPRESSIVE DISORDER - THE REVIEW

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ABSTRACT

Depression represents a mood disorder and is consideredto be one of the most common mental disorders in general. World Health Organization estimates that depression will be the leading cause of disability-adjusted life years, until 2030. Depression is a complex heterogeneous disorder whereimmune system and its regulation play an important role. Innate and adaptive immunity mechanisms are included, along with processes of immune activation and suppression.The expression of humoral factors of innate immunity, especially pro-inflammatory cytokines, is increased, whereas the intensity of cellular immune mechanisms, primarily T cells and NK cells, are impaired. The influence of pro-inflammatory cytokines on depression is reflected in their effect on certain enzymes and ensuing reduction of neurotransmitters serotonin and dopamine. They also affect the neuroendocrine function in central nervous system, resulting in increase of cortisol levels and inactivation of glucocorticoid receptors inthe periphery, which leads to neurodegeneration and decreasein neurotransmitter production. Certain cytokines affect neuroplasticity through the decreasing of concentration of neurotrophic brain factor and induction of brain cell apoptosis. Theresults are often contradictory talking about mechanisms of adaptive immunity. On one hand, an increased activity of Tlymphocytes is observed, while on the other, there are evidence of spontaneous apoptosis and impaired function of these cellsin depression. In addition, neuroprotective role of autoreactive and regulatory T cells in prevention of depression has also been demonstrated. The aim of this paper is to analyze the current knowledge on the role of immune mechanisms inthe pathogenesis of depression.

Keywords: *Depression, inflammation, immunity, cytokines.*

INTRODUCTION

Depression is one of the most significant health disorders nowadays, and one of the most common psycho-pathological conditions. The experts mostly agree that depression is one of the most painful life experiences that person can deal with (1). Depression is a part of the large group of mood disorders, which is characterized by emotional disorder and by disturbances in judgment, cognition, volition, and instincts, with possible vegetative perturbations (2). Depressive patients experience feelings of unreasonable sadness, helplessness, hopelessness, loss of interest, and satisfaction, along with loss of energy and permanent fatigue (3). According to World Health Organization (WHO) estimations, depression will be the leading cause of disability-adjusted life years (DALYs) until 2030, more than any other health disorder (4).

Etiology and pathogenesis of depression have not been completely clarified yet, however, according to the current knowledge, it has been assumed that depression is etiologically complex and heterogeneous disorder that requires multidisciplinary therapeutic approach. In the last few years there is an increased interest in influence of immune system and its disturbed regulation in the etiology and pathogenesis of depression (5, 6). So called "Cytokine theory" is more and more often mentioned in numerous papers. This theory relies primarily on the role of pro-inflammatory cytokines as important substances in mediation of neurochemical and neuroendocrine singularitiesof depression (6). Along with the infection occurrence, there is an activation of systemic immune response that consequently activates cytokines, or so called "immunetransmitters". They influence the symptoms that are frequently expressed in depressive episode: increased somnolence, irritability and anhedonia. Symptoms like social isolation, decreased libido, fatigue, cognitive disturbances, and anhedonia have been observed in patients with autoimmune or malignant diseases who are treated with proinflammatory cytokines, like alpha interferon (INF-α) (7). The activation of humoral factors of innate immunity, in other words the activation of pro-inflammatory cytokines, plays an important role in etiology and pathogenesis of depression, modifying neurotransmitters, neural plasticity, aswell as neuroendocrine performances of central nervous system (CNS) (8).

It is necessary to emphasize that microorganisms that inhabit the gastrointestinal tract interact with the host immune system and can affect psychological processes and influence neurodevelopment. Some evidence has pointed to the involvement of gut bacteria (microbiome) in the induction of depressive illnesses. Microbiome might have immunoregulatory properties in some animal models, supplementation with probiotic bacteria reduced intestinal markers of inflammation and attenuated sickness behaviours that were engendered circulating cytokine levels and microglial activation. Antibiotic-treated and germfree mice exhibit several depressive-like and anxiety-like phenotypes, as well as changes of neurotransmitters andof their receptors, neuroendocrine factors, and neurotrophins (9). There are reports showing that probiotics attenuated anxiety-like and depressive like behaviours and restricted plasma corticosterone elevations as well as the hippocampal monoamine reductions elicited by a chronic restraint stressor in rats (10). Also the observation that probiotics reduced plasma cytokine levels in inflammatoryconditions, suggests that the positive effects of these microbiota-targeting compounds on mood states could be linked to their actions on immune processes. Psychological, chemical and immunological stressors serve to form the inflammatory milieu within the gut and could negatively influence brain–immune communication, includingcytokine, hormonal, neurotransmitter and other growth factor processes, and hence favour the evolution of depressive symptomatology (11).

It is considered that etiology and pathogenesis of depression include immune activation and immune suppression processes, including innate and adaptive immunity, and all these facts may have great impact on treatment. On onehand, it has been shown that depression leads to enhancedexpression of molecules associated with inflammation, likepro-inflammatory cytokines, proteins of acute phase, and adhesive molecules. On the other hand, the decrease of thefunction of cellular immunity, primarily T-cells and naturalkiller cells (NK), influences the development of depression,its course and therapy response (12). "Immunopsychiatry"(13, 14) may be considered as field in development, and hasthe possibility to enlarge and fulfill current knowledge on mechanisms of certain psychiatric diseases development, alongside with depression. Also, it has a potential to explainminutely the specificity of pathophysiological mechanisms,which can result with most efficient therapeutic strategy on individual level. According to Blume et al, key immune-modulatory mechanisms should be emphasized, due to thefact that this may discover potential immunological biomarkers of depressed subjects that could contribute to moreefficient treatment (15).

HUMORAL FACTORS OF INNATE IMMUNITY IN DEPRESSIVE DISORDER

There is a great amount of knowledge that talks in favorof association of innate immunity mechanisms and functionality of CNS, in terms of certain psychiatric diseases development, including depressive disorder. Diverse interleukins (IL), tumor necrosis factor (TNF), and certain growth factors are included in the pathogenesis of depressive disorder (16). The knowledge on influence of inflammatory process on the development of depressive symptoms is so far described through three basic postulates: 1. Inflammation and somatic diseases, that are followed by the activation of immune system, increase risk of depression development; 2. The increased level of pro-inflammatory cytokines is verified in people suffering of depression;

Pro-inflammatory cytokines induce the development of depressive symptoms, which can be treated with antidepressants (17). Also, in some researches, it has been estimated that patients hospitalized due to autoimmune disorder or an infection are in 2.35 times greater risk of mooddisorder development (18).

Cytokines are relatively large hydrophilic molecules that cannot pass blood-brain barrier freely. Nevertheless, some studies have shown that cytokine signals may arrive to the brain through humoral, neural and cellular pathways, while certain cytokines are synthetized in CNS alone. These findings suggest that brain may not be considered as immunologically "privileged" organ (19). There are at least five mechanisms by which cytokines arrive andaccomplish their role in CNS: firstly, by passing through permeable regions of blood-brain barrier within choroid plexus and circumventricular organs; secondly, by active transport of cytokines across the blood vessel endotheliumof brain (7); the next one is by activation of endothelial cellsresponsible for transport of secondary messengers of cytokines (prostaglandins and nitrogen oxides) in brain parenchyma, passing cytokine signals across afferent nerves like vague nerve and through the entrance of peripherally activated monocytes in brain tissue (20).

When compared to people without depression, patients with clinical form of depressive disorder show signs of significant inflammation. These signs include the increase of cytokine concentration and their soluble receptors in peripheral blood and cerebrospinal liquor, as well as the increase of proteins of acute phase, chemokines, adhesive molecules, and inflammatory mediators in peripheral blood(16). An association between markers of inflammation anddepressive symptoms like fatigue, cognitive dysfunction, orsleep disorders has also been noticed (16). Sleep disturbancein patients with depression and sleep deprivation are associated with increase of IL-6 concentration, and with nuclearfactor kappa B activation (NF-kB), which represents primary transcription factor responsible for the initiation of inflammatory response (16). Studies on animals have shownthat inflammatory cytokines induce "sickness behavior" that overlaps with depression in numerous symptoms, includinganhedonia, anorexia, and locomotor activity decreasing (7). Beside aforementioned IL-6, cytokines that are playing leading role in the development of depressive disorder are IL-1,TNF, INF-γ, INF-α (6)

It has been determined that cognitive impairment is observed in approximately two-third of patients with Major depressive disorder (MDD) and persists after phasesof acute relapse in the majority of patients (21). Most serious cognitive deficits in patients with MDD include impairments in attention, memory, and executive function. Cytokines like IL-1, IL-6 and TNF might impact cognitionin various mechanisms. It has been shown that these cytokines influence memory consolidation and process of learning by affecting long-term potentiation, synaptic plasticity, and neurogenesis. Future studies should includeobjective measures of cognitive performance and at the same time the analysis of changes in indices of biologicalmechanisms of action such as inflammatory markers (22). It is important to mention that the specific polymorphism of the promoter region of IL-1beta (IL-1β) gene hasbeen associated with MDD symptoms severity in patientswith a history of childhood trauma (CT) (23). Also thismay possibly suggest a link between IL-1β gene polymorphism and the risk of MDD onset after CT exposure. InIL-1β gene studies, the negative results may be explainedby heterogeneity in the MDD participants and the consequent inability to identify a subgroup of patients withpatients characterized by inflammatory markers. The association of IL-1β with MDD may be strongest in participants with a history of CT according to qualitative results. Further studies are still needed to determine the biologicaland clinical profile of patients with MDD who may benefitfrom such greater refinement of MDD subgroups, whichcan result in designing of more personalized therapy.MDD patients with significantly increased levels of bloodIL1β or with resistant depression or those who have beenexposed to CT may be subgroups for whom IL-1 β targeted therapies may be of particular utility. (24).

On the other hand, it has been shown that in patients with major depression compared to healthy individuals, the levels of IL-10 which is an anti-inflammatory cytokine, are decreased in peripheral blood, along with the increase of CRP (25).

Antidepressant activity of anti-inflammatory therapy has also been observed in patients with autoimmune and inflammatory disorders. For example, in a large double blind, placebo-controlled trial of the TNF-alpha antagonist,etanercept, for the treatment of psoriasis, participants who received etanercept exhibited significant improvement in depressive symptoms compared with placebo treated subjects, an effect that was independent of improvement in disease activity (26).

According to meta-analysis of Goldsmith DR et al., it is important to mention that there are similarities in the pattern of cytokine alterations in MDD, bipolar disorder and schizophrenia during acute and chronic phases of illness, indicating the possibility of common underlying pathways forimmune dysfunction (27). The levels of cytokines (IL-6 andTNF), cytokine receptor antagonist (IL-1RA), and one soluble cytokine receptor (sIL-2R), were significantly elevated inall three syndromes during acute illness episodes. It is interesting to notice that all of the cytokines that were elevatedare modulated through the nuclear factor-κB, signalling pathway that is activated in inflammatory and autoimmunedisease. Acute stress increases IL-6 and other inflammatorymarkers, it can be assumed that there may be a common stress-related phenomenon occurring in acutely ill patients across these disorders (28). The treatment of acute illness may lead to resolution of inflammation and an increase in anti-inflammatory biomarkers. The common findings in chronically ill patients also might reflect the influence of chronic stress on the immune system. Chronic stress may lead to elevations in both IL-6 and sIL-2 R in chronically ill patients and persistent immune activation for some patientswith these disorders. It would be interesting to compare andcontrast inflammatory markers in the same subjects duringboth acute and chronic phases of illness. In general, treatment of these disorders led to a decrease in cytokines withproinflammatory functions and an increase in cytokines with anti-inflammatory functions (27).

THE INFLUENCE OF CYTOKINES ON NEUROTRANSMITTERS

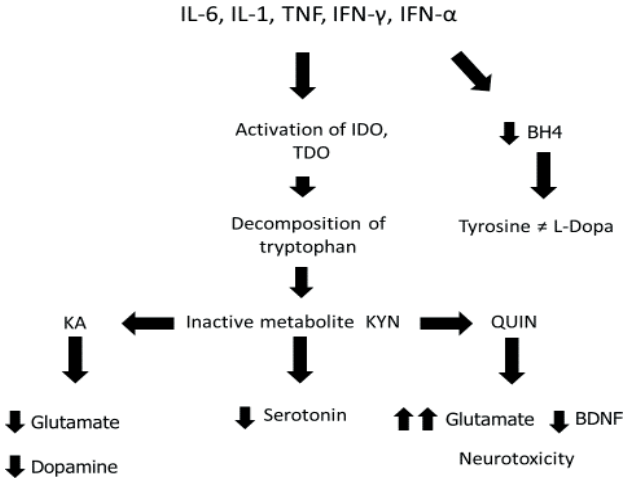
Numerous studies have shown that acute and chronic exposition to pro-inflammatory cytokines, or their synthesis inductors, like lipopolysaccharide (LPS), can changemetabolism of serotonin. This can lead to developmentof mood disorders, because this neurotransmitter is crucial for mood maintaining (29). The key event associated with the disorder of serotonin genesis and reuptake from the synaptic crack is the stimulation of indolamine-2,3deoxigenase enzyme (IDO), tryptophan-2,3-deoxigenaze(TDO), and signal molecule p38 mitogen-activated proteinkinase (MAPK) by pro-inflammatory cytokines. When activated, IDO and TDO decompose tryptophan (TRP), primary amino acid precursor of serotonin, to tryptophan catabolite kynurenine (KYN). Thus, there is a decreased production and concentration of serotonin, and his role inneurophysiological processes in CNS is compromised accordingly. KYN is transformed in kynurenine acid (KA), 3-hydrokynerenine (3-HK) and quinolone acid (QUIN) bycertain metabolic enzymes of microglia and astrocytes (8).The influence of cytokines on neurotransmitters metabolism is shown in Figure 1.

QUIN leads to increased releasing of glutamate, by activation of N-methyl-D-aspartate (NMDA) receptors leading to oxidative stress occurrence, and in combination withglutamate, it may have toxic influence on CNS (30). Whenactivated, cytokines (IL-1) in brain enhance glutamate releasing and prevent its reuptake through down regulation of glutamate transporters. Increased releasing of glutamate participates in decreased production of brain-derived neurotrophic factor (BDNF) (31).

Animal model of stress-induced depression that was presented with behavioral and biochemical alteration of already mentioned KYN pathway, has shown the antidepressant effect of IDO inhibitor (1-methyl-D-tryptophane)that is very similar to antidepressant Fluoxetine. It has been observed that 1-methyl-D-tryptophane and Fluoxetine decrease the level of pro-inflammatory cytokines in this animal model, so antidepressant effect is achieved through anti-inflammatory effect (32).

Tryptophan catabolites may develop from glucocorticoid-activated TDO, since the concentrations of glucocorticoids are higher in depression, as well as from IFNα-based immunotherapy, and that can consequently lead to depressive symptoms. Unbalanced level of tryptophan catabolites with their diverse role in neurotransmitter metabolism and in neurotoxicity, can affect the development of immunologically precipitated depressive disorder (33).

Figure 1. Cytokine influence on neurotransmitters



It has been determined that, beside serotonin, the synthesis and reuptake of dopamine are also disturbed under the influence of pro-inflammatory cytokines (34). According to observations, INF-α influences decreasing of tetrahydrobiopterin (BH4) concentrations. BH4 is an importantcofactor enzyme for tyrosine hydroxylases which convertstyrosine to L-DOPA. Just like with serotonin, p38 MAPK increases reuptake of dopamine from synaptic crack and disturbs the function of synapsis, mediated by dopamine (35). Catecholamine (noradrenaline and adrenalin) significantly interact with immune system. In vitro and in vivo researches showed that noradrenaline increased the production of TNF-α, as well as IL-6. Furthermore, acetylcholine inhibits the release of TNF, IL-1 and IL-6, while enhances the influence of anti-inflammatory cytokines, like IL-10. Since prolonged stressful reactions activate sympathetic nervous system (noradrenaline), while inhibit parasympathetic nervous system (acetylcholine), there is an increasedreleasing of pro-inflammatory cytokines which contributeto the development of depressive disorder (36).

CYTOKINE INFLUENCE ON NEURAL PLASTICITY (NEUROGENESIS)

Neural plasticity is a concept referring to different mechanisms which are crucial for brain functionality, and enables brain maintenance, adaptation and responses to the external stimuli and the most important factor of its maintenance are neurotrophic factors, first of all BDNF, whose role is exactly neurogenesis. Neural plasticity is considered to be disturbed in depression (65). Some studies have shown that in patients suffering from depression, theexpression of BDNF in hippocampus and prefrontal brain cortex is decreased. Recent researches go in favor that BDNF precursor, so called pro-BDNF, is hyper-produced in depression, while mature form of BDNF is reduced, as it is already mentioned. What has also been demonstrated is that pro-BDNF has opponent functionality, compared to BDNF, stimulating the neuron apoptosis by binding to tyrosine kinase receptor (TrkB) (38). Contributing factors of decreased expression of neurotrophic

factors in depression are certainly inflammation and pro-inflammatory cytokines, like TNF, Il-1 and IL-6. It has been shown that administration of LPS and IL-1 brings to decreased expression of genes coding for BDNF in the region of hippocampus and other cortical zones of rats, which further leads todecreased BDNF expression and decreased neurogenesis in this region. TNF and IL-6 has notable anti-proliferative influence on stem cells, through TNF receptor 1 (TNF-R) and IL-6 receptor, respectively. Over time, the decreasing of neurogenesis could contribute to the reduction of hippocampal volume, which is observed in depression (39). In physiological conditions, cytokines enable trophic support to neurons and enhance brain integrity. However, in cases of chronic distress and excessive production of proinflammatory cytokines, IL-1 in the first place, they lead todepressive symptoms through disturbances of neural plasticity and neural genesis of specific CNS regions, whichare important for etiology and pathogenesis of depression (31). The important fact that should be emphasized is thatmicroglial cells may have neuro-protective role when it is about brain inflammation with potential ensuing depressive disorder. According to certain studies, chronic activation of microglia as a result can give neuronal apoptosis, the inhibition of neurogenesis, the reduction of hippocampal volume, the decreased synthesis of neurotransmitters and increased cytotoxicity (40). In recent researches, microglia have been identified as potential target for pharmaceutic treatment. Namely, Biber et al suggested that some psychiatric conditions, including depressive disorder, can be treated with drugs which inhibit or restore specific microglial functions (41).

THE INFLUENCE OF CYTOKINE ON NEUROENDOCRINE FUNCTION

Beside the impact on neurotransmitter metabolism, cytokines have significant activating effect on hypothalamic-pituitary-adrenal (HPA) axis, primarily on the secretion ofcorticotrophin-releasing hormone (CRH) in hypothalamus and amygdales. CRH has pro-inflammatory effects, accelerating mastocyte degranulation, and stimulating increased production of TNF, IL-1 and IL-6 by macrophages (42). These inflammatory effects are inhibited by CHR1 receptor antagonists, that is, by new generation of anti-inflammatory medications, which can have potential effect in withdrawing of depressive symptoms. Numerous conditions, like autoimmune, or inflammatory diseases, aswell as certain psychiatric disorders, like depression, are followed by decreased response to glucocorticoids. That is by glucocorticoid resistance, which is believed to be the consequence of damaged function of glucocorticoid receptors (GR) (43). The stimulation of numerous inflammatorysignal molecules, like NF-kB, p38 MAPK, as well as cytokines (IFN-α, IL-1 and TNF) decreases the functionality ofGRs, by preventing their transfer from cytosol to nucleus, and their binding for DNA molecule (43). That results withhigher level of cortisol in peripheral blood and disturbances of negative feedback, which should maintain the homeostasis of HPA axis. Also, decreased expression of GR-αis noticed, which represents active form of glucocorticoid receptor, while the expression of GR-β, which is relatively inert form, is increased leading to a decreased response to glucocorticoids (43). It has been shown that antidepressantDesipramine stimulates GR translocation from cytoplasm into nucleus and promote the transcription of genes, thus returns the negative feedback into normal function that is important for subtle balance of hormonal secretion of HPA axis (44). It is significant to mention that glucocorticoids modulate the function of immunological mechanisms, affecting cytokines, the expression of adhesive molecules, transport of immune cells, as well as their proliferation and differentiation, so in that manner, they may have the role inrearranging immune process from pro to anti-inflammatory humoral response (45).

THE ADMINISTRATION OF CYTOKINES AND CERTAIN DEPRESSIVE SYMPTOMS

There are evidence that inflammatory cytokines may cause the changes in behavior, found in numerous reports of neuropsychiatric symptoms. They are induced by chronic administration of cytokines, first of all antiviral and anti-proliferative cytokines, like IFN-α, which is used in treatment of malignancy and viral infections. According to certain studies, it was found that about 30-40% of patients suffering from hepatitis C, who had been treated with IFN-α, had developed depressive disorder (46). IFN-α induces depression in 30-50% of patients, depending on administrated dose which is demonstrated in cancer patients with cytokine-induced depression (47). Depression induced by INF-α is very similar to endogenous major depression, and is used very often as paradigm for pathophysiological researches and cytokine-induced behaviorchanges (48). The levels of IL-6 and TNF, which increase after the administration of IFN-α, positively correlate withthe severity of depressive symptoms, while polymorphismsof 5-hydroxitriptamine (5-HT) transporters contributes tothe occurrence of fatigue and depressive symptoms after the applying of IFN-α (49).

REPRESENTATION OF CELLULAR FACTORS OF INNATE IMMUNITY IN DEPRESSIVE DISORDER

In a relation to cellular components of innate immunity, it is determined that the absolute number of NK cellsand their cytotoxicity are decreased in patients with depression. Decreased activity of NK cells is one of the most striking immunological changes in depression (50). In patients with depression, average age of 40 years, the duration of disease (the time passed from the first depressive episode) is a predictive factor of NK cell function. That is, the longer the duration of the disease is, the greater the chances for decreased cytotoxicity of NK cells are. As the main function of NK cells is the defense from intracellularmicroorganisms, and from certain tumor cells, depressioncould be the risk for intracellular infection and malignant disease (51). It has been shown that higher levels of cortisoldecrease the activity of NK cells, by

decreasing NKp46 density and NKp30 receptors on the surface of NK cells. Normalization of cortisol levels after the anti-depressive treatment enhances the function of NK cells. It is determined that sympathetic nervous system and neurotransmitters produced after the activation of sympathetic nervous system, primarily catecholamines, lead to the reductionof NK cell number. Namely, depressive patients during distress and physical effort show higher concentration of catecholamines and neuropeptide Y in peripheral blood, which results with the increase of cellular components of innate immunity (52). Nerve endings of sympathetic nervous system are near immune cells in those organs where their maturation occurs, so they react to certain pathogens(53). After the releasing, catecholamines through certain receptors play an important role in the suppression of NK cells and cellular immunity, in general (53). On the other hand, it is shown that physical activity stimulates good functionality of NK cells, decreasing the influence that psychomotor slowing and loss of pragmatism factors have on lowering of number and function of these cells (54). One ofthe most dominant symptoms in depression is insomnia.It also inhibits the function of NK cells by increasing the release of catecholamines which reduce NK cell number. This is the case during the transit insomnia, which is occasional wakening, or during initial insomnia, that is, impossibility to fall asleep. It is important to emphasize that the suppression of innate cellular immunity, as well as adaptive, was observed in certain patients with depression, andcorrelated with hyper-functionality of pro-inflammatory cytokines, such as IL-6. This leads to difficulties in defenseagainst intracellular pathogens and malignant disorders, particularly in this vulnerable population (55).

REPRESENTATION OF HUMORAL FACTORS OF ADAPTIVE IMMUNITY IN DEPRESSIVE DISORDER

The role of adaptive immunity is not known enough,in terms of etiology and pathogenesis of depressive disorder. However, it has been shown that chronic inflammationcorrelates with distress and decreased function of humoralimmunity, mostly because of dysfunction of T cells (56). The increasing of IL-6 concentrations, which is pro-inflammatory cytokine, is followed by decreased humoral response in depression, that is by decreased concentration of anti-bodies (57). Still, current scarce knowledge shows thathumoral immunity is not only compromised by depressivedisorder. It is also associated to other risk factors that contribute to the development of depressive disorder, but it's not the crucial factor in the depression development.

REPRESENTATION OF CELLULAR FACTORS OF ADAPTIVE IMMUNITY IN DEPRESSIVE DISORDER

Factors of innate and adaptive immunity influence the maintenance of homeostasis of immune system, by bidirectional mechanisms. Predominant secretion of pro-inflammatory cytokines (IL-12, INF-γ), or anti-inflammatory cytokines (IL-4, IL-10), in combination with higher expression of IL-2, which is the factor of proliferation and differentiation of T lymphocytes, will determine further Tlymphocytes polarization toward Type 1 (T1), Type 2 (T2)or Type 17 (T17) subpopulation (58). According to some indications, T1 response is enhanced in major depression,but the results are not consistent (59, 60).

Acute stress reaction like sleep deprivation which is commonly found in melancholic depression, activates and increases the number of antigen-presenting cells (APCs), as well as the concentration of pro-inflammatory cytokines and CRP. As the consequence, T and B cells numbertemporarily increases, in the aim of preparing the body forthe appropriate reaction. However, when stress reaction islong-lasting, like in chronic depression, these reactions become maladaptive and harmful, leading to the decrease of the number of these cells (61).

Certain researches were conducted with cellular factors of adaptive immunity, that is with T lymphocytes,and their role in pathogenesis and/or neuroprotection in depressive disorder, as well as with changes of cellular immune system in patients with depression. The greatest number of studies are about changes of cellular immunity in depressive patients. It is demonstrated that distress anddepression are influencing the decrease of T cell proliferation through the analysis of response to T cell mitogens: phytohemoglutinine (PHA) and concavalin A (Con A) (62). According to evidence, the increased concentration of IDO, glutamate and tryptophan catabolites (for example, KYN) modulates innate and adaptive immunity, in terms of blockade of T and NK cells proliferation and induction of apoptosis. Also, cytofluorimetry has shown that$CD4^+$ T lymphocytes of depressive patients are prone to accelerated spontaneous apoptosis, in terms of increased expression of Fas receptor (CD 95), which participate in the apoptotic process, together with Fas-ligand (63). Thereare some facts that inflammation, or the activity of proinflammatory cytokines, leads to down regulation of Zeta chain of T cell receptor. This is important in the transfer of T and NK cell signals that further leads to immunosuppression in context of cellular factors of adaptive immunity (64). One of the factors that is also considered when it is about reduction of the number of T cells in depression, is certainly the role of glucocorticoids. Glucocorticoids have multiple role in immunosuppression, through theparticipation in redistribution of inflammation cells, then through inhibition of inflammation and induction of apoptosis of numerous immune cells, including T lymphocytes,influencing their maturation in thymus. Due to this fact, increased concentration of glucocorticoids in peripheral blood, mainly cortisol, is important factor in the development of depressive disorder and immunosuppression by decreasing the number of T lymphocytes. It should be emphasized that certain genes play a role in T cell function and its association with depressive disorder and response to treatment. For example, SNP in genes like PSMB4 (proteasome beta4 subunit, important for antigen processing) and TBX21 (T-bet, transcription factor important for T cell differentiation, that is, for Th1

subpopulation development) increase the risk of depression (65). It could be concluded that, beside the fact that depressive disorder leads to immunosuppression in terms of decreasing T cell number, dysfunctionality of T cells caused by genetic polymorphisms may have the impact on the pathogenesis of depression. The development of anti-depressive treatment which aim is to inhibit Th 17 cell functionality is veryimportant for updating of therapeutic protocols of depressive disorder (66). The consequences of T cell function in depressive disorders are numerous, especially in terms of somatic comorbidities. Namely, it has been shown that depression have worse outcome in several diseases and conditions, due to certain level of immunosuppression by which is followed. Acquired immunodeficiency syndrome (AIDS) should also be mentioned, then different malignantdiseases, and complications related to organ transplantation (67). Reduction of regulatory T lymphocytes (for example, $CD4^+CD25^+Foxp3$ T cells) may have the influence on the development of depressive symptoms. Their immunosuppressive and anti-inflammatory features are important for inhibition of too strong immune response and pro-inflammation that is already described as significant inthe development of depressive disorders. In other words, regulatory T lymphocytes enhance the production of antiinflammatory cytokines, IL-10 first of all, and transforming growth factor-β (TGF-β). Decreased number of regulatory T lymphocytes which is found in some studies (60) may indicate the disbalance that exists between positive and negative regulatory mechanisms after chronic distress.These reactions are potentially associated with the higher susceptibility to the deterioration of depressive symptoms,especially among vulnerable population (55).

There are some studies that investigate the increased functionality of T lymphocytes in depressive disorder that is linked to higher concentration of pro-inflammatory cytokines, and to macrophages activity. According to them, activated T lymphocytes produce IFN-γ and IL-2, whereby IFN-γ activates macrophages and induces their functionality in terms of pro-inflammatory cytokines production (68). That is followed by neopterin increasing, which is a biomarker of cellular immunity activation, while IL-2 stimulates further proliferation of T lymphocytes through IL-2receptor (IL-2R). Neopterin is a molecule whose increased concentration in bodily fluids goes in favor of more expressed activity of inflammatory cells, concretely macrophages (68).

Although the greatest focus is oriented toward negative impact of depression on cellular immunity, it has beenshown that autoreactive T lymphocytes specific for CNS antigens may have important role in neuroprotection. For example, animal models showed that immunization bymodified myelin basic protein (MBP) brings the inductionof weaker autoreactive T cells leading to the decreasing of anhedonia development. Also, autoreactive T lymphocytesproduce neurotrophic growth factor (BDNF) (69).

CONCLUSION

Development of depression includes mechanisms of innate and adaptive immunity, whereby the processes of immune activation and immunosuppression are involved. That can influence the course and outcome of disease, as well as the therapy response. Inflammation is considered to be the key mechanism of innate immunity that is involved in depression development, similarly like in other diseases. Cytokines play an important role in the development of depressive disorder, through their influence on neurotransmitters, neuroendocrine function of CNS, and neural plasticity. Especially pro-inflammatory cytokineshave the influence, alongside with some other factors. Innate immunity cells show certain disorders in depression, which is particularly expressed in NK cells. Their number and cytotoxic function considerably decrease, probablyunder the influence of glucocorticoids and catecholamines.Lately, there are more researches that indicate the important role of adaptive immunity in etiology, pathogenesis, course, and prognosis of depression, depending on activation or suppression of mechanisms of adaptive immunity, especially T lymphocytes. Humoral adaptive immunity is compromised in depressive disorder, but most probably isnot one of the factors that have crucial role in its development. T lymphocytes may have neuroprotective and antiinflammatory impact on depressive disorder, while distressand depression affect T cells by decreasing their proliferation and activating their apoptosis. Also, there are diverse results about the increased concentration of certain molecules that go in favor of higher activity and proliferation of T lymphocytes in patients suffering of depression. Current knowledge on cellular factors of adaptive immunity indepression is contradictory, which leaves space for furthermore comprehensive investigations on this topic.

Multidisciplinary approach is of vital importance for detailed clarifying of complex and insufficiently explored role of immune mechanisms and immune biomarkers in etiology and pathogenesis, as well as in the outcome of depressive disorder. Depression is not a condition thatshould be observed only through psychiatric prism, but in the context of more complex picture and overlapping with other somatic disorders. We should keep in mind that immunological mechanisms represent the basis of these two seemingly separated disease groups. Understanding and clarifying all dilemmas and deploy precision of all described (and potentially new) immunological mechanisms that affect the development of depression are imperativein further researches. The aim of researches should be to better define potential therapeutic protocols with role in eliminating depressive symptoms, and in stabilization and regulation of immunological mechanisms that are in all likelihood inseparable part of etiology and pathogenesis ofdepressive disorder. Further studies should define methodswhich would single out the components of immune systemin different neuroanatomic regions in aim to complete thepalette of biomarkers which would enable personalized approach to each single patient.

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THE INFLUENCE OF SOCIAL SUPPORT ON DEPRESSION AMONG ELDERLY PEOPLE IN SERBIA

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ABSTRACT

Social factors such as social support, integration, and belonging to the community are positive resources against adverse events and living conditions. This study at examining the influence of social support on depression among elderly people in Serbia. The research was done according to the type of cross-sectional study, a mass survey of a random, representative stratified two-stage sample of the population of Serbia in 2013, which was conducted by the Ministry of Health of the Republic of Serbia. The target population consisted of 3540 respondents aged 65 and over. The Patient Health Questionnaire-8 (PHQ-8) was used to evaluate the presence of depressive symptoms and the social support score from the questionnaire Oslo-3 scale of social support. The relations between depression symptoms and social support were examined with univariate logistic regression analyses. In the univariate regression model, social support stood out as a strong predictor of depression. People with weak social support are three and a half times more likely (OR = 3.45) to have depression compared to those with strong social support. Men with small social support were more likely to have depression (OR = 5.08) than women (OR = 3.41). These results indicate the urgency of addressing depression as a public health priority to reduce the burden and disability and improve the overall health of the elderly population.

Keywords: *Social support, depression, elderly people, Serbia.*

INTRODUCTION

Geriatric depression is a health problem that is often not detected on time and requires special medical attention. Geriatric depression is generally insufficiently recognized and insufficiently treated because it is often considered a natural part of aging. Many studies have shown that about half of cases of depression in the elderly usually remain undiagnosed (39,40). Symptoms such as increased fatigue, loss of appetite, sleep deprivation, and loss of interest in everyday social activities often are not recognized as symptoms of depression. Also, older people are less likely to want to acknowledge feelings of sadness, different somatic and cognitive changes such as irritability and memory loss, disorientation, and depression. Therefore, these symptoms usually remain unrecognized and undiagnosed because they consider a normal part of the aging process. If they occur almost daily with the duration of depression for more than two weeks, they are not a normal part of the aging but symptoms of a major depressive disorder (1).

Social factors such as social support, integration, and belonging to the community are positive resources against adverse events and living conditions. They increase the ability to adapt to change and manage life. Social network and support have an independent and strong impact on health and quality of life at all stages of the life cycle, especially in the elderly. Researches have shown that the quality of life of the elderly is directly related to the degree of social interaction, the level of social engagement, and social networks. People who have a lower level of social activities and less frequent contacts have a higher level of cognitive impairment. They are more likely to feel lonely and have a higher chance of developing depression (2, 3).

Social support can affect health and well-being through modifying individual behavioral factors and adopting healthy lifestyles. It also enables better emotional adaptation to stressful and negative life events. Social support helps people to overcome stress more easily and it is associated with better mental health outcomes (4). Therefore, this study aimed at examining the influence of social support on depression among elderly people in Serbia

METHODS

The research was done according to the type of cross-sectional study, a mass survey of a random, representative stratified two-stage sample of the population of Serbia in 2013, which was conducted by the Ministry of Health of the Republic of Serbia. The target population consisted of 3540 respondents aged 65 and over. The research process itself was done following the protocol and instruments of the European Health Research (second wave). As a research instrument, it was used Standardized questionnaires consist of defined indicators/variables based on the recommendations of the World Health Organization and the European Health Research. All information obtained in the research is confidential and confidentiality is guaranteed. Respondents were provided promptly with all information about their rights as well as information related to the research, after which they signed informed consent to participate in the research.

The PHQ-8 Patient Health Questionnaire (5) was used to assess the presence of depression, which consists of eight items that address the following mental problems and ailments: decreased satisfaction or interest in doing everyday tasks or events around you; feeling of emptiness, discouragement, displeasure, depression, hopelessness; sleep problems (difficulty with falling asleep, waking up during sleep or excessive sleep); feeling that you do not have enough energy, fatigue, tiredness; decreased or increased appetite; negative opinion of themself, feelings of failure and worthlessness, self or family disappointment; difficulty with concentrating on certain activities, inability to concentrate on reading newspapers or watching TV (reduced ability to think, concentrate or indecisive - assessed subjectively or by others); slowness of physical activities or speech so that other people may notice it, or in contrast psychomotor restlessness and anxiety or a feeling that they are active more than usual (noticed by others, not just a subjective feeling).

Respondents answered how often in the last two weeks they had any of the above possible mental problems by choosing one of the four answers offered: "no problems", "a few days a week", "more than seven days" and "almost every days during the week. " The answers to each question were rated 0-3: 0 ("no problem"), 1 ("a few days a week"), 2 ("more than seven days") and 3 ("almost every day"). , and after adding up the scores for each of the eight questions, a total PHQ-8 score was obtained ranging from 0 to 24. A PHQ-8 score of zero to four indicates no symptoms of depression, a PHQ-8 score of five to nine indicates mild symptoms of depression, and a total PHQ-8 score of ten or more indicates a high probability of depression, which is further classified as moderate (PHQ-8 score of 10 to 14), moderately severe (PHQ-8 score of 15 to 19) and a severe depressive episode (PHQ-8 score 20 and above). Based on the PHQ-8 score, subjects will be classified into one of 3 categories: no depressive symptoms, mild depressive symptoms (subsyndromal depression), and depressive episode (depression). In the analysis of the results, a depressive episode meant a value of almost 10 or more.

The social support score is formed by assigning and collecting points for each answer to three possible questions from the questionnaire "Oslo-3 scale of social support": "How many people are so close to you that you can count on them when you have serious personal problems?" ranges from 1 ("None") to 4 ("6 or more"), "How many people are interested in you, in what you do, what happens in your life?" not interested ") to 5 (" Very interested "))," How easy is it to get practical help from a neighbor/neighbor if you need it? "(the number of points ranges from 1 (" Very difficult ") to 5 (" Very easy ")). By adding points to these three questions, a score of social support was established: strong social support (12-14 points), moderate (9-11 points) and poor (3-8 points) (6).

Statistical data processing was performed in the software package SPSS, version 18.0. The results of the research are presented in tables and graphs. Category variables are presented as frequencies and the Chi-square ($\chi 2$) test was used to compare differences in the frequency of these variables. A univariate and multivariate regression model was used to determine the relationship between depression and potential independent risk factors, and the probability of a relationship was expressed as the odds ratio. A probability of less than 5% was considered statistically significant.

RESULTS

PHQ-8 values recently showed that every tenth inhabitant of Serbia aged 65 and over (a total of 10%) has a depressive episode, while 17.5% of them had mild depressive symptoms (subsyndromal depression). Observed concerning the categories of depression, the largest number of the elderly population had moderate depression (5.8%), followed by moderately severe depression (2.6%), while the smallest share of respondents had a severe depressive episode (1.6%). The mean value of the PHQ-8 score in the population aged 65 and over was 3.5 (Chart 1).

In subjects with a PHQ-8 value ≥of 10 (depressive episode), the most common symptoms of depression present almost every day for the last two weeks of the study were lack of energy or a feeling of fatigue (61.8%), slow movement, or restlessness. (46.4%) and sleep problems (44.1%) (Table 1).

When it comes to social support, among the elderly who had poor social support, every fourth respondent had a depressive episode (27.3%), in contrast to only every twelfth respondent with strong social support (8.7%). Mild symptoms of depression are present in 27.3% of respondents with poor and 20.4% of respondents with moderate social support. This difference is highly statistically significant (Chart 2).

When we talk about social support, people who do not have any close person in their environment who can count on, when they have serious personal problems, the prevalence of a depressive episode is present in as many as 25.3% of respondents, or the prevalence of mild symptoms in 21.5% of respondents. When asked how much people are interested in you, depression is present in 36.1% of those who stated that there are no such people in their environment as opposed to 8.0% of those who reported a depressive episode and stated that people are very interested in them. Depression is also present in a significantly higher percentage of those respondents who stated that it is very difficult for them to get practical help from neighbors when they need it (23.5%) than those who do not have a problem with it(7.9%), (Table 2).

In the univariate regression model, social support stood out as a strong predictor of depression. People with weak social support are three and a half times more likely (OR = 3.45) to have depression compared to those with strong social support. Men with small social support were more likely to have depression (OR = 5.08) than women (OR = 3.41), (Table 3).

DISCUSSION

According to the stress relief model, a well-developed social network and positive social support can reduce the negative effects of stressful life events on the occurrence and presence of depressive symptoms in the elderly. On the other hand, the deficit in social support due to retirement, death of a spouse and friend, mental and physical limitations, can be a risk factor for the occurrence or worsening of depression in late life as well as reduced life satisfaction (7). Previous research has shown that older people with poor emotional support are more likely to be depressed, with men showing higher rates of depression than women (8).

Older people who are living with spouses and children have the lowest rates of depression (54). Similar studies have shown that frequent visits to the elderly reduce the risk of depression. The chances of depression in the elderly who are meeting with friends and relatives are reduced by less than 60% compared with the elderly who do not (9). Loss of family support and moving to a new environment affects the health of the elderly. People living in geriatric homes were more likely to suffer from depression, which could be caused by inadequate social adaptation and living in a geriatric home, which is a stressor (1). The greatest feeling of loneliness is observed among the elderly living in rural areas, in single-member households, or among elderly people who are not active in any association or organization (10).

Stressful life events and old age are significantly associated with depression, as confirmed by the results of many studies. The loss of a spouse, close relative, or friend constantly reminds the elderly of the inevitable end. This will have a particularly disturbing effect on the elderly person, especially if the deceased is a younger member of the family because then the elderly person has lost someone who could take care of him/her in old age (11).

Many studies show that social support has a strong influence on the appearance of depressive symptoms in the elderly. Retirement, in turn, has the effect of reducing income and reducing psychosocial support, which significantly affects the rate of depression in the elderly population (12). Older people who live in a family environment, who visit with friends and relatives, as well as those who nicely spend their free time, have a lower risk of depression. The poor social network, loss of a close relative, or co-worker can lead to depression in the elderly, and on the other hand, depression can contribute to a decrease in the quality of social activities (13, 14).

The results of our research suggest that the prevalence of depression is significantly higher among people who do not have adequate social support. Among the elderly who had poor social support, every fourth respondent had a depressive episode (27.3%), in contrast to only every twelfth respondent with strong social support (8.7%). Encouraging individuals to initiate, maintain, and expand social engagement in later life could help reduce depressive symptoms (15).

Depression in the elderly worsens the results of many medical diseases and increases mortality. Age-related processes, including arteriosclerosis, inflammatory and degenerative diseases, can increase vulnerability to depression. Environmental factors, such as impoverishment, isolation, change of residence, care, contribute to a further increase in susceptibility to depression or the initiation of depression in already vulnerable elderly people. Appropriate treatment of depression in the elderly reduces symptoms, prevents suicidal ideation, improves cognitive and functional status, and helps patients develop the skills needed to cope with a disability or psychosocial difficulties. Prevention of depression can significantly improve outcomes, mainly recovery of function and quality of life, as well as the risk of mortality. Therefore, the importance and possibilities for the prophylaxis of depression should be considered, especially in those circumstances where the risk of depression is markedly increased, such as stroke, cancer, institutionalization, etc. (16).

It is crucial to provide screening for early depression using routine diagnostic tools such as questionnaires for older people who are living in the community, in order to detect depression early and to reduce the burden on their families and society as a whole. It would also be useful to improve the physical health, psychological health, and health of the elderly. Older people are expected to live with their family or relatives, to participate in religious activities and group communities (17).

Stats from many studies suggest the importance of screening for depression in the elderly because of its high prevalence and risk of increased morbidity and mortality. Many factors, biological and psychosocial, affect variability in both clinical presentation and response to treatment. There is significant evidence that prevention can significantly reduce the incidence rate of depression. A recent meta-analysis studied the effects of preventive interventions with individuals without confirmed depression revealed a 21% reduction in incidence over 1-2 years in prevention groups compared with control groups (18).

In addition to proactive and effective control of primary diseases, it is necessary to make efforts to improve the psychological and social function of patients. Depressive symptoms in the elderly are associated with greater difficulties in the treatment of comorbidities, poorer prognosis, and should be thoroughly investigated and early treatment of this vulnerable population should be provided. Therefore, it is necessary to identify depressive symptoms early, to relate them with other diseases, in order to intervene on time with preventive measures and thus prevent the evolution of the disease to full depression (19).

Aging-related depression can be controlled by creating living conditions and environments that increase the presence and activity of older people in social situations. Also, attempts to secure their financial assistance and independence will have a positive effect on their mental well-being (20, 21).

Moreover, public awareness of the mental problems of the elderly population, early diagnosis, and proper management of these groups of people will not only alleviate their suffering but represent a way to improve the quality of life of this category of society, which certainly represents a great contribution to society as a whole. Public health professionals as well as primary care physicians need to be trained to recognize depression and take appropriate treatment. Also, social protection providers, from the formal and informal sectors, should be included in the teams that should take steps at the national level and direct more resources to support the elderly population (22, 23).

Depression significantly affects not only the quality of life of the patient but also the family and the community and contributes to a significant increase in health care costs, so the prevention of depression deserves special attention. Improved recognition and treatment of geriatric depression has great potential for improving physical and mental health later in life, reducing disability, and improving quality of life (24).

Graph 1. Prevalence of depression in the population aged 65 and over

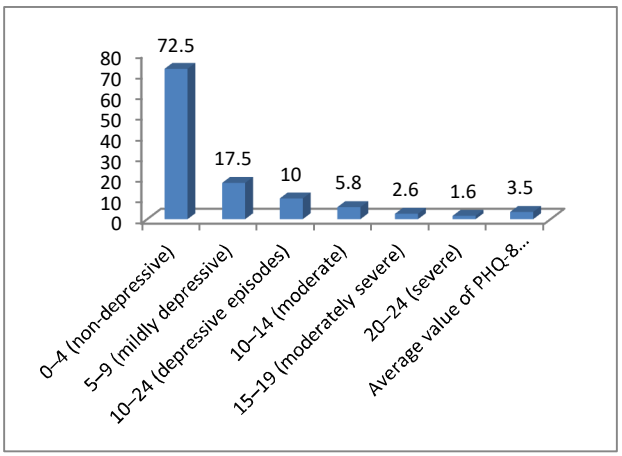

Table 1. Depressive symptoms in people with PHQ-8 ≥10

Symptoms of depression	not at all		few days		more than 7 days		almost every day	
	n	%	n	%	n	%	n	%
Poor interest or pleasure in doing something	19	5.4	113	31.9	90	25.4	130	36.9
Discouragement, hopelessness	24	6.9	93	26.6	103	29.4	130	37.1
Sleep problems	22	6.2	64	18.1	112	31.6	156	44.1
Feeling tired or lacking in energy	6	1.7	27	7.6	102	28.9	218	61.8
Poor appetite or overeating	87	24.6	111	31.4	76	21.5	80	22.6
Bad opinion of yourself	115	33.9	100	29.5	56	16.5	68	20.1
Difficulty concentrating	54	15.4	121	34.6	72	20.6	103	29.4
Slow motion or restlessness	65	18.7	69	19.9	52	15	161	46.4

Table 2. Prevalence of depression and social support

Social support	Without depressive symptoms		Mild depressive symptoms		Depressive episode		p*
	n	%	n	%	n	%	
How many people are so close to you that you can count on them when you have serious personal problems?							
None	42	53.2	17	21.5	20	25.3	< 0.0005
1 and 2	1013	66.7	304	20	202	13.3	
3 and 5	1120	76.6	233	15.9	109	7.5	
6 or more	390	81.3	67	14	23	4.8	
How many people are interested in you, in what you do, what happens in your life?							
Very interested	1208	77	234	14.9	126	8	< 0.0005
Somewhat interested	973	73	243	18.2	116	8.7	
Neither interested nor uninterested	262	65.3	89	22.2	50	12.5	
Little interested	85	54.5	39	25	32	20.5	
Not interested	37	44.6	16	19.3	30	36.1	
How easy is it to get practical help from a neighbor/neighbor if you need it?							
Very easy	510	75.8	110	16.3	53	7.9	< 0.0005
Easy	1000	76.5	206	15.7	102	7.8	
Possible	726	71.5	179	17.6	111	10.9	
Difficult	262	63.7	92	22.4	57	13.9	
Very difficult	67	50.8	34	25.8	31	23.5	

*** Chi-square test**

Graph 2. Prevalence of depression and social support

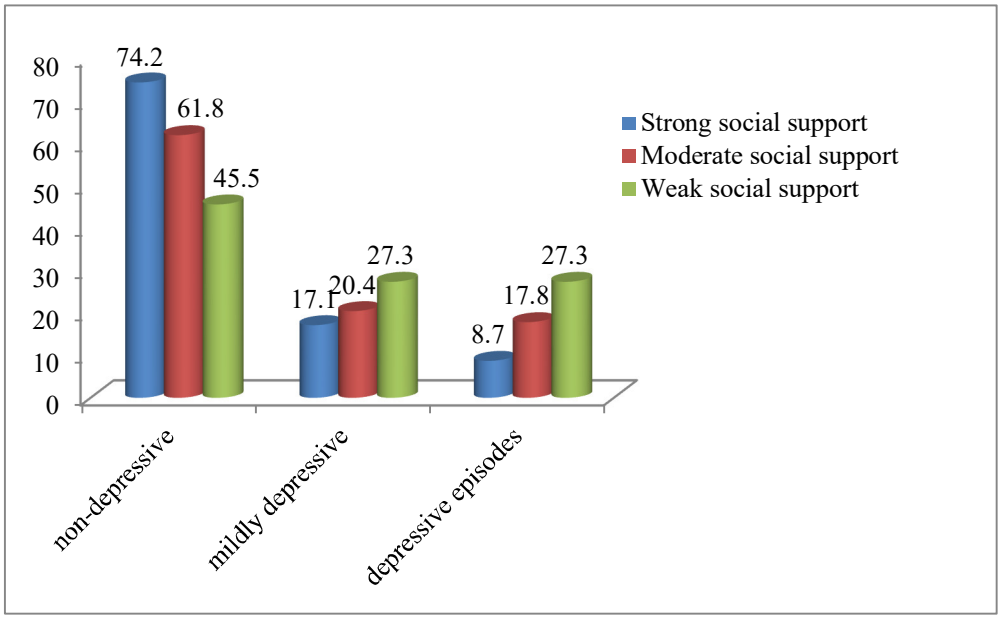

Table 3. Odds ratios (OR) and 95% confidence intervals (CI) for the presence of depression in the elderly in relation to social support

Social support	Univariate analysis, **OR (95% CI)**		
	Total	**Males**	**Females**
Strong	1	1	1
Moderate	1.78 (1.45-2.17)*	2.635 (1.34-5.50)*	1.347 (0.82-2.11)
Weak	3.45 (1.05-11.34)*	5.08 (2.21-11.62)*	3.41 (2.01-5.83)*

*** p<0.001**

CONCLUSION

These results indicate the urgency of addressing depression as a public health priority to reduce the burden and disability and improve the overall health of the elderly population. It is necessary to identify on-time subpopulations that are at high risk of developing depression. By identifying predictors of depression, a focused, targeted intervention could be organized to preserve the mental health of the individual and the community.

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CHARACTERISTICS OF HOSPITALIZED COVID-19 PATIENTS IN SUMADIJA DISTRICT IN 2020

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ABSTRACT

The number of hospitalized COVID patients varies in accordance with the waves of the pandemic. The aim of the study was to examine the characteristics of hospitalized COVID-19 patients and predictors of in-hospital died with special reference to the importance of comorbidity. A retrospective cohort study that included all COVID patients hospitalized at the Clinical Center Kragujevac in 2020. The data contained in the Hospitalization Report are described, and the predictors of hospital mortality are defined by binary logistic regression. 1336 COVID patients were hospitalized. The average age of the hospitalized patients was 58.1 ± 16.5 years, 2/3 of them were males. The largest number of hospitalized patients live in Kragujevac - 62.8%. During hospitalization, 19.4% (n = 206) of patients died, who were on average 13 years older (t = 14.13, df = 504.3, p <0.01), and stayed in the hospital 2 days shorter (Z = -5.36, p <0.01) when compared to discharged patients. 86.5% (n = 1155) of hospitalized patients had comorbidities, most often hypertension and other heart diseases, diabetes mellitus and renal failure. Statistically significant predictors of the lethal outcome of hospitalization were patients' age (OR = 0.94 95% CI = 0.93-0.95), residence (OR = 0.84, 95% CI = 0.75-0.95), length of hospitalization (OR = 1.06, 95% CI = 1.04-1.09) and the presence of comorbidity as the strongest predictor (OR = 5.31, 95% CI = 2.37-11.89). COVID Patients with comorbidities require special attention because comorbidities affect the outcome of hospitalization.

Keywords: *SARS-CoV-2 infection, comorbidities, intrahospital mortality.*

INTRODUCTION

At the end of 2019, in the city of Wuhan, in the Republic of China, several cases of respiratory infections in humans were recorded, and on January 7th, 2020. a causative agent called the new corona virus, SARS-Cov-2 was isolated, and the disease was named Covid-19. The new coronavirus is a highly contagious strain of coronavirus that is spread by droplets and airborne particles, so the epidemic quickly grew into a pandemic. The first registered case of coronavirus in Serbia was reported on March 6, 2020. From then until today, the pandemic lasts with its variations and peaks called epidemiological waves (1).

Covid-19 disease most commonly causes upper respiratory tract problems and flu-like symptoms (2). Typical symptoms are fever, chills, runny nose, sore throat, cough and malaise. Most of those infected have a mild clinical picture. However, in 5-10% of cases, most often in elderly, immunocompromised patients and patients with comorbidities, the disease can spread to the lower respiratory tract. These patients usually require hospitalization, and a number of them develop a very severe clinical picture, with pneumonia, worsening of gas parameters, the need for oxygen therapy and mechanical ventilation. The outcomes of such hospitalizations are often fatal (3). The most common health disorders encountered in covid patients requiring hospitalization are cardiovascular diseases, primarily hypertension, diabetes mellitus, lung and kidney diseases (4). Treating patients with comorbidities is more complex and challenging, and mortality is higher (5).

The scientists around the world are facing with the COVID pandemic for the first time and it leaves many questions open. One is questions directly related to infection, the association with comorbidities, the impact of regular therapy that patients take on the course and outcome of covid infection, while the other group of questions is related to the health care system (6). COVID-19 has further increased the challenges that health systems around the world have long faced. Managing the health system and providing sufficient funds for the operation and modernization of medical services in order to increase efficiency and effectiveness, with 100% coverage of the population with health care for many years is a challenge for all countries. In 2018, the Republic of Serbia allocated 8.5% of gross domestic product (GDP) for health, according to the World Bank, which is a significantly higher percentage compared to most other European countries. However, the value of GDP per capita in the Republic of Serbia in 2020 is almost 7 times lower than the average of the European Union (7,666.24 vs. 47,095 USD) (7). Therefore, the question of the readiness of the health system, in terms of capacity to treat patients, spatial and technical, but also human resources to respond to such a great challenge remains open.

The aim of the study was to analyze the predictors of the lethal outcome within the hospitalization of covid patients, with special reference to the influence of comorbidities.

METHODS

The University Clinical Center of Kragujevac is a regional clinical center where the population of Sumadija and Western Serbia is treated -about 2 million people and where more than 50,000 hospitalizations are done, annually. The databases on hospitalization reports, which the University Clinical Center of Kragujevac submits to the Institute of Public Health in Kragujevac, are part of the Integrated Health Information System of the Republic of Serbia.

This is a retrospective cohort study, which includes all hospitalized patients at the Clinical Center Kragujevac in the period 1.1. - December 31, 2020 with confirmed, or probable pneumonia caused by SARS-CoV-2 infection as the final diagnosis. Hospitalizations due to SARS-CoV-2 infection were identified by the ICD-10 codes B34.2, B34.9, U07.1 and U07.2. The dataset included patient characteristics such as: sex, age, secondary diagnoses based on International Classification of Diseases-10 (ICD-10) codes, hospital stay and type of hospital discharge. After their discharge, patients were not further monitored.

Statistical Analysis

Continuous data are described by arithmetic mean and by the measure of variability, whereas categorical data are presented in the form of percentages. Continuous data that had a normal data distribution were tested with the Student's t test, or the Mann Whitney U test when the distribution was not normal. Categorical data were tested by Chi-square test. Binary logistic regression was applied to observe the predictors of the lethal outcome of hospitalization.

For the purposes of the research, the following variables were transformed:

1. Address of the place of residence - patients living in the territory of the City of Kragujevac were in the category of Kragujevac, patients living in the municipalities of Batocina, Lapovo, Raca, Topola and Knic were classified in the category of Sumadija district, and all others were classified in the category of Western Serbia.
2. Admission/discharge ward-patients admitted /discharged from the Emergency Center and Intensive Care Unit are classified in one category - ER Center. Hospitalized in wards outside the Emergency Center, Infectious and Pulmonology Clinic and Day Hospital are classified in the Other category.

SPSS statistical software for Windows, Version 19 were used (IBM, Armonk, NY, USA). A p-value ≤ 0.05 was considered significant.

RESULTS

In 2020, 1336 covid patients were treated at the University Clinical Center Kragujevac. The mean age of the hospitalized patients was 58.1 ± 16.5 years. Two/thirds of the hospitalized patients were male patients (n = 860, 64.4%). Hospitalized female patients are on average 2.5 years older than hospitalized male patients (t = -2.48, df = 868.8, p <0.05). The largest number of hospitalized patients lives in the territory of the City of Kragujevac, 62.7% (n = 838). At the UCC, patients were most often admitted and discharged from the Clinic for Infectious Diseases. Patients stayed in CC Kragujevac for an average of 12.1 ± 7.8 days and had 16.4 ± 85.6 hours of ventilatory support.

19.4% (n = 206) of patients died during hospitalization. The deceased patients were 13 years older (t = 14.13, df = 504.3, p <0.01). Mortality is similar in both sexes (χ^2 = 0.01, df = 1, p> 0.05). There is a significant difference in the outcome of hospitalization of covid patients in relation to residence (χ^2 = 9.47, df = 2, p <0.05). Every fourth patient from Western Serbia, every seventh from Šumadija and every sixth from the territory of the City of Kragujevac died during hospitalization. The largest number of deceased covid patients were admitted to the Emergency Center, 60% of the total admitted to the Emergency Center and the Pulmonology Clinic, 57.3% of the total admitted to it (χ^2 = 141.1, df = 4, p <0.01). Analysis of the outcomes of hospitalization and of the ward on discharge shows that the largest number of patients died in the Emergency Center, 91.8% of the total discharged covid patients from the same ward, and at the Clinic of Pulmonology every fourth of the total discharged covid positive patients (χ^2 = 463.4, df = 4 , p <0.01). Before the lethal outcome, patients spent 10.5 ± 8.6 days in the hospital, which is statistically significantly shorter compared to discharged patients (Z = -5.47, p <0.01). Deceased covid patients had 4 times more hours of ventilatory support compared to discharged patients (Z = -7.88, p <0.01) (Table 1.).

In addition to the underlying cause of hospitalization, 86.5% (n = 1155) of covid patients had other health disorders. The most common comorbidities in hospitalized patients were diseases of the circulatory system, endocrine system and genitourinary system, among them hypertension, diabetes mellitus and renal failure. Of the total hospitalized patients who had diseases of the circulatory system as comorbidity, 38.1% of them died during hospitalization, i.e. 41.4% of patients who had endocrine system disorders. Almost every other patient who had genutourinary tract diseases as a comorbidity died during hospitalization caused by covid infection (Figure 1.).

The lethal outcome of hospitalization is influenced by various factors. In our study, statistically significant predictors of the lethal outcome of hospitalization were age of patients (OR = 0.94 95% CI = 0.93-0.95), their place of residence (OR = 0.84, 95% CI = 0.75-0.95), length of hospitalization (OR = 1.06, 95%). CI = 1.04-1.09) and the presence of comorbidity (OR = 5.31, 95% CI = 2.37-11.89). The strongest predictor was the presence of accompanying health disorders. Hospitalizations of covid-positive patients who had comorbidities 5 times more often ended in lethal outcome compared with hospitalizations of patients who did not have associated diseases (Table 2.).

DISCUSSION

A pandemic caused by SARS-Cov-2 infection with a mortality rate of 2% is a serious threat to public health, causing major socio-economic changes whose impact on the global level will be felt long after it is over. In the first phase, the system under the greatest load, the health system (8.9), suffers the greatest changes. During the pandemic, the University Clinical Center of Kragujevac underwent organizational and structural changes. At first, COVID-positive patients were admitted based on the severity of the clinical picture at the Emergency Center, Infectious Diseases and Pulmonology Clinic. However, as the need for hospitalization grew, other clinics also switched to the so-called covid regime. This meant that non-covid patients were transferred from that clinic to an adequate other clinic, in order to receive covid-positive patients. This was most often the case with clinics that had the technical capacity for oxygen support, such as the Neurology Clinic.

Similar to the results of our study and in a study conducted in China and the United States, the largest number of hospitalized COVID-19 patients were male patients of older age (10,11). A study of nearly 4,000 COVID-19 patients treated in intensive care units in northern Italy shows that these are mostly elderly men, with additional health disorders required by invasive mechanical ventilation. Predictors of hospitalization lethal outcome similar to our study were age, gender (male), hours of mechanical support, and the presence of certain comorbidities (COPD, hypercholesterolemia, type 2 diabetes) (12). Numerous other studies also point to the importance of gender, age, and comorbidity. Chronic health disorders that are most common in the general population are also found in hospitalized Covid patients, cardiovascular disease and diabetes mellitus. Among cardiovascular diseases, hypertension predominates. In our study, the prevalence of hospitalized COVID patients with comorbidities was 86.5%, which coincides with a study conducted in Spain in which the prevalence was 81.9% (13).

Chronic health disorders require the application of appropriate therapy, so doctors are concerned that there could be drug interactions and further deterioration of the clinical picture. So, Angiotensin-Converting Enzyme Inhibitor and Angiotensin II Receptor Blocker, as the most commonly used groups of drugs in the treatment of hypertension, can increase the mRNA expression of cardiac angiotensin-converting enzyme. This is particularly significant given the fact that the meta-analysis showed that cardiovascular health disorders were associated with a more frequent lethal outcome of Covid infection, while kidney, cerebrovascular, cardiovascular and respiratory diseases and diabetes were associated with a higher risk of developing severe COVID-19 (14-16).

Table 1. Characteristics of hospitalized covid patients

Variables	Discharged		In-hospital died		p
	n	%	n	%	
Age	55.5±16.4		68.4±12.3		<0.01*
Sex					
Men	692	80.5	168	19.5	>0.05
Femen	385	80.9	91	19.1	
Place of residence					
Kragujevac	687	82	151	18	
Šumadijski okrug	152	84.4	28	15.6	<0.05*
Zapadna Srbija	238	74.8	80	25.2	
Admission ward					
ER	22	40	33	60	
Infectious Diseases Clinic	873	85	154	15	
Pulmonology Clinic	32	42.7	43	57.3	<0.01*
Day hospital	118	84.3	22	15.7	
Other	32	82.1	7	17.9	
Discharge ward					
ER	9	8.2	101	91.8	
Infectious Diseases Clinic	603	92.9	46	7.1	
Pulmonology Clinic	359	76.2	112	23.8	<0.01*
Day hospital	90	100	/	/	
Other	16	100	/	/	
Stay in hospital	12.47±7.5		10.48±8.6		<0.01*
Hours of ventilatory support	10.34±73.8		41.81±119.9		<0.01*

Figure 1. The most common comorbidities in discharged and deceased patients

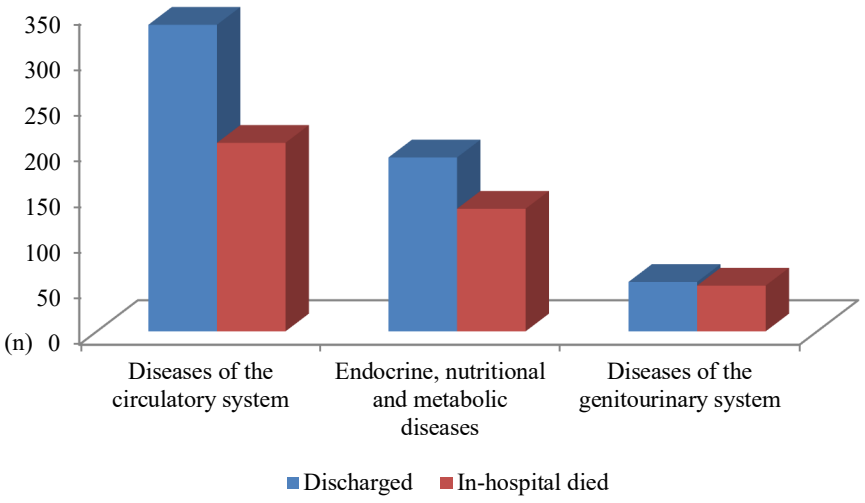

Table 2. Predictors of hospital mortality in patients

Predictors	Exp(B)	95%CI	p
Age	0.94	0.93-0.95	<0.01*
Place of residence			
Kragujevac (ref)	1		<0.05*
Other	0.84	0.75-0.95	
Hospital stay	1.06	1.04-1.09	<0.01*
Comorbidities			
No (ref)	1		<0.01*
Yes	5.31	2.37-11.89	

LIMITATIONS

This study has several limitations. First, the study population included patients hospitalisated only in Clinical Centre Kragujevac. Second, the data were collected from the electronic hospital database Institute of Public Health Kragujevac, so it has only the general data.

CONCLUSION

The lethal outcome of hospitalization is influenced by various factors. In addition to the severity of the underlying disease(s) and the patient's age, the most significant risk factors for the fatal outcome of hospitalization are comorbidities. However, hospitalized elderly patients with SARS-CoV-2 infection and comorbidities require special care.

CONFLICT OF INTEREST

None.

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THE ROLE OF ULTRASONOGRAPHY IN THE DIAGNOSIS OF FUNCTIONAL DYSPEPSIA

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ABSTRACT

Functional dyspepsia (FD) is a syndrome mostly diagnosed by subjective patients' symptoms after excluding organic, systemic and metabolic diseases. Aim: The goal of this study is to evaluate gastric emptying in patients with functional dyspepsia, by measuring the antral area (cm2) after the intake of a test meal using ultrasonography as an objective and widely applicable method. Material and Methods: This study included 30 patients (mean age of 46.53 ±9.73 years) with symptoms of FD according to the ROMA IV criteria and 30 healthy individuals (mean age of 42.87 ±4.42 years). A 5 MHz ultrasound probe was used to measure the stomach antral area at 6 different time points: in the fasting state, following the meal intake at 5, 30, 60, 90 and 120 min postprandially. Results: The antral area was statistically significantly larger after a 30-minute postprandial period in patients with FD comparing to healthy controls (p<0.05). There was a statistically significant difference in the rate of gastric emptying at 120 minutes in patients with functional dyspepsia, compared to healthy subjects (p <0.01). Patients with postprandial distress syndrome had the average value of gastric emptying 48.25 compared to 56.09 in patients with epigastric pain syndrome (p <0.05). The slowest emptying was observed in patients with nausea and postprandial fullness (p <0.05). Conclusion: Functional dyspepsia is associated with delayed gastric emptying. Using ultrasonography to measure the antral area helps us to assess gastric emptying and therefore to assess patients with functional dyspepsia. The antral area was significantly larger in patients with functional dyspepsia compared to healthy subjects after the test meal, suggesting slower gastric emptying in the dyspeptic patients. Since the diagnosis of functional dyspepsia is based mostly on diverse patients' symptoms, using ultrasonography to measure the antral area helps us to objectively assess this problem.

Keywords: *Functional dyspepsia, ultrasonography.*

ABBREVIATIONS

FD - Functional dyspepsia
PDS - Postprandial Distress Syndrome
EPS - Epigastric Pain Syndrome
MRI - Magnetic resonance imaging
GI – Gastrointestinal

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INTRODUCTION

Functional dyspepsia (FD) is a syndrome originating from the gastroduodenal segment of the digestive tube and it is the most common functional disorder of the digestive tube. It is estimated that 40% of adults have dyspeptic symptoms at least once a year, most often diagnosed in the fifth decade of life and equally present in both sexes.

According to the Rome IV criteria, functional dyspepsia is defined as the presence of postprandial fullness, early satiety, epigastric pain and burningthat are severe enough to interfere with the usual activities. The diagnostic criteria imply the presence of one or more of these symptoms during 3 days per week in the last 3 months, with the onset, at least, 6 months ago. The upper gastrointestinal symptoms such as nausea, belching, or abdominal bloating can also occur. According to the burden symptoms, patients can be classified into two subgroups of functional dyspepsia (1,2):

- Postprandial Distress Syndrome (PDS)
- Epigastric Pain Syndrome (EPS)

The pathophysiology of FD is complex. The key mechanism for the onset of functional dyspepsia is impaired gastrointestinal motility and sensitivity. Slow gastric emptying, impaired gastric accommodation and visceral hypersensitivity are most commonly associated with functional dyspepsia. These three mechanisms are related and difficult to examine each. The symptoms induced by gastric distension are not region-specific. The measurement of gastric emptying is most accessible and is a good indicator of gastric motility disorders (3-6).

The percentage of dyspeptic patients with delayed gastric emptying ranges from 20% -50%. However, most of the studies were performed in small groups of patients and small control groups. In the largest studies, gastric emptying of solids was delayed in about 30% of patients with functional dyspepsia (3,7). The measurement of gastric emptying may be useful in understanding the severity of dyspeptic symptoms. The gold standard for measuring gastric emptying is scintigraphy. Other reliable methods include the breath test, magnetic resonance imaging (MRI), gastric aspiration techniques, epigastric impedance measurements, electrogastrography and other methods. Ultrasound of the stomach was initially performed to detect and investigate organic diseases. Ultrasound can be used to evaluate the antral contractility, gastric emptying, transpyloric flow, gastric configuration, intragastric distribution of meals, gastric accommodation and strain of the gastric wall. Several studies have demonstrated a good correlation between scintigraphy and ultrasonography in the evaluation of gastric function (6,7,8). The antrum is the most accessible part of the stomach for the ultrasound measurement. It is often used for assessing gastric emptying. Measuring the area of the antrum and change of its size in time is associated with the patient's perception of fullness (9,10). Ultrasound is a non-invasive, safe, reproducible, widely available method and does not require expensive equipment. Ultrasonography does not change the physiology of the gastrointestinal tract and is widely available in the outpatient settings (11). We used ultrasonography to evaluate gastric motility since this method is available in most institutions and is easy to apply and can be recommended in the diagnosis of FD.

It is difficult to correctly diagnose functional dyspepsia according to the subjective assessment of the patient, considering the variety of symptoms. The aim of this paper is to show how ultrasonography can disclose organic disease and functional behavior of the GI tract. The significance of this paper is to show that using ultrasound measurement of the antral surface could help in the functional dyspepsia diagnosis in an objective way.

MATERIALS AND METHODS

This study included a total of 60 participants: the group of patients with symptoms of FD according to the ROMA IV criteria (21 patients with PDS (the average age 46.5) and 9 patients with EPS (the average age 46.5)) and 30 (the average age 42.7) healthy individuals without any symptoms. The group of participants was recruited at the Medical Center "Bezanijska kosa" from January 2019 to September 2019.

The inclusion criteria for the study were patients with FD according to the ROMA IV criteria. The exclusion criteria were pathological laboratory findings (liver or kidney failure), all patients who were Helicobacter pylori-positive, patients who were treated for Helicobacter. pylori infection in the previous six months, patients with a gallbladder stone, diabetics as well as patients with other metabolic disorders, organic or systemic diseases and all patients who took the therapy that affects the motility of the digestive tract. Patients should not take the medications such as metoclopramide, erythromycin and anticholinergic drugs for 48 hours before the testing. All patients were asked about the dyspeptic symptoms before the testing.

All procedures conducted in patients were in accordance with the ethical standards of the Institutional Research Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

A 5 MHz ultrasound probe (Hitachi EUB-5500) was used to measure the area of the antrum of the stomach. The antral area was visualized in the sagittal plane scan between the left lobe of the liver, pancreas, aorta or vein cava inferior and superior mesenteric vein. The measurements were performed in the supine position, in the right decubitus. The outer profile of the antrum was measured using the built-in caliper and calculation program of the ultrasound apparatus. The minimal amount of force was applied during each reading to prevent compression of the antrum (10,11). To ensure the accuracy of the results, the measurements were taken twice and then averaged.

Before starting the test, each participant fasted for eight hours prior to the study. The antral area was measured at 6 different time points: in the fasting state, following the meal intake at 5, 30, 60, 90 and 120 minutes postprandially. Each participant took a 150Kcal meal (17g protein, 15g fat and 27g carbohydrate) with 200 ml of water. At each time point, the participants were asked to record the severity of each dyspeptic symptom (epigastric pain, burning in the stomach, postprandial fullness, early satiety, bloating, nausea, vomiting and belching).

Statistical analysis

Numerical variables were expressed as the mean ± SD for normal distribution or median for non-normal distribution, or with frequency and percentage for the categorical data. The comparison of groups was performed using Student's t-test and ANOVA for normally distributed data. $\chi 2$ analysis was used to test the relation between non-continuous variables. P-value below 0.05 was considered significant. The whole analysis was performed with the SPSS statistical analysis software, Version 20.0 (SPSS, Chicago, Illinois, USA).

RESULTS

According to the ROMA III classification criteria for functional dyspepsia, there were 21 patients with postprandial distress syndrome (70%) and 9 with epigastric pain (30%) in the study group. The patients reported the following symptoms: epigastric pain in 3 (10%), bloating in 5 (16.7%), postprandial fulness in 7 (23.3%), nausea in 5 (16.7%), belching in 10 (33.3%) patients. The most intensive symptoms were reported by 8 (26.7%) patients in the first five minutes, 16 (53.3%) patients from 6-30 minutes and 6 (20%) patients from 31-60 minutes.

There was no significant statistical difference between the antral area surface in patients with functional dyspepsia (3.44 ± 1.50; 14.40± 5.15) and healthy controls (3.26 ± 0.64; 12.47± 1.96) during fasting, and at 5 minutes after the test meal ($p > 0.05$, respectively). The antral area was statistically significantly larger over a 30-minute postprandial period in patients with FD comparing to healthy controls (12.03± 4.28 vs 9.54±2.04) ($p<0.05$). Measurements of the antral surface in the study and control group through every time point were depicted in Table 1.

Table 1. Measurements of the antral surface area in the study population.

Antral area surface	group	N	mean	Standard deviation	p
0. minutes	study	30	3.44	1.50	>0.05
	control	30	3.26	0.64	
5. minutes	study	30	14.40	5.15	>0.05
	control	30	12.47	1.96	
30.minutes	study	30	12.03	4.28	<0.05
	control	30	9.54	2.04	
60.minutes	study	30	10.38	4.16	<0.05
	control	30	8.01	1.72	
90.minutes	study	30	8.80	3.60	<0.05
	control	30	5.63	1.33	
120.minutes	study	30	7.17	3.04	<0.05
	control	30	3.72	0.66	

There was a statistically significant difference in the rate of gastric emptying at 120 minutes in patients with functional dyspepsia (60.60±9.95), compared to healthy subjects (69.62±6.66), ($p<0.01$). There was a significant difference comparing gastric emptying between the subgroups of patients with FD. Patients with postprandial distress syndrome had the average value of gastric emptying 48.25 compared to 56.09 in patients with epigastric pain syndrome ($p<0.05$).

Also, there was a significant difference between the study and control group when measuring 50% gastric emptying ($p<0.5$). There was significantly faster gastric emptying in healthy subjects. All healthy subjects (100%) had gastric emptiness over 50% at 120 minutes, while 53.3% of dyspeptic patients had emptiness less than 50% at 120 minutes, ($p<0.01$), (Table 2).

Table 2. Measurement of gastric emptying in the study population.

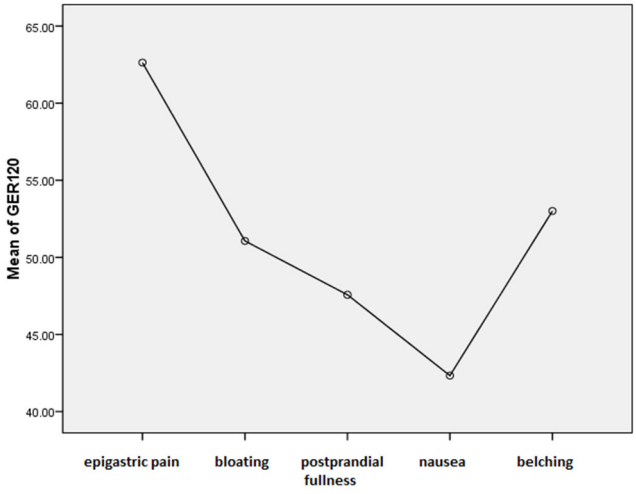
Group		p50 <=50%	p50 >50%	Total	p
study	Number	16	14	30	
	%	53.3%	46.7%	100.0%	
	% p50	100.0%	31.8%	50.0%	<0.01
control	Number	0	30	30	
	%	0.0%	100.0%	100.0%	
	% p50	0.0%	68.2%	50.0%	
All	Number	16	44	60	
	% group	26.7%	73.3%	100.0%	
	% p50	100.0%	100.0%	100.0%	

ANOVA test showed significant differences in the mean values of the rate of gastric emptying in the 120th minute compared to the most intensive symptoms during the test. The slowest emptying was observed in patients with nausea and postprandial fullness ($p<0.05$), (Table 3 and Figure 1).

Table 3. GER by symptoms.

GER	N	Mean	SD	Minimum	Maximum
epigastric pain	3	62.63	13.66	49.30	76.60
bloating	5	51.06	9.67	35.84	62.38
Postprandial fullness	7	47.57	7.85	35.41	56.35
nausea	5	42.33	10.96	27.35	55.97
belching	10	53.01	6.42	43.06	60.70
Total	30	50.60	9.95	27.35	76.60

Figure 1. Mean GER by the most intensive symptoms.



DISCUSSION

Our study showed that ultrasonography, as an easy, effective and non-invasive modality, could be used in clinical practice to evaluate gastric emptying in both, healthy participants and those with gastrointestinal disorders, such as FD. As the antrum is the most accessible part of the stomach, we used the ultrasonography method to determine the size of the antrum after the test meal as a measure of gastric emptying.

Dyspepsia is a persistent or recurrent feeling of discomfort or pain in the upper abdomen. The pathophysiology of FD is complex. Gastrointestinal motility disorders (slowed gastric emptying and impaired gastric accommodation) are

the most common cause of functional dyspepsia. The measurement of gastric emptying may be useful in understanding the severity of dyspeptic symptoms. Numerous studies have examined the association between delayed gastric emptying and dyspepsia. Depending on the type of study, the percentage of dyspeptic patients with delayed gastric emptying ranges from 20%-50%. In a meta-analysis of 17 studies involving 868 patients and 397 healthy, significantly slower gastric emptying was observed in 40% of patients with functional dyspepsia (3). In some studies, it has been found that slowed gastric emptying is associated with a feeling of postprandial fullness, nausea and vomiting (12,13). In an early study by HauskennT.et others, it was shown that the mean antral area was wider in patients with functional dyspepsia than in healthy controls (14).

In this study, the ultrasound examination of the antrum function (change in the size of the antrum per unit time) was performed, which is otherwise often used in examinations of the degree of gastric emptying. Although scintigraphy is the gold standard in the examination of gastric emptying, the ultrasound technique has advantages, because it is non-invasive, reproducible and does not require expensive equipment. The worldwide studies of the validity of ultrasound monitoring of gastric function in relation to scintigraphy have been performed and it has been found that there is a good correlation between these two methods. There is also no significant difference in the position the patient assumes during the testing, whether lying down or sitting, there is also agreement in the findings between different examiners when measurements are performed on the same patients under the same conditions (8,17,18).

In this study, it was shown that the average antrum area observed per unit time in the FD group and the control group did not differ in fasting and 5 minutes after the intake of the test meal. From 30 minutes up to 120 minutes, the average area of the antrum in the control group was smaller. The level of gastric emptying at 120 minutes after the test meal, was less in patients with functional dyspepsia than in healthy subjects. Observed at 120 minutes, 16 of 30 patients (53.3%) had the level of gastric emptying less than 50% in the FD group , while all healthy subjects had the level of gastric emptying over 50%. There was a statistically significant difference in the distribution of subjects with functional dyspepsia and control groups concerning gastric emptying of 50%, after the test meal.

The obtained data are consistent with the previous publications, where it has been found that the percentage of patients with functional dyspepsia and slowed gastric emptying depends on the study, ie. the test conditions varied in the range of 20%-50% (3,7).

In this study, slower gastric emptying was found in a patient with postprandial distress syndrome, compared to subjects with epigastric pain syndrome. In the literature, delayed gastric emptying has been associated with postprandial distress syndrome (19,20).

The complaints reported by patients as the most intense ones during the testing were: belching, postprandial fullness, nausea, bloating, and epigastric pain. None of the patients reported early satiety, heartburn, and vomiting during the testing. Most patients reported their symptoms in the interval between 5 minutes and 30 minutes during the testing, which coincides with the time when the antrum area in the group of patients with functional dyspepsia begins to differ significantly from the antrum area in the control group. There are no similar data in the literature.

The gastric emptying level at 120 minutes in relation to the most intense discomfort that occurred during the testing, was slowest in patients with nausea and postprandial fullness, which agrees with the data from the literature (15).

CONCLUSION

The patient who is suspected to have functional dyspepsia has to do numerous laboratory tests, imaging and endoscopic examinations but the diagnosis is mostly made upon the variety of subjective symptoms. It is known that the ultrasound measuring of the area of the antrum and change of its size in time is useful in assessing gastric functional disorders. We showed that the ultrasound measured antrum area in patients with functional dyspepsia, was statistically significantly larger starting from 30 minutes up to 120 minutes postprandially, which coincided with the appearance of symptoms. Also, the level of gastric emptying was significantly slower in patients with postprandial distress syndrome. Delayed gastric emptying was observed in subjects with nausea and postprandial fullness. Numerous methods for testing the function of the digestive tube are reserved for clinical studies and are not available in daily practice. Most of the methods are invasive, requiring expensive equipment and well-trained staff. Ultrasonography correlates well to other methods. It is reproducible, simple, available and non-invasive, and is therefore, the best option having in mind the patient's compliance. Ultrasonography has its disadvantages, it is a subjective method and gases in the stomach can significantly reduce visibility. Despite some limitations of ultrasonography, we suggest its use to be part of the diagnostic algorithm for the diagnosis of functional dyspepsia.

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EVOLUTION OF IMPAIRED RENAL FUNCTION IN CHILDREN AFTER CONTINENT VESICOSTOMY

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ABSTRACT

The aim of this study was to determine whether early commencement of clean intermittent catheterisation can improve impaired kidney function in children with continent vesicostomy as well as to analyse the effect of different types of continent vesicostomy and the observed outcome. All children with continent urinary derivation performed at the University Children's Hospital in Belgrade, Serbia, during the period 1990-2016, were included in this retrospective-prospective study. The participants were divided into three groups with respect to type of continent vesicostomy (appendicovesicostomy, preputial continent vesicostomy, and vesicostomy using distal ureter). Clean Intermittent Catheterisation with continent vesicostomy, oxybutynin, and antibiotic prophylaxis were used in a standard way. Renal function was monitored by the value of glomerular filtration rate taken before the start of the therapy and three years afterward. The significance of differences was tested by the paired-samples t-test and ANOVA test. We analysed 74 patients aged 3 to 10 years (5.5-y average) of which 80% were boys. Renal function improved in 60.5% patients. A highly significant improvement in kidney function three years after the commencement of combined treatment was shown irrespective of the performed method of vesicostomy ($p<0.01$). Using the ANOVA test, we have proved that there is no difference in the efficiency of therapy between particular groups ($p= 0,256$). The timely started therapy lead to significant kidney function improvement. The type of continent vesicostomy did not affect kidney function.

Keywords: *Clean intermittent catheterisation, continent vesicostomy, renal function, children.*

INTRODUCTION

Neurogenic bladder caused by various pathoanatomical substrates is one of the frequent causes of chronic kidney insufficiency, mostly because of the accompanying vesicoureteral reflux) (VUR) and recurrent pyelonephritis that leads to kidney tissue deterioration (1, 2). Solving urinary incontinence and preservation of renal function in children with congenital neurogenic bladder have posed a major challenge for paediatric surgeons and paediatric urologists for years (3). Practically, until the mid-70s, the incontinent intestinal conduits had been the only solution to these problems. Then, J. Lapides introduced the concept of clean intermittent catheterization through the native urethra, which significantly resolved these problems (4). However, in a relatively high percentage of cases, the method did not function due to: difficulty while performing (self) catheterisation (pain in males, orthopaedic problems) or continuous urinary leakage between catheterisations.

To overcome this, in 1976, Mitrofanoff recommended alternative ways for bladder emptying, continent appendicovesicostomy or ureterovesicostomy, with bladder neck closure (in the majority of cases) (5). These methods have been widely used especially after the works of Duckett and Snyder, and after the introduction of various other alternative routes (5, 6). These have been deservedly named as Mitrofanoff principle. For this purpose, besides the appendix and ureter, a part of the small intestine, sigma, preputium, urachus, etc. are also used (7-9). Apart from continence, stoma must be easily and painlessly catheterized, with no need for bandaging or urine bags. The extraperitoneal and extravesical approach is the least aggressive with a lower number of complications (10). Stoma must be placed as closely as possible to the bladder reservoir in order to provide the shortest possible path without curves. When using the appendix or Monti ileal conduit, it is usually the right lower abdominal quadrant or the left lower abdominal quadrant if left ureter or sigma conduit is used. Some authors suggest that the umbilicus is an ideal stoma site in order to achieve the less frequent occurrence of stenosis (11). Clean Intermittent Catheterisation (CIC) is not always simple to apply in children, as a large number of these patients are paraplegics or quadriplegics, so they need considerable parents' help (12).

The study aims to determine whether correct and early Clean Intermittent Catheterisation combined with antimuscarinic drugs and antibiotic prophylaxis can improve impaired kidney function in children with continent vesicostomy as well as to analyse the influence of different types of continent vesicostomy on the observed outcome.

PATIENTS AND METHODS

We performed a retrospective study of children (aged 3–8 years) who underwent continent urinary diversion at the University Children's Hospital in Belgrade and Clinical Centre Kragujevac in the period 1990–2016. The indications for continent vesicostomy were various types of neurogenic and myogenic bladder dysfunctions with urinary incontinence due to various pathoanatomical substrates. The patients with other bladder-emptying methods (Credé maneuver) or those emptying through the native urethra were excluded. The age at the moment of formation of continent vesicostomy was 3 to 10 years (5.5 years average). The monitoring period was at least 36 months.

Clean Intermittent Catheterisation was used in a standard way together with oxybutynin and prophylaxis for urinary infections first- and second-generation of cephalosporins or co-trimoxazole) (7). Renal function was monitored by the value of glomerular filtration rate (GFR) taken before the start of therapy and three years afterward. The GFR is the amount of fluid filtered from the glomerular capillaries into Bowman's capsule per unit of time (13). Total GFR was calculated as total plasma clearance 99mTc diethylenetriaminepentaacetic acid (DTPA) and corrected to 1.73 m^2 body surface area. Radioisotope was given as a one-time injection. A significant decline of renal function was considered when GFR was lower for more than 25% compared to preoperative value during the related period (13).

According to continent vesicostomy, all patients were classified into 3 groups depending on whether appendix vermiformis, distal ureter, or preputium was used. Burry tunnelling model was most often used as an anti-reflux (continent) mechanism and Lich-Gregoire technique.

Based on urodynamic testing or measuring bladder capacity, oxybutynin was occasionally eliminated.

This study was approved by the Ethics Committee of the Kragujevac Clinical Centre, decision number 01-1629.

STATISTICAL ANALYSES

The data have been processed by statistical analysis. Following the character of numerical variables, parametric t-test and ANOVA test were used. Statistical significance was set to 0.05 or more. The study ensured the statistical power of 0.8. PASW Statistics 18 was used for statistical analysis.

RESULTS

Seventy-four patients were included in the study. Eighty percent of these patients were males.

Continent vesicostomy using the appendix was done on 31 (40.5%) children, with distal ureter on 28 (39%), and with preputium on 15 (20.5%) children. The main diseases were anomalous development of the brain and spinal cord in 26 patients (35.1%), bladder exstrophy in 10 (12.2%), posterior urethral valve in 19 (22.4%), expansive processes in 6 (7.3%), and other diseases in 24 (27.6%) (sacral agenesis, prune belly syndrome, imperforate anus).

Table 1 shows the classification of patients based on their main diseases and the type of surgery performed.

Before the start of therapy, the GFR average value was 71.57 ml/min/ 1.73m^2, and three years later it was 81.9 ml/min/1.73m^2 which showed a significant statistical difference (t-test, p< 0.001; Table 2). The lowest GFR value was 59 ml/min in a patient with posterior urethral valve. Among the patients in our research group, there was no statistically significant difference in GFR value (ANOVA test, p =0.256; Table 3). Renal function decreased in 9 patients (9.5%), it remained the same in 25 (29%) and it improved in 52 (60.5%) patients.

Table 1. Number and percentage of patients based on disease and type of surgery

Main disease	GIT segment	Urotract segment	Preputial segment	Sample total
Anomalous development of brain and spinal cord	10 (38.5%)	4 (15.4%)	12(46.1%)	26 (35.1%)
Exstrophy-epispadias complex	8 (80%)	2 (20%)	0 (0%)	10 (12.2%)
Posterior urethral valve	5 (15.8%)	9 (47.4%)	7 (36.8%)	19 (22.4%)
Expansive process	2 (33.3%)	0 (0%)	4 (66.7%)	6 (7.3%)
Other diseases	6 (33.3%)	6 (50%)	1 (16.7%)	13 (17.6%)
Total	31 (40.5%)	27 (39.0%)	25 (20.5%)	74 (100%)

Table 2. Parameters of renal function before and after surgery

Paired Samples Statistics	Mean	N	SD	t	df	p
GFR before stoma opening mL/min/1.73m^2	71.57	73	10.39	**-8,881**	45	**<0,001***
GFR after three years mL/min/1.73m^2	81.9	73	12.908			

* Highly significant difference

Table 3. Parameters of renal function before and after surgery between groups

ANOVA (Tukey HSD)		Sum of Squares	df	Mean Square	F	Sig
GFR before stoma opening mL/min/1.73m^2	between groups	175.313	2	87.656	.801	.458
GFR after three years mL/min/1.73m^2	between groups	462.519	2	231.260	1.423	.256

DISCUSSION

Uropathy caused by bladder dysfunction, if not treated, can lead to damage in the kidney tissues (11). Protection of the upper urotract is achieved through the preservation of both bladder functions: control of intravesical pressure during urine collection and adequate bladder emptying at low pressures (12). Back in 1972, Lapides and associates presumed that 'maintenance of a good blood supply to the renal pelvis, ureter, bladder, and urethra by avoiding high intraluminal pressures and over-distension is the key to prevention of urinary tract infection' (13). To start with CIC (clean intermittent catheterisation) combined with antimuscarinic drugs as soon as possible is the key to the prevention of high intravesical pressure (11-13).

In their series of patients with myelomeningocele, Luis and associates reported renal parenchymal damage in 19.4% of cases (DMSA scintigraphy), with high prevalence in patients older than 10 (27.3%) which is twice as high as in the patients under the age of 5 (13.3% (14). The cause of such a higher incidence of kidney damage could be explained by delay, irregular using the advised treatment, and the lack of good medical follow-up of this group of patients (12, 14).

The difference of 5 years in the patients' age during the continent vesicostomy period had no significant effect on total GFR value (14).

Determination of urea and creatinine concentration in serum is imprecise for this purpose. First of all, because the increase of the concentration in blood is a late sign of renal dysfunction, and secondly, if one kidney is healthy, it can maintain normal concentration for a very long time (13, 14). Determination of the total and separate GFR values is the most precise estimation of kidney function (13). Slightly decreased GFR does not necessarily correlate with renal dysfunction, and, therefore, a 25% decrease in renal function is considered significant (13). A disadvantage of this method can be in partial absorption of a marker in the intestinal segment (in augmented bladders or in appendix); however, this absorption is so low that it has almost no effect on the real GFR value (12-14).

All our patients presented as pathoanatomical substrate developed neurogenic or myogenic bladder dysfunction and incontinency with frequent urotract infections. (Table 1) . We advised starting a therapeutic strategy with Clean Intermittent Catheterisation and antimuscarinic drugs as soon as possible, designing an individual therapeutic strategy for each patient separately. CIC through continent urinary diversion was more acceptable for parents than catheterization through the native urethra.

The above-said therapy gave results fast through statistically significantly improved GFR values in the third year after the beginning of treatment. There was a statistically significant difference between the values of these parameters before and three years after the beginning of therapy (P-0.000).

Statistical significance was not confirmed between the three groups of patients regarding the values of the parameters (ANOVA P-0.256) which indicates that the continent derivation type is not of decisive importance for determination or improvement of renal function.

In the group of 74 patients (148 renal units), 52 patients had significant improvement in renal function (60.5%). In 9 (9.5%) patients, renal function deterioration was reported. Those were the patients with considerably decreased kidney function before the start of therapy. Three patients did not receive or received the advised treatments irregularly. Three patients with bladder augmented by a GIT segment had numerous complications (stomal stenosis, calculosis, frequent infections). Thus, renal function deteriorated over time inevitably leading to kidney transplantation. In two patients, the therapy started rather late when renal function had already considerably deteriorated. Timely initiation of therapy is essential for the preservation of kidney function (15).

In one patient, despite adequate and timely therapy application, there was no kidney function improvement. Hopps and Kroop reported a small percentage of kidney function decrease (3.2%) in a retrospective study dealing with intercontinent vesicostomy and CIC, but the therapy started in the neonatal period. However, this study was not an attempt to achieve continence, unlike our patients' series, but the therapy was started earlier. Lews's study reported the incidence of kidney insufficiency of 18% in patients whose treatment started before puberty in comparison to 30% in patients commencing treatment after puberty (16, 17).

For the preservation of renal function, it is very important to continue patient monitoring using ultrasound check-ups and creatinine concentrations (18).

CONCLUSION

Clean intermittent catheterisations combined with antimuscarinic drugs and antibiotic prophylaxis, if timely started, lead to significant improvement in renal function. The type of continent vesicostomy had no statistical significance for kidney function. Multidisciplinary medical monitoring, even the existence of a clinic for these patients, would certainly contribute to faster diagnosis, earlier initiation of therapy, and preservation of kidney function.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Kragujevac Clinical Centre, decision number 01-1629.

CONFLICTS OF INTEREST

None.

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POPULATION PHARMACOKINETICS OF VALPROATE IN CHILDREN: THE IMPORTANCE OF TOTAL DAILY DOSE, COMPLIANCE AND CO-TREATMENT

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ABSTRACT

Valproate represents one of the most commonly used anticonvulsants worldwide, whose narrow therapeutic range and high potential for drug-drug interactions leads to pronounced intra- and inter-individual variability in plasma concentration and response. The aim of our study was to apply population pharmacokinetics analysis to comprehensively investigate and detect the most important factors affecting pharmacokinetics of valproate in Serbian children with epilepsy. This retrospective observational study was based on demographic and medical data retrieved from the medical records on epileptic patients treated with valproate at the pediatric department of the Clinical Centre, Kragujevac, Serbia. Valproate serum concentrations were obtained as a part of routine medical practice. Population pharmacokinetics analysis was performed by MonolixSuite 2019R1 (Lixoft, Antony, France) software, using one-compartment model with first order absorption and linear elimination. The study included 1642 valproate concentrations obtained from 232 patients, of which 201 (1420 concentrations) were included in the index set used for the modelling, while the other 31 (222 concentrations) were the validation set used for external validation of the final model. Covariate testing based on the whole index set revealed that only total daily valproate dose significanly affected the clearance of valproate: $Cl\ (l/h) = 0.135 \times 1.002^{DD}$. When only compliant patients were included, co-treatment with carbamazepine was shown to be of significance as well: $Cl(l/h) = 0.121 \times 1.002^{DD} \times 1.2^{CBZ}$. Our study demonstrated that valproate clearance correlates with total valproate daily dose. The influence of co-treatment with carbamazepine on valproate pharmacokinetics can be observed and used for clearance estimation only in compliant patients.

Keywords: *Valproate, children, population pharmacokinetics, total daily dose, carbamazepine.*

INTRODUCTION

Epilepsy is a chronic disease characterized by simultaneous irregular discharge of neurons in the brain. The goal of anticolvusive therapy is to achieve complete control of the onset of seizures, with as little side effects as possible (1). Choosing appropriate medication for each individual patient is a hard task, which neurologists are facing in everyday clinical practice. The decision is influenced by many factors, including the type of epilepsy, age of the patient, concomitant therapy, pharmacological drug profile, as well as the pharmacoeconomic aspects (2). The treatment should start with a single drug, which can be later replaced or combined with another, if it turns out to be insufficient to control seizures (1, 3).

Based on the available literature, valproate (VPA) represents one of the most commonly used anticonvulsants worldwide (4). It's narrow therapeutic range and high potential for drug-drug interactions at several pharmacokinetics levels often leads to pronounced intra- and inter-individual variability in VPA plasma concentration and response (5). Polytherapy that combines VPA with other anticonvulsants, such as carbamazepine (CBZ), phenytoin or barbiturates, usually results in increased VPA clearance in both adults and children, mostly due to induction of drug metabolizing enzymes (6). The effect on VPA pharmacokinetics of newer anticonvulsants, such as clobazam, ethosucimide, levetiracetam, or topiramate, has been investigated too, yet with limited or inconclusive results (6-8).

It has been reported that the pharmacokinetics of VPA also strongly depends on age, especially during the infant period and in childhood, and even more if used in children in combination with other anticonvulsants (9). In Tunisia, Ben-Mahmoud et al. showed that the frequency of subtherapeutic serum VPA levels increased significantly at younger age, as well as in the presence of several enzyme inducing anticonvulsants (10, 4). In Mexico, Correa et al. found that body weight, total daily dose and concomitant use of phenobarbital, but not CBZ, were of greatest importance for the disposition of VPA in children (11, 6). The study by Jankovic et al., as the only one available in the literature that included Serbian pediatric population treated with VPA, demonstrated that body weight and concomitant administration of CBZ may have significant effect on VPA clearance (12, 2). However, this study did not examine the possible effect of concomitant use of drugs other than CBZ or lamotrigine.

Besides age and concomitant use of other drugs, it has been observed that patients prescribed with anticonvulsants frequently fail to comply with drug regimen, which often reduces treatment efficacy. Factors that can increase noncompliance could be adverse reactions to drug and inconvenient drug regimens affecting everyday life, as well as the lack of family support, or inadequate relationship with the clinician [13]. In addition, VPA pharmacokinetic profile may be affected by various other demographic and medical factors, such as total daily dose, body weight, and comorbidities (14).

Having in mind that highly variable response to VPA among pediatric epileptic patients has been frequently met in routine practice in Serbia, and that the available literature does not clarify the role of many of the proposed VPA-affecting factors in this population, the aim of our study was to apply population pharmacokinetics (PPK) analysis to comprehensively investigate and detect the most important factors affecting pharmacokinetics of VPA in Serbian children diagnosed with and treated for epilepsy.

MATERIALS AND METHODS

Patients and data collection

This was a retrospective observational study based on the data retrieved from the medical records on epileptic patients treated with VPA at the pediatric department of the Clinical Centre, Kragujevac, Serbia, between March 1st 2010 and March 30th 2020. To be included, patients had to meet the following criteria: 1) age between 1 and 18 years, 2) confirmed diagnosis of epilepsy, 3) VPA treatment lasting for at least 12 months, and 4) availability of relevant demographic and medical data, including at least 3 steady-state VPA serum concentrations. The exclusion criteria were: 1) any concomitant non-anticonvulsive therapy that could significantly affect pharmacokinetics of VPA, and 2) pregnancy or breastfeeding. For each patient, the following information was collected: age, sex, weight, the data on VPA treatment, including drug formulation, total daily dose, compliance with the recommendations on VPA treatment (routinely obtained from patients and/or their parents as a part of anamnesis), and available steady-state serum concentrations, as well as the information on concomitant use of other anticonvulsants, including their total daily dose. VPA steady-state serum concentrations were obtained at least 2 weeks[15] after beginning of the treatment or adjustment of the dose, as a part of routine medical practice. In brief, 3ml blood samples were taken half an hour before the morning dose, and the trough valproate concentrations[15] were determined using the immunoassay technique (Emit® 2000 Valproic Acid Assay, Beckman Coulter, Brea, California), with the linear range between 3.98 and 150 μg/mL, limit of quantification of 3.98 μg/mL, and an assay precision defined by ≤ 5% total coefficient of variation. The study was approved by the ethics committee at the Clinical Centre, Kragujevac, Serbia (approval No 01/20-402, obtained on April 3rd 2020), and conducted in accordance with the Declaration of Helsinki and its subsequent revisions. Given the record-based type of the study, the anonimization of the data, and the risk of authorisation bias, written information consent has not been obtained from the patients[16].

PPK modelling

PPK analysis was performed using MonolixSuite 2019R1 (Lixoft, Antony, France) software, which combines stochastic approximation expectation maximization with a Markov chain Monte Carlo procedure for maximum likelihood

estimation of the pharmacokinetic (PK) parameters in non-linear mixed effect model.

To determine the structural model, one- and two- compartment PPK models with first order absorption and linear elimination were compared (15), and the selection was made based on the precision of the parameter estimation expressed as the relative standard error (RSE), correlation between parameters from the correlation matrix of the estimates, collinearity between parameters expressed as the condition number, as well as on goodness of fit (GOF) plots, including observations vs. predictions plot, distribution of the population- and individual-weighted residuals (PWRES and IWRES, respectively), and the visual predictive check (VPC) plot (17, 18). For the residual variability, combined, proportional and constant error models were assessed, and the selection was based on the estimate for the combined residual error and the value of corrected Bayesian information criteria (BICc) (17). PK parameters were assumed to be lognormally distributed.

To determine the statistical model, covariates that were tested included age, sex, weight, compliance, VPA formulation, VPA total daily dose, as well as concurrent use and total daily dose of other anticonvulsants. To select the informative covariates, all were screened by Pearson's correlation test (for continuous covariates) or ANOVA (for categorical covariates) vs individual parameters and random effects, and those deemed to be potentially significant were assessed by addition to the structural model in order of significance. The covariates were considered relevant and kept as a part of the final model if they decreased the interindividual variability of the affected parameter (expressed as standard deviation of the inter-individual variability i.e. ω), and decreased the values of −2 log-likelihood (−2LL) and BICc. Similarly, correlations between random effects that were screened for using the Pearson correlation tests were selected and added to the final model if they decreased the values of -2LL and BICc (17).

The ability of the final model to capture the data was evaluated by assessing GOF plots, especially observation vs individual and population predictions, and VPC plot. The predictive performance of the final model was tested externally using a validation set, which consisted of data on pediatric epileptic patients that were subsequently collected and not included in the index set used for PK modelling (19). Bias, expressed as mean prediction error (MPE), and precision, expressed as mean absolute prediction error (MAPE), mean squared prediction error (MSE) and root mean squared prediction error (RMSE), were calculated with 95%CI based on the observed VPA concentrations and the mode of the predicted VPA concentrations for each individual (19, 20).

RESULTS

Patients and data collection

The study was based on a total of 1642 VPA concentrations (observations) obtained from 232 patients, of which 201 (1420 observations) were included in the index set used for PPK modelling, while the other 31 (222 observations) were the validation set used for external validation of the final model. Demographic and medication characteristics of the patients are presented in Table 1. Of them, all patiens from the index set and 30 from the validation set were deemed to be compliant with the recommendations on VPA treatment in 1273 and 177 occasions (observations), respectively, and their characteristics are presented separately in Table 2.

PPK modelling

The fitting of the compared structural models without covariates suggested that one-compartment PPK model with first order absorption and linear elimination adequatelly described the available VPA data, and it was subsequently applied as a base model. The constant error model was the most accurate for assessment of inter-individual and inter-occasion variability.

Covariate testing based on the whole index set revealed that only total daily VPA dose significanly affected the clearance (Cl) of VPA, decreasing -2LL and BICc each by approximately 15%, i.e. from 13209.47 to 11252.13, and from 13283.45 to 11333.37, respectively. There was no significant correlations observed between random effects. GOF plots of observed vs. population predicted VPA concentrations by the base and the final model in the whole index set (presented in Figure 1) reveals that the final model improves the data fitting and adequately describes the obtained VPA concentrations, with the proportion of outliers of 5.28%. The values of the PPK final model estimates for VPA Cl in all patients included in the modelling are summarized in Table 3, and the model based on the data from the whole index set can be described as follows:

$$Cl\ (l/h) = 0.135 \times 1.002^{DD}$$

where DD represents the total VPA daily dose in mg. Based on the equation, VPA Cl ranged from 0.201 l/h in patients daily receiving the minimal 200mg VPA dose to 7.341 l/h in those receiving maximal, i.e. 10 times higher dose, with the Cl of 0.604 l/h corresponding to the 750mg VPA daily dose, i.e. the median VPA daily dose in our index set. External validation of the predictive performance of the final model was carried out using a validation set, and the prediction errors of validation set are presented in Table 4. GOF plots of observed vs. population predicted VPA concentrations by the base and the final model in the validation set are presented in Figure 2.

Stratification of the GOF plot of observed vs. population predicted VPA concentrations in the whole index set according to compliance with the recommendations on VPA treatment revelaed that the data fit better for the group of patients/observations that were compliant (Figure 3). Therefore, the data were split by compliance, and only compliant patients/observations were included in the index subset used for additional PPK modelling. One-compartment PPK model with first order absorption and linear elimination and the constant error model were used as a base for covariate testing.

In the index subset consisted of compliant patients/observations, in addition to daily VPA dose that was confirmed to significantly affect VPA PK (decreasing -2LL and BICc each by approximately 18%, i.e. from 13995.27 to 11480.31, and from 11553.38 to 14061.20, respectively), co-treatment with carbamazepine (CBZ) was shown to be of significance as well: it additionally decreased -2LL and BICc to 10082.79 (approx. 13%) and 10163.02 (approx.12%), respectively. There was no significant correlations between random effects. GOF plots of observed vs. population predicted VPA concentrations for comparison of the base and the final model in the index subset of compliant patients/observations (Figure 4) confirmed the suitability and accuracy of the final model, with the proportion of outliers decreased to 4.63%.

The values of the PPK final model estimates for VPA Cl in compliant patients/observations included in the modelling are summarized in Table 3, and the model based on the data from the index subset of compliant patients/observations can be described by the following equation:

$$Cl(l/h) = 0.121 \times 1.002^{DD} \times 1.2^{CBZ}$$

where DD represents the total VPA daily dose in mg, and CBZ has a value of 1 if a patient is co-treated with CBZ, and 0 if not.

Based on the equation, a) if CBZ is not used, VPA Cl values correspond to those obtained by the PPK model based on the whole index set and range from 0.201 l/h to 7.341 l/h, depending on the VPA daily dose; b) if CBZ is used, VPA Cl range from 0.216 l/h to 7.896 l/h in patients daily receiving 200mg to 2000mg VPA, respectively, with the value of 0.650 l/h associated with the median VPA daily dose of 750mg. External validation of the predictive performance of the final model was carried out using a validation subset of compliant patients/observations, and the corresponding prediction errors are presented in Table 4. The corresponding GOF plots of observed vs. population predicted VPA concentrations by the base and the final model in the validation set are presented in Figure 5.

Table 1. Demographic and medication characteristics of all patients included in the study: index set included in PPK modelling and the corresponding validation set

		All patients/observations	
		Index set	Validation set
Demographic data			
No of patients		201	31
Sex (male/female)		84/117	16/15
No of observations		1420	222
Age (years)	range	1 - 17	1 - 17
	median (IQR)	8 (5 - 11)	7 (4 - 11)
Weight (kg)	range	4 - 94	10 - 88
	median (IQR)	29 (20 - 42)	28 (18 - 43)
Medication data			
Valproate			
Daily dose (mg)	range	200.0 - 2000.0	200.0 - 1500.0
	median (IQR)	750.0 (550.0 - 1000.0)	700.0 (500.0 - 1000.0)
Weight-adjusted daily dose (mg/kg)	range	0.3 – 79.6	14.3 - 50.0
	median (IQR)	27.3 (23.1 - 33.3)	27.8 (23.6 – 31.6)
Drug formulation (syrup/retard tbl.)		604/816	119/106
Serum concentration (mg/l)	range	3.7 - 128.0	18.9 - 120.0
	median (IQR)	76.8 (64.0 - 87.0)	75.6 (62.8 - 87.6)
Co-treatment			
Carbamazepine	yes/no	57/1363	11/211
daily dose (mg)	range	100.0 - 800.0	100.0 - 800.0
	median (IQR)	400.0 (300.0 - 600.0)	360.0 (270.0 - 540.0)
Clobazam	yes/no	104/1316	12/210
	range	5.0 - 20.0	10.0 - 20.0

		All patients/observations	
		Index set	Validation set
daily dose (mg)	median (IQR)	15.0 (10.0 - 15.0)	17.5 (15.0 - 20.0)
Ethosuximide	yes/no	69/1351	7/215
daily dose (mg)	range	500.0 - 1000.0	600.0 - 900.0
	median (IQR)	700.0 (600.0 - 800.0)	600.0 (600.0 - 800.0)
Levetiracetam	yes/no	116/1304	10/212
daily dose (mg)	range	300.0 - 1500.0	300.0 - 1000.0
	median (IQR)	1000.0 (675.0 - 1000.0)	750.0 (600.0 - 1000.0)
Topiramate	yes/no	37/1383	7/215
daily dose (mg)	range	50.0 - 200.0	75.0 - 150.0
	median (IQR)	75.0 (60.0 - 100.0)	100.0 (100.0 - 100.0)
Lamotrigine	yes/no	14/1406	5/217
daily dose (mg)	range	50.0 - 75.0	50.0 - 1500.0
	median (IQR)	50.0 (50.0 - 50.0)	50.0 (50.0 - 100.0)

Table 2. Demographic and medication characteristics of compliant patients included in the study: index set included in PPK modelling and the corresponding validation set

		All patients/observations	
		Index set	Validation set
Demographic data			
No of patients		201	30
Sex (male/female)		84/117	16/14
No of observations		1273	177
Age (years)	range	1 - 17	1 - 17
	median (IQR)	8 (5 - 11)	7 (5 - 11)
Weight (kg)	range	4 - 94	10 - 88
	median (IQR)	29 (20 – 42)	30 (20 – 44)
Medication data			
Valproate			
Daily dose (mg)	range	200.0 - 2000.0	200.0 - 1500.0
	median (IQR)	750.0 (600.0 - 1000.0)	700.0 (600.0 - 1000.0)
Drug formulation (syrup/retard tbl.)		518/754	85/92
Serum concentration (mg/l)	range	8.0 - 128.0	55.3 - 120.0
	median (IQR)	79.0 (68.9 - 88.0)	80.0 (70.6 - 91.0)
Co-treatment			
Carbamazepine	yes/no	47/1226	6/171
daily dose (mg)	range	100.0 - 800.0	300.0 - 800.0
	median (IQR)	400.0 (360.0 - 600.0)	500.0 (325.0 - 750.0)
Clobazam	yes/no	96/1177	11/166
daily dose (mg)	range	5.0 - 20.0	10.0 - 20.0
	median (IQR)	15.0 (10.0 - 15.0)	20.0 (15.0 - 20.0)
Ethosuximide	yes/no	55/1218	7/170

		All patients/observations	
		Index set	Validation set
daily dose (mg)	range	500.0 - 1000.0	600.0 - 900.0
	median (IQR)	800.0 (600.0 - 800.0)	600.0 (600.0 - 800.0)
Levetiracetam	yes/no	110/1163	8/169
daily dose (mg)	range	300.0 - 1500.0	300.0 - 1000.0
	median (IQR)	1000.0 (625.0 - 1000.0)	750.0 (675.0 - 1000.0)
Topiramate	yes/no	33/1240	4/173
daily dose (mg)	range	50.0 - 200.0	100.0 - 150.0
	median (IQR)	100.0 (60.0 - 100.0)	100.0 (100.0 - 112.5)
Lamotrigine	yes/no	12/1261	5/172
daily dose (mg)	range	50.0 - 75.0	50.0 - 150.0
	median (IQR)	50.0 (50.0 - 50.0)	50.0 (50.0 - 100.0)

Table 3. PPK model estimates for valproate clearance in patient included in the final PPK model**

	All patients/observations		Compliant patients/observations	
	Estimated value	RSE(%)	Estimated value	RSE(%)
Pop. estimate*	0.135	6.47	0.121	4.9
β_Cl_DD	0.000794	4.66	0.000861	3.72
β_Cl_CBZ	/	/	0.0792	28.5
ω_Cl*	0.059	13	0.046	21.5
γ_Cl*	0.019	26.3	0.022	24.5

RSE(%) – relative standard error; * - l/h; CBZ - carbamazepine co-treatment; DD - daily dose of VPA; ω - standard deviation of the inter-individual variability; γ - standard deviation of the inter-occasion variability; ** - in the base model, parameters of variability, i.e. ω_Cl and γ_Cl, were estimated to 0.317 and 0.130, respectively, in the whole index set, and to 0.304 and 0.133, respectively, among compliant subjects

Table 4. Prediction errors of the final PPK models in validation sets

	All patients/observations		Compliant patients/observations	
	Error	95% CI	Error	95% CI
MPE	-0.098	-1.037, 0.841	-0.185	-1.121, 0.751
MAPE	5.429	4.822, 6.037	5.036	4.468, 5.605
MSE	50.737	37.481, 63.994	40.157	32.285, 48.029
RMSE	7.123	6.122, 8.000	6.337	5.682, 6.930

MPE - mean prediction error; MAPE - mean absolute prediction error;
MSE - mean squared prediction error; RMSE - root mean squared prediction error

Figure 1. Goodness-of-fit plots of observed vs. population predicted VPA concentrations by the base (a) and final (b) models in all patients and observations included in the index set

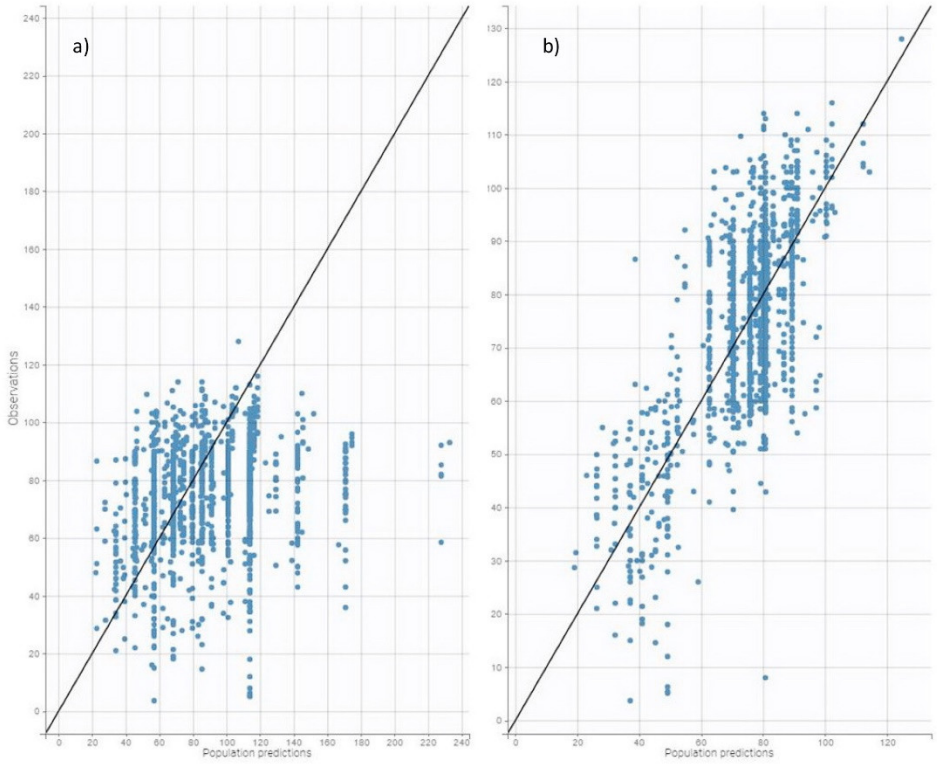


Figure 2. Goodness-of-fit plots of observed vs. population predicted VPA concentrations by the base (a) and final (b) models in all patients and observations included in the validation set

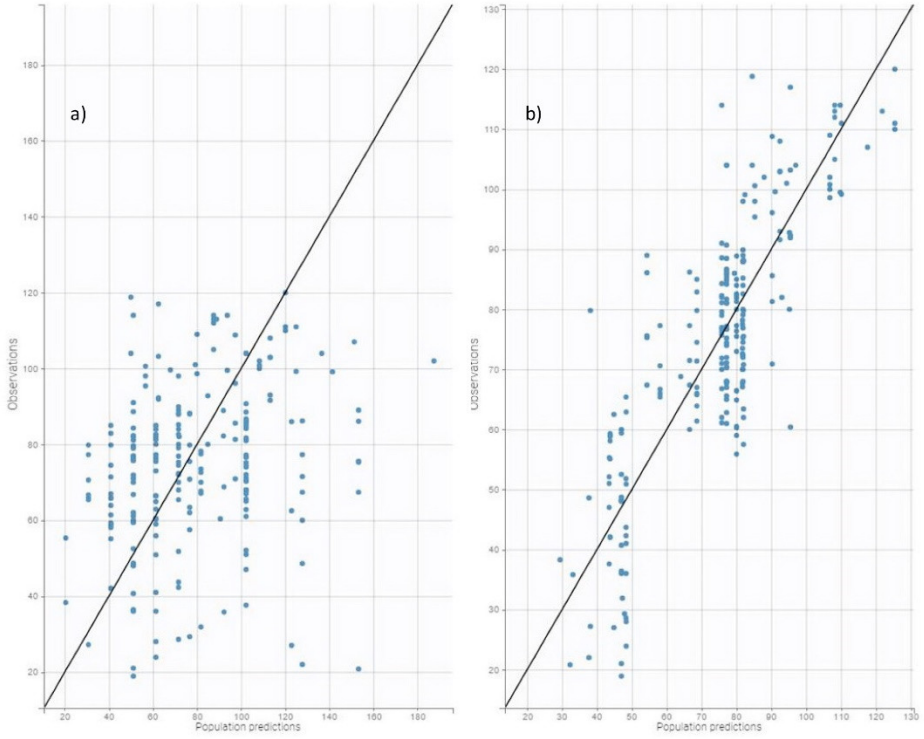
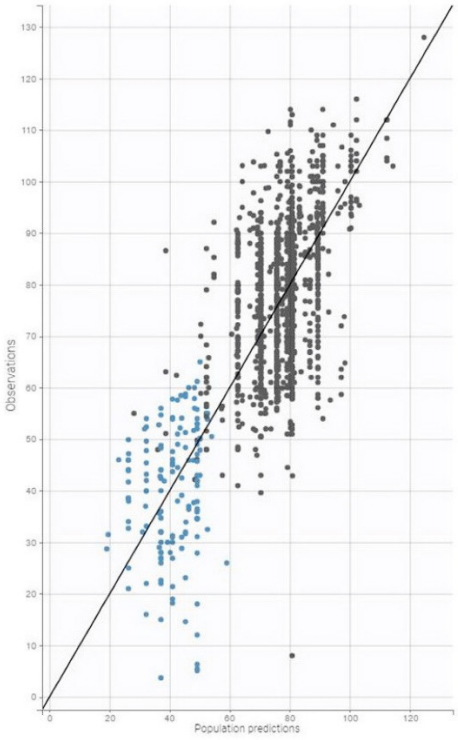


Figure 3. Goodness-of-fit plot of observed vs. population predicted VPA concentrations by the final models in all patients and observations included in the index set, stratified by compliance



compliant patients are marked with black, and non-compliant with blue dots

Figure 4. Goodness-of-fit plots of observed vs. population predicted VPA concentrations by the base (a) and final (b) models in compliant patients and observations included in the index set

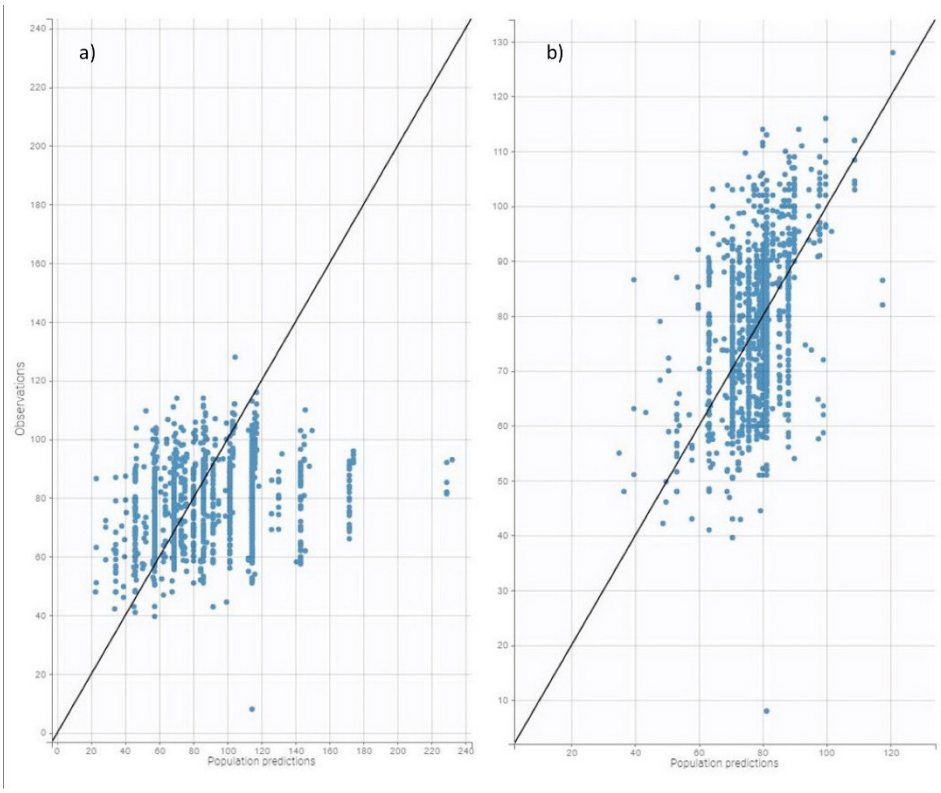
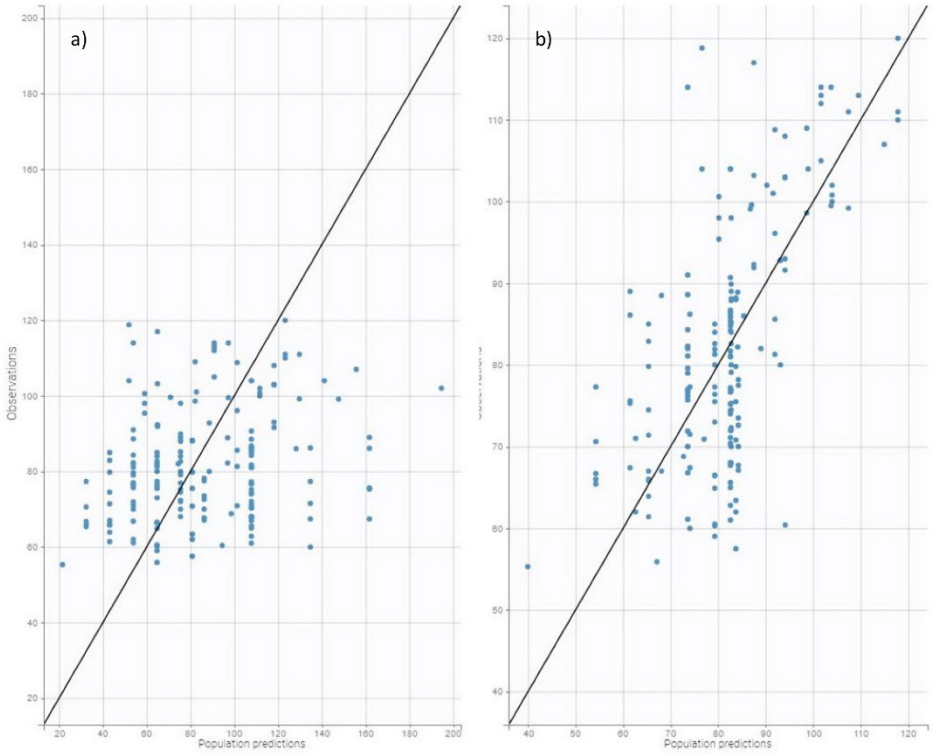


Figure 5. Goodness-of-fit plots of observed vs. population predicted VPA concentrations by the base (a) and final (b) models in compliant patients and observations included in the validation set



DISCUSSION

In the present study, we investigated the influence of several demographic and drug-related factors on VPA pharmacokinetics in Serbian pediatric epileptic patients. PPK analysis revealed that VPA Cl depends on total VPA daily dose, while co-treatment with CBZ exhibits additional effect that can be detected only in compliant patients. Given the pronounced variability in response to VPA observed among pediatric epileptic patients in Serbia that renders the need for better and more informative drug dosing decisions, we hope our findings will add to clinicians' considerations related to VPA-based anticonvulsive treatment.

The total daily dose is generally recognized as an important factor affecting drug pharmacokinetics. In both adults and children, VPA is usually administrated at a low starting dose, which is thereafter increased slowly until the optimal clinical response is achieved, or a maximum tolerated dose reached [1]. Serum drug levels, which serve as a reliable indirect indicator of VPA efficcacy, can be assessed by therapeutic drug monitoring (TDM), which is widely recommended and used as a guide for drug dose adjustment (21, 5). The reference range for the total serum VPA concentration has been set between 50 mg/L and 100 mg/L (350 μmol/L to 700 μmol/L) [6], although seizure control has been reported at concentrations as low as 8.7 mg/L (22). In our study, both the daily dose (total and weight-adjusted) and the observed VPA serum concentrations varied considerably, and spanned the range much wider than the recommended. While the administered dose was usually higher than recommended for pediatric population (9), serum drug concentrations mainly corresponded well to the previous reports (23, 24). Although the difference in age could be at least partly responsible for these findings, the observed variability clearly suggests the presence of other factors that could affect drug concentration and response.

In childhood, VPA pharmacokinetics displays significant variations, one of the most important being related to gradual decrease of VPA Cl with age. Namely, it has been observed that infants may need a dose that is 2-3 times higher than recommended for older children, to achieve the same serum concentration (25, 10). However, our analysis revealed that the main determinant of VPA Cl in children was not the age, but the total daily dose. In spite of the opposite results described as well (12, 26), our finding complies with previous studies reporting higher VPA Cl rate in children treated with higher VPA doses (11, 15, 24). It seems there are two possible explanations for this finding: one related to VPA protein binding and the other to so-called "TDM effect" (15, 24). Namely, it is well known that VPA is up to 95% protein bound (17), with binding rate being concentration-dependent: higher VPA doses lead to binding saturation, resulting in higher unbound VPA fraction (6, 11), which is the only one that can undergo elimination processes and whose increase

accelerates dose-dependent drug Cl. On the other hand, "TDM effect" implies that patients having higher VPA Cl for other reasons will be, due to lower measured serum concentrations, prescribed with higher dose, thus the association between VPA dose and Cl might be easily misunderstood. While most probably both phenomena take a part in the observed (15, 24), inclusion of the total daily dose in the model certainly has a merit in estimating VPA clearance.

Another important feature of VPA is its susceptibility to drug-drug interactions, which usually take place during metabolism. VPA is metabolized through at least three different routes in humans, including glucuronidation, β oxidation, and cytochrome P450 (CYP)-mediated oxidation, responsible for biotransformation of approximatelly 50%, 40% and 10% of the administered dose, respectively (27). While any of the described pathways might endure alterations due to concomitant administration of other drugs, enzyme-inducing anticonvulsants, such as CBZ, phenytoine, or barbiturates, most probably affect VPA pharmacokinetics by inducing relevant UDP-glucuronosyltransferases (UGTs) and CYPs (6). Due to enzyme induction, anticonvulsive polytherapy has a potential to accelerate VPA Cl and the formation of its (frequently toxic) metabolites, decresing its half-life while at the same time increasing the risk of VPA-related adverse effects (6, 27). In the present study, concomitant therapy with CBZ was detected as another determinant of VPA pharmacokinetics, but the association became evident only after non-compliant patients were excluded from the analysis. Having in mind that most of the earlier studies based their analyses on the data from apparently compliant patients (4, 24, 28), our findings correspond well to the previosly published reports. It should be noted that the CBZ-related increase in VPA clearance, as both drugs are highly protein bound, could be at least partly also due to competition in protein binding (29). Namely, CBZ-driven displacement of VPA from protein binding sites will increase unbound VPA fraction and subseqeuntly its clearance. Since increased elimination of unbound fraction will return it to its previous level, the alteration of VPA unbound concentration, as well as the clinical consequences of this type of CBZ-VPA interaction, is expected to be transient. However, CBZ-dependent increase in VPA clearance, even if caused by disruption of VPA protein binding, is expected to last as long as both drugs are taken - not only concomitantly, but also in a regular manner. Regardless of the mechanism, our results imply that concomitant therapy with CBZ should be taken into account when assessing VPA pharmacokinetics, but only after compliance has been established.

It is worth noting that we did test for, but did not identified as important for VPA pharmacokinetics, the following: age, sex, weight, VPA formulation, and concomitant use of clobazam, ethosuximide, levetiracetam, topiramate and lamotrigine. All of the investigated factors were previously attended in VPA-related studies, and more or less frequently associated with at least one of the VPA pharmacokinetic parameters (15). We believe that the lack of significant associations in our study could be explained by the fact that we estimated only Cl, and not the absorption rate constant or volume of distribution, which were previously found to be affected by age, sex, weight, or formulation. In addition, some of the factors, such as co-treatment with anticonvulsants other than CBZ, were not very frequently observed in our study sample, thus there is a possibility that we were underpowered to detect their possible influence.

In conclusion, the present study demonstrated that VPA Cl correlates with total VPA daily dose. The influence of co-treatment with CBZ on VPA pharmacokinetics can be observed and used for Cl estimation only in compliant patients.

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CONFLICT OF INTEREST STATEMENT

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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OXIDATIVE STRESS IN ACUTE MYOCARDIAL INFARCTION PATIENTS

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ABSTRACT

The aim of this study was to assess and compare the oxidative stress status in STEMI and NSTEMI patients by measuring markers of free radical production and levels of antioxidant. The research was designed as an observational study with a cohort of 83 AMI hospitalized patients divided into a case (STEMI) and control (NSTEMI) group. In the study, we used the data from the patients' electronic medical records and paper files and two scores (TIMI, and GRACE) analyzed routine biochemical parameters and biochemical assays for ROS. The statistical analysis included descriptive methods, hypothesis testing and bivariate correlation. In our research, the results clearly showed that patients with STEMI had an increased activity of vSOD compared with NSTEMI patients ($p<0.01$). Also, the levels of TBARS in plasma of patients with STEMI were significantly increased compared to the NSTEMI patients ($p<0.01$). Importantly, there were no differences between STEMI patients and NSTEMI patients, for the levels of O_2^-, H_2O_2, NO, CAT, GSH. In STEMI patients, we found a positive correlation between TBARS and troponin level and a positive correlation between SOD and troponin level. In addition, our results also showed a positive correlation between AST levels and TBARS and a positive correlation between AST levels and SOD. We also found a positive correlation between GRACE score values and TBARS and SOD. Our study demonstrated that the levels of TBARS and SOD were significantly higher in STEMI patients than in NSTEMI.

Keywords: *Acute myocardial infarction, oxidative stress, free radical, troponin.*

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INTRODUCTION

Acute myocardial infarction denotes the presence of acute myocardial injury with the detection of a rise and/or fall of cTn values with at least one value above the 99th percentile URL and with at least one of the following: symptoms of acute myocardial ischaemia, new ischaemic ECG changes, development of pathological Q waves, imaging evidence of new loss of viable myocardium or new regional wall motion abnormality in a pattern consistent with an ischaemic aetiology, and/or identification of a coronary thrombus by angiography including intracoronary imaging or by autopsy (1).

In general, myocardial infarction is divided into STEMI (infarction with ST segment elevation) which requires the reperfusion therapy as soon as possible and NSTEMI (infarction without ST segment elevation), which does not require the reperfusion therapy, except in the following situations: hypotension or shock, repeated and progressive chest pain refracted on the medicamentous therapy, malignant arrhythmias or cardiac arrest, mechanical complications of acute MI, acute heart failure, intermittent ST segment elevation (2). The pathophysiological substrate is represented by the acute, unstable atherosclerotic plaque complicated by occlusive thrombosis (complete interruption of the blood flow in myocardial infarction) (3). STEMI occlusion is long-lasting and necrosis is transmural (it covers the entire wall) and with NSTEMI, the occlusion is of shorter duration, with less necrosis that does not include the entire wall (2,4).

ROS are spontaneously produced as by-product during the electron transfer in oxidative phosphorylation (5,6). ROS molecules include superoxide, peroxide, hydroxyl radicals, and reactive nitrogen species (RNS) (7). Furthermore, ROS oxidize various cellular and extracellular components, including nucleotides, DNA, proteins, polysaccharides, and lipids (5,8). The antioxidant activity is accomplished as a result of the antioxidant scavenger system which includes enzymes superoxide dismutase, catalase and glutathione peroxidase and antioxidant vitamins C, E, A (5,9).

The oxidative stress process involving the increased oxidant production with impairment of antioxidant mechanisms plays an essential role in the pathogenesis of atherosclerosis, and it is increased by the cardiovascular risk factors such as smoking, diabetes mellitus, dyslipidemia, etc (10,11).

The aim of this study was to assess and compare the oxidative stress status in two main groups of acute MI (STEMI and NSTEMI) by measuring markers of free radical production and levels of antioxidant, as well as to analyze patients' redox state considering the diseases activity and other clinical characteristics. We also recorded further treatment strategies after the diagnosis, primary percutaneous coronary intervention, thrombolytic therapies, delayed rescue PCI, or conservative therapy.

MATERIALSAND METHODS

Patients

The research was designed as an observational study which included a cohort of 83 patients

with AMI hospitalized in the Clinic of Cardiology, University Clinical Center "Kragujevac", Kragujevac, between September the 17th and December the 30th, 2019. The study group was divided into case (patients with STEMI) and control patients (patients with NSTEMI). We extracted the data retrospectively from the patients' electronic medical records and paper files (such as years, sex, risk factors, hypertension, hyperlipidemia, diabetes, smoking, heredity, previous vascular disease, Killip classification, etc). The diagnosis of STEMI was defined as the concurrence of prolonged chest pain or discomfort with persistent ST-segment elevation in 2 or more consecutive leads or with the presumed new left or right bundle-branch block with increased cardiac enzymes and presence of symptoms of myocardial ischeamia (12).

The diagnosis of NSTEMI was defined as the concurrence of prolonged chest pain or discomfort without ST segment elevation, (ECG changes can be presented as ST segment depression, transitory ST-segment elevation, T wave changes, or normal ECG findings) and with increased values of cardiac enzymes (13).

The routine biochemical parameters, troponin, CK, CK-MB, hemoglobin, platelets, CRP, lipid status, pro-BNP, AST were analyzed in patients. In our study, we used two scores (Thrombolysis In Myocardial Infarction – TIMI, and Global Registry of Acute Coronary Events – GRACE) in order to assess the prognosis of recurrence of myocardial ischaemia and mortality, and also to select patients who needed urgent revascularization (14). NSTEMI patients with GRACE score of 140 or higher underwent early revascularization (within 24h) and were hospitalized in the ICU, those with GRACE score between 109 – 140 were hospitalized in the hospital ward, and required revascularization within 72 hours of diagnosis, and those with GRACE score less than 109 underwent the conservative treatment (15).

An echocardiographic examination was also performed to determine the ejection fraction and segments mobility (16).

Biochemical Assays

Blood samples were taken from an antecubital vein into the Vacutainer test tube containing sodium citrate anticoagulant. Blood samples were analyzed immediately. Blood was centrifuged to separate plasma and red blood cells (RBCs). Biochemical parameters were measured spectrophotometrically.

Superoxide anion radical determination

The level of superoxide anion radical (O_2^-) was measured using nitro blue tetrazolium (NBT) reaction in TRIS-buffer combined with plasma samples and read at 530 nm.

Hydrogen peroxide determination

The protocol for measurement of hydrogen peroxide (H_2O_2) is based on oxidation of phenol red in the presence of horseradish peroxidase. 200 µl sample with 800 µl PRS (phenol red solution) and 10 µl POD (Horseradish Peroxidase) were combined (1:20). The level of H_2O_2 was measured at 610 nm.

Nitric oxide determination

Nitric oxide (NO) decomposes rapidly to form stable metabolite nitrite/nitrate products. Nitrite (NO_2^-) was determined as an index of the nitric oxide production with Griess reagent. 0.1 ml 3 N PCA (Perchloride acid), 0.4 ml 20 mM ethylenediaminetetraacetic acid (EDTA), and 0.2 ml plasma were put on ice for 15 min, then centrifuged 15 min at 6,000 rpm. After pouring off the supernatant, 220 µl K_2CO_3 was added. Nitrites were measured at 550 nm. Distilled water was used as a blank probe.

Index of lipid peroxidation (thiobarbituric acid reactive substances, TBARS)

The degree of lipid peroxidation in plasma was estimated by measuring of TBARS using 1 % TBA (thiobarbituric Acid) in 0.05 NaOH, incubated with plasma at 100 ^{0}C for 15 min and read at 530 nm. Distilled water was used as a blank probe. TBA extract was obtained by combining 0.8 ml plasma and 0.4 ml trichloroacetic acid (TCA), then the samples were put on ice for 10 min, and centrifuged for 15 min at 6,000 rpm. This method was described previously.

Determination of antioxidant enzymes

Isolated RBCs were washed three times with three volumes of ice-cold 0.9 mmol/l NaCl, and hemolysates containing about 50 g Hb/l (prepared according to McCord and Fridovich) were used for the determination of CAT activity. The CAT activity was determined according to Beutler. Then 50 ll CAT buffer, 100 µl sample, and 1 ml 10 mM H_2O_2 were added to the samples. Detection was performed at 360 nm. Distilled water was used as a blank probe. The SOD activity was determined by the epinephrine method of Misra and Fridovich. A hundred µl lysate and 1 ml carbonate buffer were mixed, and then 100 µl of epinephrine was added. Detection was performed at 470 nm.

Statistics

The statistical analysis was performed with SPSS 15.0 for Windows. The results are expressed as the mean ± standard error of the mean. The data distribution was checked with the Shapiro–Wilk test, and depending on its results, the appropriate parametric or nonparametric test was used. The differences between two groups were assessed using T test or Man-Whitney test, while the differences between the values of means from more than two groups were assessed by ANOVA or Kruskal–Wallis test. The correlation between various variables was found using Bivariate correlation, i.e., Spearman's coefficient of correlation.

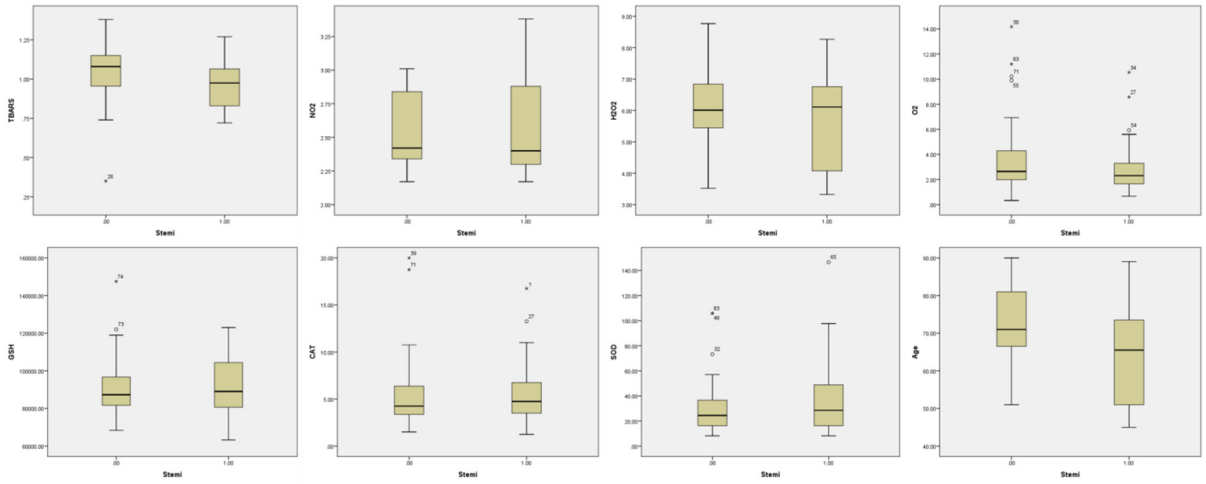
Table 1. Demographic and clinical characteristics of patients with NSTEMI (control) and STEMI. *p<0.01

	NSTEMI	STEMI
Number of patients	34 (41%)	49 (59%)
Age	71.46±1.64	64.5±2.27
AST	49.59±6.52	158.46±30.42*
Troponine	3.64±0.95	42.17±10.57*
CK-MB	29.44±4.83	151.53±21.42*
CK	397.14±96.97	1738.25±287.19*
ProBNP	5769±1271	5162±1457
Haemoglobin	123.72±3.59	128.65±3.68
thrombocytes	242.91±14.54	247.53±15.84
CRP	44.9±9.94	40.96±9.83
Total Chloesterol	5.12±0.22	5.15±0.23
LDL	3.07±0.16	3.27±0.2
HDL	1.1±0.04	1.06±0.04
Triglycerides	1.69±0.16	1.68±0.13
GRACE Score	147.06±4.55	143.37±6.21

Table 2. Parameters of oxidative stress in NSTEMI and STEMI patients.

	TBARS	NO_2	H_2O_2	O_2^-	GSH	CAT	SOD
NSTEMI	1.08±0.03	2.42±0.06	6.01±0.97	3.51±2.54	87278±1492	5.42±4.25	24.42±22.82
STEMI	0.97±0.02	2.4±0.1	6.1±2.21	2.99±2.17	91138±1735	5.53±4.75	28.49±32.49

Figure 1. Oxidative stress parameters in NSTEMI (0) and STEMI (1) patients. *p<0.01.



RESULTS

Eighty-three patients with AMI (mean age 71.46 years, SD ± 11.29, range 51 - 90) had a clear diagnosis of STEMI or NSTEMI based on the clinical characteristics, troponin values and ST segment deviation on ECG. The median duration of hospitalization for STEMI was 4 days (2 to 6 days) and for NSTEMI was 8 days (6 to 10 days). The demographic and clinical characteristics of patients with STEMI and NSTEMI (controls) are summarized in Table 1. STEMI was diagnosed in 49 patients (59%), while NSTEMI was diagnosed in 34 patients (41%). The parameters of oxidative stress are shown in Table 2 (comparison of the status of free radicals and antioxidant in STEMI and NSTEMI).

In terms of the oxidative stress parameters, STEMI and NSTEMI patients differed in two out of seven investigated parameters which included the index of lipid peroxidation, and activity of SOD (Figure 1). In our research, the results clearly showed that patients with STEMI had an increased activity of SOD compared with NSTEMI patients ($p<0.01$). Also, the levels of TBARS in plasma of patients with STEMI were significantly increased compared to the NSTEMI patients ($p<0.01$). Importantly, there were no differences between STEMI patients and NSTEMI patients, for the levels of O_2^-, H_2O_2, NO, CAT, GSH (Figure 1).

In STEMI patients, we found a positive correlation between TBARS and troponin level and a positive correlation between SOD and troponin level. In addition, our results also showed a positive correlation between AST levels and TBARS and a positive correlation between AST levels and SOD (Suppl. 1).

We also found a positive correlation between GRACE score values and TBARS and SOD (Suppl. 1).

DISCUSSION

Many studies have demonstrated a role of ROS in the pathogenesis of acute coronary syndrome (17, 18). In the basis of formation of atherosclerotic plaque, there is a complex process involving several mechanisms, including the endothelial dysfunction, neovascularization, vascular proliferation, apoptosis, matrix degradation, inflammation, and thrombosis (19,20,21). The key process in atherosclerotic plaque formation includes destruction of well-balanced homeostatic mechanisms, which incurs the oxidative stress (11,22).

For that reason, it is essential to investigate the role of oxidative stress in cardiovascular disease processes, such as atherogenesis, ischemic-reperfusion injury and cardiac remodeling (23,24). Currently, an increasing number of studies suggest that the levels of oxidative stress markers in body fluids had a positive correlation with the atherosclerotic disease activity (25, 26).

Koprivica et al. study demonstrated that ACSs patients had a significantly less efficient antioxidative defense system

compared to healthy subjects, significantly lower levels of SOD and CAT activity which resulted in significantly increased levels of lipid peroxidation. Moreover, this study clearly showed that the oxidative status between STEMI and NSTEMI patients differed only in the SOD level (27). Our results also undoubtedly showed that the SOD level is higher in STEMI patients compared to NSTEMI patients which is analogous to the previously mentioned research.

Lavall et al. study included the oxidative profile of patients with ST segment elevation and patients with non-ST-segment elevation (NSTEMI). The results of this study showed that plasma TBARS and SOD activity were significantly higher in STEMI patients compared to the control (NSTEMI) (28). Also, Yosri Noichri et al. results showed that patients with AMI compared to healthy subjects had a significantly lower catalase activity and significantly higher TBARS levels (29). Our research showed that STEMI patients had a higher level of SOD activity and also had a higher level of plasma TBARS compared to NSTEMI patients which is also in correlation with the previously mentioned researches.

Bagatini et al. results demonstrated an increase in substances reactive to thiobarbituric acid (TBARS) and carbonyl protein levels in AMI and the risk groups. In addition, the research showed a positive correlation between TBARS, carbonyl protein levels, and troponin in AMI patients (30). Moreover, in our study, the results clearly showed a positive correlation between TBARS and troponin, which is also in correlation with the previously mentioned study. These findings reinforce the fact that the increased lipid peroxidation is associated with the infarct size and degree of cellular damage (31, 32).

The previous studies have clearly indicated that AMI patients are under higher oxidative stress compared with the control (healthy subjects), no matter which type of AMI they have (27,33). The results of our study demonstrated that the oxidative profile generated by STEMI and NSTEMI is similar regardless of the size of arterial occlusion. The results associated with the investigation of oxidative stress in STEMI and NSTEMI patients suggest that the antioxidant supplementation is needed to improve the antioxidant defense, since the antioxidant defense is compromised and lipid peroxidation is increased in those patients (34, 35).

The study of Shahzad et al found a positive correlation between the level of oxidative stress markers and Grace score, which is in agreement with our results that TBARS and SOD rise were associated with high Grace score values (36).

CONCLUSION

Our study demonstrated that the levels of TBARS and SOD were significantly higher in STEMI patients than in NSTEMI. They were also statistically significantly higher in STEMI patients with high values of AST and troponin. In addition, the value of Grace score and age of a patient were associated with the value of mentioned markers of oxidative stress. Other parameters of oxidative stress didn't show a significant correlation with AMI type.

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CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

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PHARMACOECONOMIC ASPECTS OF TREATING CHILDHOOD PNEUMONIA - COST OF ILLNESS STUDY BASED ON DATA FROM SERBIA

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ABSTRACT

The pharmacoeconomic aspect of the treatment of pneumonia in the pediatric population is scarce analyzed in the countries of the Balkan region. This research aimed to determine the value of total hospital costs of treating childhood pneumonia from the Republic Fund of Health Insurance perspective. This retrospective cost of illness study was performed using the "from the bottom to the top" approach. It included 82 patients with childhood pneumonia who were treated at Clinical for pediatrics at Clinical Centre Kragujevac. The total costs of hospital treatment of pneumonia for the examined population were 4,095,293.73 RSD. The largest share in total hospital costs has the length of patient hospitalization (22%). The median total hospital costs per patient per year amounted to 40,249.91 RSD (4,793.10 - 142,149.63 RSD). Since the main determinants of overall costs of treating childhood pneumonia are the length of stay and noninvasive ventilation rate, therapeutic strategies should provide not only efficient treatment in compliance with current guidelines as also decreasing these determinants to offer a better pharmacoeconomic profile of childhood pneumonia.

Keywords: *Childhood pneumonia, direct costs, cost of illness study.*

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INTRODUCTION

Pneumonia represents the most severe form of acute lower respiratory infection and also one of the most frequent reasons for hospital treatment among the children population in modern health systems (1). The incidence of pediatric pneumonia has shown decreasing tendency, but this medical condition remains one of the most significant causes of childhood morbidity (2). The occurrence of pediatric pneumonia positively correlates with diverse risk factors as chronic medical conditions, malnutrition, acquired infections, exposure to smoke, and low socioeconomic status (3). Clinical presentation of pediatric pneumonia diverse regarding age, immunological status of patient, severity of infection as well etiological cause of pneumonia (4). Although treating pneumonia in children nowadays correlates with positive outcomes, this medical condition persists as a serious illness, mainly because pneumonia is the leading cause of mortality in children younger than 5 years.

Childhood pneumonia is an illness that mostly requires treatment in the hospital. Approximately 3 to 18% of hospital admissions among the pediatric population are due to pneumonia (5, 6). Treating childhood pneumonia in hospitals generates a substantial burden of this medical condition, especially in low- and middle-income countries. Results from pharmacoeconomic studies indicate that treating childhood pneumonia encompasses direct, "obvious" costs due to hospital care, laboratory and imaging diagnostic, i.v. administration of antibiotics are, but also "hidden" fees derived from the inability of parents to work or to take care of family due to care of the ill child (7). It is estimated that the total costs of treating one episode of pneumonia are 5 to 13 fold higher in developed countries comparing to countries with low- and middle-income countries. Despite these facts, the pharmacoeconomic impact of childhood pneumonia in low- and middle-income countries shouldn't be underestimated since direct medical costs due to hospitalization and indirect costs due to parents` inability to work still represent a significant part of monthly income (5, 7).

Cost of illness studies could contribute to better understanding not only size but also the structure of total costs of treating a medical condition of interest and also provide data that can be used for adequate allocation of resources within health systems, especially in countries with a recent history of socioeconomic transition as countries from Balkan region are (8).

This study aimed to estimate the total costs of treating childhood pneumonia from the perspective of the Republic Health Insurance Fund. Also, evaluating the structure of total costs and its main determinants was encompassed with the aim of this study.

PATIENTS AND METHODS

Study settings and perspective

To evaluate and analyze pharmacoeconomic aspects of treating childhood pneumonia in a hospital, we performed a cost of illness study, using the "from bottom to the top" approach. This retrospective cost of illness study was conducted from the perspective of the Republic Fund of Health Insurance. This research complied with Consolidated Health Economic Evaluation Reporting Standards (CHEERS) for performing pharmacoeconomic studies established by the International Society for Pharmacoeconomics and Outcomes Research (ISPOR) (9). Performing of this study was approved by the Ethics Committee of Clinical Center Kragujevac (N°of approval -01/18-843).

Study population

In order to estimate the total direct costs of treating childhood pneumonia, we included patients who were treated due to pneumonia at the Pediatric Clinic of Clinical Center Kragujevac for one year (January 2018 to - December 2018). Inclusion criteria were patients who were treated in hospital with diagnosis at admission or discharge of pneumonia (code of disease: J12.0, J12.1, J12.2, J12.3, J12.8, J12.9, J13, J14, J15.0, J15.1, J15.2, J15.3, J15.4, J15.5, J15.6, J15.7, J15.8, J15.9, J16.0, J16.8, J17.0, J17.1, J17.2, J17.3, J17.8, J18.0, J18.1, J18.2, J18.8, J18.9). Patients with incomplete medical documentation were excluded from the study.

The study population was calculated using formula N = (1.96)2*4* SD2/d2, where SD is standard deviation and d desirable confidence interval) and data available from the study of Madsen HO et al. The estimated sample size was 25 patients, and to increase the power of the study, we finally included 82 children randomly chosen in consolidation within inclusion criteria (10, 11).

Demographic and Clinical Data

Demographic and clinical data were extracted from medical records of patients who were assigned to the study population. We included general demographic data such as age and sex of patients and also medical data regarding pneumonia: number of episodes, number of hospitalizations, the length of hospitalizations on general word, length of hospitalizations on intensive care unit, laboratory and imaging tests performed during hospitalizations, specialists' exams, pharmacotherapy, and hospital care.

Costs

All medical data were translated into pharmacoeconomic data using Tariff Book and the price of drugs available in electronic files at the Republic Fund of Health Insurance web pages (12, 13). This pharmacoeconomic study was performed from the Republic Fund of Health Insurance perspective, meaning that only direct costs were extracted from medical files and evaluated.

All data were inserted into the Excel file and processed using descriptive statistics in the SPSS program, version 21 (14). Multiple linear regression analysis was used to determine the main determinant of the total cost of treatment of childhood pneumonia. Data about costs are presented as median costs and ranges in Republic Serbian Dinars (RSD) and also in Euros (EUR) (15).

RESULTS

The study population included 82 patients, and among them, 49% were male, and 51% were female. Regarding age, 2.4% were newborns, 53.7% were infants, 40.2% were toddlers and 2.43% were adolescents. Community-acquired pneumonia was detected in 68% of patients, while hospital-acquired pneumonia was presented in 32% of patients. According to the etiological cause of pneumonia, 12.19% of patients had bacterial pneumonia; virus as an etiological factor was detected in 15.85% of patients, while 8.53% and 63.4% of patients had atypical and non-classified pneumonia, respectively. The median number of pneumonia episodes per patient per year was 1 (1-4). The median length of hospitalization was 6 (0-33) days on the general ward and 0 (0-7) days on intensive care units. There were 49 (59.8%) patients who required noninvasive ventilation ≤ 24 hours and 33 (40.2%) who did not.

Total costs of treating childhood pneumonia for the study population per one year were estimated at 4,095,293.73 Dinars (RSD), which is 34,829.55 EUR.

The median of total costs of treating childhood pneumonia in the hospital was 40,249.91 RSD (4,793.10 RSD - 142.149,63 RSD), respectively 342.31 EUR (40.76 EUR - 1208.95 EUR).

Table 1 contains data on total hospital costs of treating childhood pneumonia regarding gender of patients.

Total hospital costs of treating childhood pneumonia regarding age of patients are presented in Table 2.

Table 3 contains data on total hospital costs of treating pneumonia according to the classification of pneumonia.

Pharmacoeconomic aspects of treating pneumonia regarding cause are presented in Table 4.

The structure and percent representation of direct costs in total costs of treating childhood pneumonia is presented in Figure 1.

Table 1. Total hospital costs of treating childhood pneumonia per patient per year regarding gender

Gender	Total hospital costs per patient per year	
	Median	Range
Male	38,170.30 RSD 324.63 EUR	13,151.52 RSD - 142,149.63 RSD 111.85 EUR -1,208.95 EUR
Female	47,454.22 RSD 403.58 EUR	4,793.10 RSD - 127,388.97 RSD 40.76 EUR - 1,083.41 EUR

Table 2. Total hospital costs of treating childhood pneumonia per patient per year regarding age

Age of patients	Total hospital costs per patient per year	
	Median	Range
Newborns	36,878.94 RSD 313.67 EUR	25,787.02 RSD - 47,970.85 RSD 219.33 EUR - 408.01 EUR
Infants	45,261.76 RSD 384.96 EUR	13,151.52 RSD - 142,149.63 RSD 111.85 EUR - 1,209.53 EUR
Toddlers	34,760.43 RSD 295.65 EUR	4,793.10 RSD – 137,732.37 RSD 40.76 EUR - 1,171.48 EUR
Adolescents	44,798.51 RSD 381.0 EUR	40,316.62 RSD – 45,837.31 RSD 342.0 EUR - 389.86 EUR

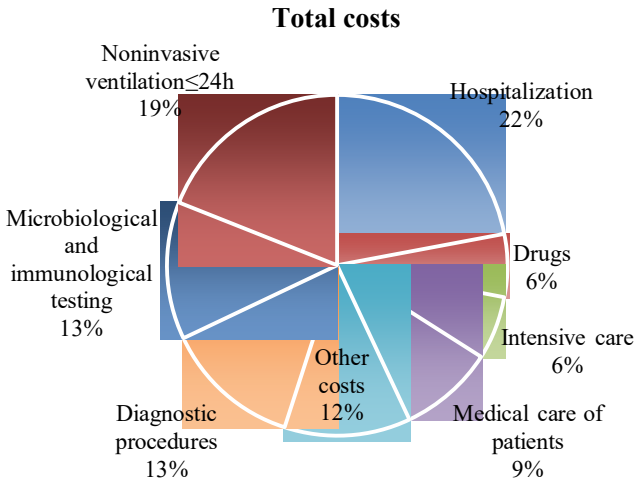
Table 3. Total hospital costs of treating childhood pneumonia per patient per year regarding classification of pneumonia

Type of pneumonia	Total hospital costs per patient per year	
	Median	Range
Hospital-acquired pneumonia	43,101.56 RSD 366.56 EUR	13,151.52 RSD – 142,149.63 RSD 111.85 EUR – 1,208.95 EUR
Community acquired pneumonia	40,018.25 RSD 340.34 EUR	4,793.10 RSD – 137,722.37 RSD 40.76 EUR – 1,171.29 EUR

Table 4. Total hospital costs of treating childhood pneumonia per patient per year regarding etiological cause of pneumonia

Type of pneumonia	Total hospital costs per patient per year	
	Median	Range
Bacterial	39,787.51 RSD 338.38 EUR	6,956.58 RSD – 80,507.83 RSD 59.16 EUR – 684.70 EUR
Viral	43,539.68 RSD 370.28 EUR	13,128.28 RSD – 142,139.53 RSD 111.65 EUR – 1,208.86 EUR
Atypical	46,937.59 RSD 399.18 EUR	23,137.67 RSD – 136,832.09 RSD 196.77 EUR – 1,163.72 EUR
Non-classified	35,943.71 RSD 305.69 EUR	4,793.1 RSD – 137,722.37 RSD 40.76 EUR – 1,171.29 EUR

Figure 1. Main determinants of total costs of treating childhood pneumonia



Multiple regression analysis resulted in the model that explained 73.0% of the cost variance (F(3,78)=70,297, p=0.00). The main contributors to the total costs of treating childhood pneumonia are: use of non-invasive ventilation (B=16.244,83, 95% CI (7.953,51-24.536,14), p<0.05), followed by length of hospitalizations at intensive care units (B=11.078,55, 95%CI (8.376,84-13.780,26, p<0.05) and length of hospitalization at general ward (B=3.030,54, 95% CI (2.386,62-3.674,45), p<0.005). Regarding collinearity diagnostics, Variance Inflation factor (VIF) is estimated on 1,217, 1,010 and 1,233 for use of non-invasive ventilation, length of hospitalization at general ward and length of hospitalizations at intensive care units respectively indicating no collinearity (moderate collinearity) among these variables.

DISCUSSION

The cost of illness studies encompasses different aspects of treating a medical condition of interest and provides transparent insight into the economic sphere and clinical and socioeconomic consequences of disease of interest (15). Results from these kinds of pharmacoeconomic studies could contribute to better allocation of the health budget, which is crucial for decision-makers within health systems, especially in countries with a recent history of socioeconomic transition as countries in the Balkan region are. We believed that costs of treating childhood pneumonia in the Balkan region are presented in a limited manner and that this study could provide new insight in treating childhood pneumonia to key stakeholders in the Balkan region.

Total costs of treating childhood pneumonia for the examined population were estimated at 34,829.55 €, with a median of costs of 342.31 EUR per patient per year. These results were similar to data from a study conducted by Ceyhan M et al., where the median of costs of treating childhood pneumonia was about 330.09 € (16). Ayieko P. et al. indicated that treating childhood pneumonia within the health system in Kenya demanding 104.2 € per patient, which is lower than in Serbia. Similarly, results from a pharmacoeconomic study from the Gambia pointed that treating outpatient and inpatient pneumonia was estimated at 8 USD (6.68 €) and 34 USD (28.42€) per patient from the provider perspective (17, 18). On the other hand, all direct costs derived from treating one episode of childhood pneumonia were significantly higher in Germany, with a median of all direct costs of 1,133.00 USD (947.16€) (19). These discrepancies among values of direct costs are derived from differences within specifics of the organization of presented health systems, especially in the sphere of valuing and pricing of medical services, which mainly depends on these countries' economic position and healthcare policies. Yet, despite these differences among costs of treating childhood pneumonia within different countries, the economic burden of this medical condition remains significant for most health systems (5, 7).

Recent pharmacoeconomic studies indicate that the length of hospital stay is one of the dominant contributors to the overall costs (20). According to the World Health Organization (WHO) guidelines, the recommended length of hospitalization for the treatment of pneumonia in the pediatric population is 5 days (20-22). In our study, the length of hospitalization in the general care department, which is also the determinant with the largest share (22%) in total treatment costs, had a median value of 6 hospital days, which is longer than the WHO recommendations. Our findings are consistent with the results of Zhang S. et al. from 2006, where the median of the length of stay in the hospital for severe pneumonia was 7.9 days (5.5-9.2) for high-income countries and 6.4 days (4.1-7.1) for low- and middle- income countries (23). Our results have pointed that one additional day of treatment at intensive care units generates an increase of the total costs of treating childhood pneumonia for 94.22 EUR. One additional day on the general ward will increase the total costs of treating childhood pneumonia by 25.77 EUR.

The length of hospital treatment correlates positively with the value of the total cost of treating pneumonia. These kinds of results are expected since hospitalization per se generates new medical services and procedures, which promote an increase in the total costs of treating disease. Our results have shown that noninvasive ventilation ≤24 hours is the second-largest determinant in total costs of treating childhood pneumonia with 19% participation in total costs, especially in infants, which complies with guidelines of treating acute respiratory failures (24). Prices derived from the use of noninvasive ventilation ≤24 hours are not only crucial regarding its volume but also in the manner of significant contribution to total costs. Our results have shown that one additional use of noninvasive ventilation ≤24 hours augments total costs of treating childhood pneumonia for 138.51 EUR.

Therefore, hospital treatment should be more efficient in diagnostic and therapeutic terms to rationalize cost and better distribution of economic resources. On the other hand, lower prices of health services which are typical for low- and middle- income countries, cannot be a justification for a more significant number of hospital days because hospitalization itself is a risk for the emergence of new clinical conditions that can be important in medical and pharmacoeconomic terms (17).

Our study has few limitations. Our study was retrospective. We included only direct costs due to reason we had only access to the medical data of patients, so we could expect that the total costs of treating childhood pneumonia are higher. Also, the total size of our population was 82 patients, which don't represent all population of patient with childhood pneumonia, but it is almost 4 fold time higher than the calculated sample size.

CONCLUSION

To decrease the overall costs of treating childhood pneumonia in hospital settings, it is necessary to reduce the length of stay and noninvasive ventilation rate through more efficient diagnostics and timely implementation of therapeutic measures confirmed as effective and safe in previous clinical trials. Identifying the costs underlying the management of childhood pneumonia could provide essential information for all stakeholders and contribute to better allocation of health services for child health, which is of great concern in countries with a recent history of socioeconomic transition as countries from the Balkan region are.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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ASSESSMENT OF THE INFLUENCE OF POSTDILUTION ONLINE HEMODIAFILTRATION ON THE RATE OF REMOVAL OF MIDDLE MOLECULAR WEIGHT UREMIC TOXINS

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ABSTRACT

Hemodiafiltration is a method of treatment used to replace kidney function, which effectively removes uremic toxins of middle molecular weight from the blood of patients with the end-stage of chronic kidney disease. The aim of this study was to examine the effect of postdilution online hemodiafiltration on the degree of β_2-microglobulin removal. Thirty patients treated with postdilution online hemodiafiltration were examined. The main parameter for assessing the removal efficiency of uremic toxins of middle molecular weight was the concentration of β_2-microglobulin in the serum before and after a single session of postdilution online hemodiafiltration. The following tests were used for statistical analysis: Shapiro-Wilk test, Student's T test for bound samples and Wilcoxon test. The average total convective volume is 21.38 ± 2.97 liters per session. The reduction index of β_2-microglobulin during a single session of postdilution online hemodiafiltration is 70.86 ± 6.87%. The average loss of albumin during a single postdilution online hemodiafiltration is 2.50 ± 0.92 g/4h, and the albumin reduction index is 6.20 ± 2.12%. Postdilution online hemodiafiltration effectively removes β_2-microglobulin from the blood of patients with end-stage chronic kidney disease. The reduction index of β_2-microglobulin is ~ 71.00% and the loss of albumin is less than 4.0 g/4h. This dialysis modality prevents the development of dialysis-related amyloidosis and atherosclerotic cardiovascular diseases in the population of patients treated with regular hemodiafiltration.

Keywords: *Hemodiafiltration, uremic toxins, β_2-microglobulin, albumin.*

INTRODUCTION

Cardiovascular diseases are the leading cause of death in patients treated with regular dialysis (1, 2). In patients with end-stage chronic kidney disease treated with dialysis, there is an increase in the concentration of uremic toxins in the serum. Depending on the molecular weight, uremic toxins can be divided into three categories: uremic toxins of low molecular weight (MW < 500 Da) that are soluble in water, uremic toxins that bind in a high percentage to plasma protein (degree of binding to plasma protein > 90%), which are mainly of low molecular weight (MW < 500 Da) and uremic toxins of middle molecular weight (MW = 0.5-60 kDa) (3). Middle molecular weight uremic toxins include pro-inflammatory cytokines (interleukin-1β, interleukin-6, interleukin-18, tumor necrosis factor alpha – TNFα), proteins (pentraxin-3, YKL-40) and adipokines (leptin) (3). Pro-inflammatory cytokines and proteins play a significant role in the development of microinflammation, while leptin plays a significant role in the development of malnutrition in patients treated with regular dialysis (3). Microinflammation, malnutrition and oxidative stress are significant non-traditional risk factors, resulting in the development of atherosclerosis (atherosclerotic cardiovascular disease), amyloidosis, malnutrition, erythropoietin resistance and anemia (3-10).

Hemodiafiltration (HDF) is a method of treatment used to replace kidney function, which has a protective effect on the cardiovascular system and significantly improves the outcome of patients with the end-stage chronic kidney disease (11-15). Postdilution online hemodiafiltration combines diffusion and convection. The diffusion process removes uremic toxins of low molecular weight, while the process of convection (convective transport) removes uremic toxins of middle molecular weight. The diffusion rate depends on the blood flow strength (Qb, mL/min) and the flow rate of the dialysis solution (Qd, mL/min), while the convection rate depends on the blood flow rate (Qb, mL/min) and the ultrafiltration rate (Quf, mL/min) (11-15). The convective flow rate is the sum of the flow rate of the substitution solution (Qsubs, ml/min) and the net ultrafiltration rate (Qnuf, mL/min). The rate of net ultrafiltration is the actual loss of fluid from the patient's body during hemodiafiltration treatment (11-15).

Postdilution online hemodiafiltration effectively removes uremic toxins of middle molecular weight, and its efficiency depends on the total convective volume (Vconv), that is, on the characteristics of the dialyzer, blood flow through vascular hemodialysis (Qavf ≥ 600 mL/min) and the blood flow rate (Qb ≥ 350 mL/min) (11-15). The main characteristics of the dialyzer are: high ultrafiltration coefficient (Kuf > 40 mL/h x mmHg), sieving coefficient for beta-2-microglobulin greater than 0.60, sieving coefficient for albumin less than 0.01 (loss of albumin per session less than 4.0 g), capillary density greater than 11.000 allows the flow of dialysis solution Qd = 400-500 mL/min, internal diameter of dialyzer capillaries greater than 200 μm, sterilization without ethylene oxide, absence of bisphenol A (BPA) and good biocompatibility. Dialyzers with dialysis membranes with an area of ≥ 2.0 m^2 (11-15) should be used to optimize the filtration fraction.

The total convective volume (Vconv) is the sum of the volume of substitution (Vsubs) and the volume of net ultrafiltration (Vnuf): Vconv = Vsubs + Vnuf (4, 5). The volume of substitution is calculated from the formula Vsubs = Qsubs x T, where: Qsubs is the rate of the flow of substitution solution (mL/min), and T is the duration of individual hemodiafiltration treatment (4.0 h = 240 min). The volume of net ultrafiltration is calculated from the formula Vnuf = Qnuf × T, where: Qnuf is the rate of net ultrafiltration (mL/min), and T is the duration of individual hemodiafiltration treatment (4.0 h = 240 min) (4, 5). The target total convective volume in patients treated with postdilution online hemodiafiltration should be ≥ 22 liters per session (11-15).

In clinical practice, measurement of serum β_2-microglobulin and albumin concentrations, before and after treatment, is used to assess the efficacy of removal of middle molecular weight uremic toxins during the treatment of postdilution online hemodiafiltration. Based on the concentration of β_2-microglobulin in the serum before and after the treatment of postdilution online hemodiafiltration, the reduction index of β_2-microglobulin (RR - Reduction Ratio) is calculated. It is calculated from the following formula: RR (%) = [1-(Cpost/Cpre)] × 100, where: Cpre is the serum β_2-microglobulin concentration before hemodiafiltration treatment (mg/l), and Cpost is the β_2-microglobulin concentration in serum after treatment of postdilution online hemodiafiltration (mg/L). During the treatment of postdilution online hemodiafiltration, less than 4.0 g of albumin (≤ 4.0 g/4h) is lost, which is of great importance in order to prevent the development of malnutrition (11-15). According to the recommendations of the JSDT (Japanese Society for Dialysis Therapy), the predialysis concentration of β_2-microglobulin in the serum should be less than 30 mg/L, or less than 25 mg/L (11-15).

MATERIALS AND METHODS

The study examined 30 patients treated with regular postdilution online hemodiafiltration at the Center for Nephrology and Dialysis of the Clinical Center Kragujevac. The examination was conducted in compliance with the Helsinki Declaration on Medical Research, obtained the consent of the Ethics Committee of the Clinical Center Kragujevac (Decision of the Ethics Committee No. 01-20-765) and the consent of patients.

Patients that were examined were treated with regular postdilution online hemodiafiltration, three times a week for 4 hours (12 hours per week), "high-flux" biocompatible membrane (Diacap Pro 19H; α Polysulfone Pro; surface area 1.9 m^2; gamma sterilization; sieving coefficient for β_2-microglobulin – SC = 0.7; sieving coefficient for albumin – SC < 0.001; mass transfer for urea – KoA = 1145; ultrafiltration coefficient – Kuf = 97 mL/h/mmHg; manufacturer B. Braun Medical Inc., U.S.A. Corporate Headquarters), on machines with controlled ultrafiltration type Fresenius 5008S, Gambro

Artis and B. Braun, with average blood flow rate – Qb = 257.00 ± 18.65 mL/min and average dialysate flow rate – Qd = 500.00 ± 0.00 mL/min. A standard ultrapure hemodiafiltration solution (number of bacterial colonies < 0.1 CFU/mL, endotoxin concentration – E < 0.03 EU/mL) was used, with a calcium concentration of 1.75 mmol/L (PGS21), 1.50 mmol/L (PGS25) and 1.25 mmol/L (PGS27). The concentration of sodium Na^+ in the solution for online hemodiafiltration was 140 mmol/L, the concentration of bicarbonate was 35 mmol/L, and the concentration of potassium K^+ was 2.0 mmol/L. The average total convective volume was Vconv = 21.38 ± 2.97 liters per session. Unfractionated heparin was used for anticoagulation of extracorporeal circulation. The average monthly dose of unfractionated heparin was 4508.32 ± 541.92 IU. All patients were treated with agents that stimulate erythropoiesis (short-acting: epoetin-α, epoetin-β; long-acting: darbepoetin-α). The study did not include patients with active infection (average leukocyte count was 6.18 ± 1.72 × 10^9/L), proven active bleeding, uncontrolled malignancies, as well as patients treated with immunosuppressive medicines.

In order to assess the degree of removal of uremic toxins of medium molecular weight and the degree of protein loss during a single session of postdilution online hemodiafiltration, the concentration of β_2-microglobulin and albumin in serum was examined before and after the hemodiafiltration session with Diacap Pro® 19H dialysis membrane. Based on the measured concentration of β_2-microglobulin, the Reduction Ratio – RR was calculated using the formula: RR (%) = [1- (Cpost/Cpre)] × 100, where: Cpre is the concentration of β_2-microglobulin in serum before postdilution online hemodiafiltration treatment (mg/L), and the Cpost is the serum β_2-microglobulin concentration after post-dilution online hemodiafiltration treatment (mg/L).

A blood sample for laboratory analysis was taken before the start and after the end of the average weekly single postdilution online hemodiafiltration, before heparin administration. Routine laboratory analyses were determined by standard laboratory tests and were calculated as the average value of three measurements over three consecutive months.

Serum albumin concentration was determined by turbidimetric method, on a Beckman Coulter AU680 analyzer. In patients treated with regular dialysis, hypoalbuminemia is defined as a serum albumin concentration that is less than 35 g/L.

Serum albumin concentration after an online hemodiafiltration session is calculated from the equation: $Albumin_{post}$ = Calb post/{1 + [(UF)/0.2 × (BWpre - UF)]}, where: UF = BWpre - BWpost. BWpre - body weight of patients before dialysis (kg), BWpost - body weight of patients after dialysis (kg). Calb - serum albumin concentration (g/L), UF - neto-ultrafiltration flow rate (L/4h).

Serum β_2-microglobulin concentration was determined by turbidimetric method, on a Beckman Coulter AU680 analyzer. In patients treated with regular dialysis, the predialysis serum β_2-microglobulin concentration should be less than 25 mg/L.

Serum ferritin concentration was determined by turbidimetric method, on a Beckman Coulter AU680 analyzer. In patients treated with regular dialysis, the normal serum ferritin concentration is 100-500 ng/mL.

Serum CRP concentration was determined by turbidimetric method, on an Olympus AU680 analyzer, and was calculated as the average of two measurements over two consecutive months. The normal serum CRP concentration is ≤ 5 mg/L. Microinflammation is defined as a serum CRP concentration higher than 5 mg/L.

The concentration of vitamin D in the serum was determined by the electrochemiluminescence method, on the Cobas e 411 analyzer. The normal concentration of vitamin D in the serum is 20-40 ng/mL. In patients treated with regular dialysis, the normal vitamin D concentration is ≥ 30 ng/mL (30-80 ng/mL). Severe deficiency is defined as a vitamin D concentration < 10 ng/mL, and the vitamin D deficiency exists if the concentration is 10-20 ng/mL, and finally, insufficiency is defined as a serum vitamin D concentration of 20-30 ng/mL.

Concentration of intact parathyroid hormone in serum was determined by immunoradiometric method (IRMA) on a WALLAC WIZARD 1470 gamma counter. Normal concentration of intact parathyroid hormone in serum is 11.8-64.5 pg/mL. In patients treated with regular dialysis, the upper normal limit is 300 pg/mL.

Prealbumin and transferrin were detemined on Abbott Architect analyzer using immunoturbidimetric method. In patients treated with regular dialysis, the normal serum prealbumin concentration is ≥ 0.30 g/L (≥ 30 mg/dL).

Normalized degree of protein degradation – nPCR was calculated based on the formula: nPCR = (PCR × 0.58)/Vd, where: PCR is the degree of protein degradation, and Vd is the volume of fluid in the body. PCR is calculated from the formula: PCR = [(9.35 × G) + (0.29 × Vd)], where: G is the degree of urea production, and Vd is the volume of fluid in the body. The degree of urea production is calculated from the formula G = [(C1-C2)/Id] × Vd, where: C1 is the serum urea concentration before dialysis (mmol/L), C2 is the serum urea concentration after dialysis (mmol/L) and Id is the time between two dialyzes (h). The volume of fluid in the body is calculated from the formula: Vd = 0.58 × DW, where DW is the dry body weight of the patient after dialysis (kg).

Percentage of interdialysis yield in the patient's body weight – % IDWG was calculated using the formula: % IDWG = [(body weight of the patient before dialysis) (kg) - "dry body weight" of the patient) (kg)/"dry body weight" of the patient (kg)] × 100].

Dialysis adequacy was assessed based on the single-pool Kt/Vsp index, calculated according to the Daugridas second-generation formula: Kt/Vsp = -ln (C_2/C_1 - 0.008 × T) + (4 - 3.5 x C_2/C_1) × UF/W, where: C_1 is the urea value before dialysis (mmol/L), C_2 is the urea value after dialysis (mmol/L), T is the hemodialysis duration (h), UF is the interdialysis yield (L) and W is the body weight after hemodialysis (kg). According to K/DOQI guidelines, hemodialysis is adequate if Kt/Vsp ≥ 1.2.

The degree of urea reduction – URR index was calculated using the following formula: URR = (1-R) × 100%, where R represents the ratio of serum urea concentration after and before dialysis treatment. Dialysis is adequate if the URR index = 65-70%.

Blood flow through the vascular approach – Qavf was determined by Color Doppler ultrasound examination, on a Logic P5 machine, using a 7.5 MHz probe. Blood flow through the vascular approach that provides adequate hemodialysis is 500-1000 mL/min.

Kolmogorov-Smirnov test, Student's T test for bound samples and Wilcoxon test were used for statistical analysis of the obtained data. Significance thresholds were probabilities of 0.05 and 0.01.

RESULTS

A cross-sectional study was conducted at the Center for Nephrology and Dialysis of the Clinical Center of Kragujevac, which included patients treated with postdilution online hemodiafiltration, three times a week for 4 hours. The basic parameters of postdilution online hemodiafiltration are shown in Table 1. Thirty patients (23 men, 7 women), average age 54.87 ± 11.66 years, average length of hemodialysis treatment 4.95 ± 5.40 years, average nutrition 23.49 ± 3.75 kg/m^2 and the average adequacy index of postdilution online hemodiafiltration – spKt/V 1.41 ± 0.25 were examined. General patient data are shown in Table 2.

For the treatment of anemia in the examined patients, short-acting and long-acting erythropoietins, intravenous iron preparation, i.v. vitamin B preparation and folic acid (per os) were used. The average monthly dose of short-acting erythropoietin was 18857.14 ± 8234.65 IU, long-acting erythropoietin 102.94 ± 52.54 μg, the average monthly dose of intravenous iron was 244.44 ± 104.16 mg, the average monthly dose of i.v. vitamin C was 6000.00 ± 0.00 mg, the average monthly number of ampoules of Beviplex (complex of B vitamins) was 360.00 ± 0.00, the average monthly dose of vitamin B12 was 2500.00 ± 0.00 μg, and the average monthly dose of folic acid was 180.00 ± 60.00 mg. Secondary hyperparathyroidism in the examined patients was treated with calcium-containing phosphate binders, active metabolites of vitamin D and paricalcitol. The average monthly dose of rocaltrol was 4.89 ± 4.28 μg. A combination of angiotensin I converting enzyme blockers, angiotensin II receptor blockers, beta blockers, calcium channel blockers and Henle's loop diuretics was used to treat arterial hypertension. Renin-angiotensin system blockers (mainly angiotensin I converting enzyme blockers) were used in 18 (60.00%) patients, beta blockers also in 18 (60.00%) patients, in 8 (26.70%) patients Henle loop diuretics were used, and in 15 (50.00%) patients calcium channel blockers.

The average values of the parameters of anemia, iron status, microinflammation, malnutrition, secondary hyperparathyroidism, hypervolemia and β_2-microglobulin extraction index are shown in Table 3.

Mean serum albumin and β_2-microglobulin values before and after a single session of postdilution online hemodiafiltration are shown in Table 4. Serum β_2-microglobulin concentration before a single session of postdilution online hemodiafiltration less than 25 mg/L was found in 11 (36.67%) patients, and less than 30 mg/L in 21 (70.00%) patients. There is a high, statistically significant difference between serum β_2-microglobulin concentrations before and after a single session of postdilution online hemodiafiltration ($p < 0.01$). The average decrease in β_2-microglobulin concentrations during a single session of postdilution online hemodiafiltration is 19.45 ± 4.66 mg/L, while the average reduction index of β_2-microglobulin during a single session of postdilution online hemodiafiltration is 70.86 ± 6.87%.

There is a high, statistically significant difference between serum albumin concentration before and after a single session of postdilution online hemodiafiltration ($p < 0.01$). The average decrease in albumin concentration during a single session of postdilution online hemodiafiltration is 2.50 ± 0.92 g/L, and the albumin reduction index is 6.20 ± 2.12%. All examined patients had a serum albumin concentration greater than 35 g/L (39.77 ± 2.64 g/L) before the postdilution online hemodiafiltration session. After a single session of postdilution online hemodiafiltration, serum albumin concentration was also higher than 35 g/L in all patients (37.27 ± 2.11 g/L).

Table 1. Data on the treatment of patients with postdilution online hemodiafiltration

Parameters	Average value
Qnuf (mL/min)	11,74 ± 3,90
Qsubs (mL/min)	80,64 ± 14,20
Qconv (mL/min)	92,37 ± 13,39
Vsubs (L)	18,58 ± 3,20
Vconv (L)	21,38 ± 2,97
FF (%)	36,00 ± 5,00

Qnuf – net ultrafiltration rate, Qsubs – substitution flow rate, Qconv – convective flow rate, Vsubs – substitution volume, Vconv – total convective volume, FF – filtration fraction

Table 2. General patient data

GENERAL DATA		Statistical parameters
		Xsr ± SD
Number (N)		30
Gender (m/f, %)		23/7 (76,66/23,34)
Age (years)		54,87 ± 11,66
Length of dialysis treatment (years)		4,95 ± 5,40
Body mass index – BMI (kg/m^2)		23,49 ± 3,75
Systolic arterial blood pressure – SBP (mmHg)		128,00 ± 8,72
Diastolic arterial blood pressure – DBP (mmHg)		77,32 ± 5,12
Mean arterial blood pressure – MAP (mmHg)		94,22 ± 5,30
Dry body weight of the patient – W (kg)		71,82 ± 12,54
Interdialysis yield in BM – IDWG (kg)		2,35 ± 0,94
Percentage of interdialysis yield in BM – IDWG (%)		3,44 ± 1,58
Ultrafiltration rate – UF (mL/h)		587,50 ± 235,20
Ultrafiltration rate – UFR (mL/kg/h)		8,57 ± 3,92
Blood flow through the vascular approach – Qavf (mL/min)		936,00 ± 460,90
Hemodialysis adequacy index – Kt/V		1,21 ± 0,24
Single pool hemodialysis adequacy index – spKt/V		1,41 ± 0,25
Degree of urea reduction – URR (%)		69,41 ± 7,06
Primary kidney disease	Glomerulonephritis chronica (N, %)	7 (23,32)
	Nephropathia hypertensiva (N, %)	11 (36,66)
	Nephropathia diabetica (N, %)	1 (3,34)
	Nephropathia obstructiva (N, %)	1 (3,34)
	Nephropathia chronica (N, %)	6 (20,00)
	Renes polycystici (N, %)	4 (13,34)
Comorbidities		
Hypertensio arterialis (N, %)		21 (70,00)
Cor hypertensivum compensatum (N, %)		7 (23,32)
Cardiomyopathia dilatativa (N, %)		1 (3,34)
Diabetes mellitus complicatus (N, %)		1 (3,34)

Table 3. Average values of test parameters

TEST PARAMETERS	Statistical parameters
	Xsr ± SD
Hb (g/L)	101,20 ± 7,06
Hct (%)	30,45 ± 1,78
Fe (μmol/L)	11,20 ± 5,50
TSAT (%)	33,52 ± 15,20
FER (ng/mL)	568,42 ± 267,85
CRP (mg/L)	4,57 ± 5,48
UP (g/L)	66,57 ± 3,02
ALB (g/L)	39,77 ± 2,64
PALB (g/L)	0,34 ± 0,09
TRSF (g/L)	1,60 ± 0,40
UA (μmol/L)	368,00 ± 68,72
nPCR (g/kg/24h)	2,00 ± 0,60
VitD (ng/mL)	16,57 ± 7,70
iPTH (pg/mL)	220,22 ± 189,45
RR-β_2M (%)	70,86 ± 6,87
RR-Alb (%)	6,20 ± 2,12

Hb – hemoglobin, Hct - hematocrit, Fe – serum iron concentration, TSAT – iron transferrin saturation, FER – serum ferritin concentration, CRP – serum C-reactive protein concentration, UP – serum total protein concentration, ALB –serum albumin concentration, PALB – serum prealbumin concentration, TRSF – serum transferrin concentration, UA – serum uric acid concentration, nPCR – normalized protein degradation, VitD – serum vitamin D concentration, iPTH – serum intact parathyroid hormone concentration, RR – Reduction Ratio (β_2-microglobulin, albumin)

Table 4. Influence of a single session of postdilution online hemodiafiltration on serum albumin and β_2-microglobulin concentration

Test parameters	Statistical parameters		Significance (p)
	Before OL-HDF	After OL-HDF	
	Xsr ± SD	Xsr ± SD	
Albumin (g/L)	39,77 ± 2,64	37,27 ± 2,11	Zemp = -4,776, p < 0,001
β_2-mikroglobulin (mg/L)	27,34 ± 5,89	7,88 ± 2,24	Zemp = -4,782, p < 0,001

OL-HDF - postdilution online hemodiafiltration

Statistical test: Wilcoxon test

DISCUSSION

Cardiovascular diseases are the leading cause of death in patients treated with regular dialysis. Uremic toxins, microinflammation, malnutrition, oxidative stress, endothelial dysfunction, erythropoietin resistance and anemia are significant non-traditional risk factors for the development of cardiovascular disease (16-19). Early detection and optimal control of non-traditional risk factors play a key role in preventing the development of cardiovascular disease in this patient population (20, 21).

β_2-microglobulin is a middle molecular weight uremic toxin (MW – 11.8 kDa), soluble in water, and an increase in its serum concentration results in the development of Dialysis-Related Amyloidosis (DRA) (22-26). In patients treated with regular dialysis, the serum β_2-microglobulin concentration before a single dialysis session should be < 30 mg/L (22-26). In the examined patients, the serum β_2-microglobulin concentration before a single session of postdilution online hemodiafiltration less than 25 mg/L was present in 11 (36.67%) patients, and less than 30 mg/L in 21 (70.00%) patients. During a single session of postdilution online hemodiafiltration, the average decrease in serum β_2-microglobin concentrations was 19.45 ± 4.66 mg/L. The average extraction index of β_2-microglobulin during a single session of postdilution online hemodiafiltration was 70.86 ± 6.87%. The results of the research done so far have shown that during a single session of highly permeable "high-flux" hemodialysis, the reduction index for β_2-microglobulin is 50-60%. In MCO hemodialysis ("medium cut-off" dialysis membrane) the reduction index for β_2-microglobulin is 70%, and in high-volume (Vconv > 22 liters per session) postdilution online hemodiafiltration it is 80-85% (RR ≥ 80%) (22-26). In the examined patients, the average total convective volume (Vconv) was 21.38 ± 2.97 liters per session, and the average blood flow rate – Qb = 257.00 ± 18.65 mL/min, which may explain the slightly lower reduction index of β_2-microglobulin. Patients with total convective volume – Vconv < 22 liters per session have statistically significantly lower blood flow rate (Qb) and higher filtration fraction (FF) compared to patients with Vconv ≥ 22 liters per session. Total convective volume – Vconv ≥ 22 liters per session is achievable in clinical practice in 75% of patients. High-volume postdilution online hemodiafiltration effectively removes uremic toxins of middle molecular weight, primarily due to high convective transport, without significant albumin loss. In the examined patients, the average decrease in albumin concentration during a single session of postdilution online hemodiafiltration was 2.50 ± 0.92 g/L, and the albumin extraction index was 6.20 ± 2.12%. There is a high, statistically significant difference between serum albumin concentration before and after online hemodiafiltration ($p < 0.01$). The results of this study are consistent with the results of other authors, who showed that the albumin reduction index – RR-Alb < 11% indicates the loss of albumin by dialysate in an amount less than 3.5 g/4h (27). Albumin loss during a single session of online hemodiafiltration in the examined patients can be explained by the high filtration fraction (FF = 36.00 ± 5.00%). High filtration fraction (FF) results in high transmembrane pressure, increased albumin loss during a single session of online hemodiafiltration, and an increased risk of blood clotting in the dialyzer (28). After a single session of postdilution online hemodiafiltration, the serum albumin concentration in all patients was higher than 35 g/L (37.27 ± 2.11 g/L). During the treatment of postdilution online hemodiafiltration, less than 4.0 g of albumin (≤ 4.0 g/4h) is lost, which is of great importance in order to prevent the development of malnutrition (22-28). Microinflammation and increased serum concentration of leptin in patients treated with regular dialysis play a significant role in the development of malnutrition. Leptin is a middle molecular weight adipokin (MW – 17 kDa), which

reduces the appetite of patients treated with regular dialysis (energy intake < 30 Kcal/kg/ day, protein intake less than 0.8 g/kg/day). Body mass index – BMI < 20 kg/m^2, serum albumin concentration < 35 g/L, serum prealbumin concentration less than 0.30 g/L and normalized protein degradation rate – nPCR > 1.0 g/kg/day are risk factors for adverse outcome in patients treated with regular dialysis (28). Studies show that high-volume postdilution online hemodiafiltration effectively removes pro-inflammatory cytokines and leptin, reduces microinflammation, reduces serum leptin concentrations, prevents the development of malnutrition, and improves the outcome of these patients (30, 31).

Uremic toxins induce the development of microinflammation in patients treated with regular dialysis. Pro-inflammatory cytokines (interleukin-6) stimulate hepcidin synthesis in liver cells. Hepcidin is a middle molecular weight uremic toxin (MW – 2.7 kDa), which blocks the release of iron from cells of the reticuloendothelial system. This results in the development of functional iron deficiency, resistance to the action of erythropoietin and the development of anemia. High-volume postdilution online hemodiafiltration reduces microinflammation, effectively removes hepcidin, reduces erythropoietin resistance, and enables optimal control of anemia (achieving and maintaining the target hemoglobin value in the blood of patients treated with regular dialysis) (32, 33).

Optimization of total convective volume (Vconv) depends on patient-related factors (hematocrit, serum total protein concentration) and factors associated with postdilution online hemodiafiltration session, such as: blood flow rate (Qb), duration of individual postdilution online hemodiafiltration session (T) and dialysis membrane type (34). The target blood flow rate should be – Qb ≥ 350 mL/min. The rate of blood flow depends on the blood flow through the vascular approach for hemodialysis (Qavf > 600 mL/min) and the diameter of the arterial and venous needle (15G/16G) (34). High hematocrit and increased concentration of total serum proteins increase the filtration fraction and decrease the total convective volume (Vconv). High-volume postdilution online hemodiafiltration (Vconv ≥ 22 liters per session) requires constant education and training of medical technicians (34). The main limitations of postdilution online hemodiafiltration are: not being widely available to patients treated with regular hemodialysis, high treatment costs, constant education of medical technicians, and achieving high total convective volume in clinical practice (34, 35).

CONCLUSION

Postdilution online hemodiafiltration effectively removes uremic toxins of middle molecular weight in the range of 0.5-50 kDa. Total convective volume depends on the rate of blood flow, blood flow through the vascular approach and the type of dialysis membrane. High-volume postdilution online hemodiafiltration (total convective volume ≥ 22 liters per session) reduces microinflammation, oxidative stress, malnutrition, erythropoietin resistance, prevents the development of amyloidosis associated with dialysis, prevents the development of accelerated atherosclerosis and improves the outcome of patients treated with regular dialysis.

CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

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RUTHENIUM(II) COMPLEXES AS POTENTIAL APOPTOSIS INDUCERS IN CANCER THERAPY

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ABSTRACT

The compound cis-diamminedichloroplatinum(II) (cisplatin) is the most widely used anticancer drug, but due to its serious side effects (including gastrointestinal symptoms, renal tubular injury, neuromuscular complications, and ototoxicity), clinical applications of cisplatin are limited. Therefore, these limitations have provided an encouragement for further research into other transition metal complexes, with an aim to overcome the disadvantages related with cisplatin therapy. In the search for effective complexes that can be targeted against tumor cells, many research groups synthesized various ruthenium(II) complexes with different ligands. Also, newly synthesized ruthenium(II) complexes showed selective anticancer activity against different types of cancer cells. Activity of ruthenium(II) complexes in some cases was even higher than that of cisplatin against the same cells. Precise mechanism of action of ruthenium(II) complexes is not fully understood. The different examples mentioned in this review showed that ruthenium(II) complexes decreased viability of cancer cells by induction of apoptosis and/or by cell cycle arrest which implies their different mechanism of action against different types of cancer cells.

Keywords: *Ruthenium complexes, apoptosis, cancer therapy.*

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INRODUCTION

The compound *cis*-diamminedichloroplatinum(II) (cisplatin) is the most widely used anticancer drug, but due to its serious side effects (including gastrointestinal symptoms, renal tubular injury, neuromuscular complications, and ototoxicity), clinical applications of cisplatin are limited (1). Therefore, these limitations have provided an encouragement for further research into other transition metal complexes, with an aim to overcome the disadvantages related with cisplatin therapy (1, 2). Ruthenium compounds proved to be the most promising ones in the search for anticancer agents containing metals other than platinum (3). Reduced toxicity, good selectivity for tumors, inhibition of antimetastatic progression and antiangiogenic properties are advantages of ruthenium based drugs (4). This is believed to be due to the ability of ruthenium to mimic the binding of iron to serum transferrin, which solubilises and transports iron in plasma. Therefore, ruthenium-based drugs may be delivered more efficiently to cancer cells as cancer cells overexpress transferrin receptors, to satisfy their increased demand for iron (1, 5). After binding to the transferrin receptor on cell membranes, the inert complex Ru(III) in tumor cells can be activated and reduced to a more reactive Ru(II) complex (4). The redox potential of the ruthenium complex allows for more effective antitumor therapy. Glutathione, ascorbate and proteins have the ability to reduce, while molecular oxygen and cytochrome oxidize Ru(II). In malignant cells there is a lower concentration of oxygen and an increased glutathione concentration, which results in the reduction of the Ru (III) complex in a more reactive Ru (II) complex (6).

Potential targets of RU(II) complexes

Cancer cells divide relentlessly due to a loss of control of normal restraints on cell cycle division. Proliferation of cancer cell is regulated by DNA, and many ruthenium complexes have high selectivity for binding to DNA (7). Ruthenium(II) compounds can be bonded to the DNA covalently and non-covalently (1, 7). For example, ruthenium(II) complexes can bind covalent to the N7 atom in guanosine (8, 9). Ru–DNA covalent binding distorts the DNA backbone, which impairs processes of replication and transcription (7-9). The non-covalent interaction of complexes with DNA includes electrostatic interactions, intercalation and groove binding (7, 10). Intercalation occurs when compounds are added between adjacent base pairs in the DNA double helix. Despite the fact that ruthenium complexes can bind to DNA, recent researches also indicate that certain proteins may be molecular targets for ruthenium compounds (11).

Ruthenium complexes as protein kinase inhibitors

Ru(II) complexes have been demonstrated to be promising agents as protein kinase inhibitors (12). Protein kinases are known regulators of various aspects of cellular life and moreover, they are one of the main targets for different anticancer drugs (13). They are large enzyme family with homologous active sites and have highly conserved ATP binding sites (13-15). Platinum complexes are reported as protein kinase inhibitors (14). However, various ruthenium complexes are also reported as protein kinase inhibitors (15-20). In general, inert metal complexes, such as ruthenium complexes, can be promising scaffolds for the design of different enzyme inhibitors (15). Maksimoska et al. designed ruthenium complexes that are selective inhibitors of p21-activated kinase 1 (PAK1). PAK1 have significant roles in metastasis and tumorigenesis and for that reason these compounds are potential candidates for cancer therapy (15). Bregman and Meggers synthesized ruthenium half-sandwich complexes that are inhibitors of glycogen synthase kinase 3 (GSK3) and proto-oncogene serine/threonine-protein kinase Pim-1 (16). Both GSK3 and Pim-1 play important role in the regulation of apoptosis and cell cycle progression, but Pim-1 has also been implicated in numerous cancers including Burkitt's lymphoma and prostate cancer (16-18). Meggers et al also investigated inhibitory activities of the ruthenium compounds against the protein kinases GSK-3, Pim-1, MSK-1 and CDK2/CyclinA and these results were also promising (18). Mitogen- and stress-activated protein kinase-1 (MSK1) and Cyclin-dependent kinase 2 (CDK2) are also involved in cell cycle progression (18, 21, 22).

Ruthenium(II) complexes and their role in endoplasmic reticulum stress pathway and ROS generation

Another approach for the treatment of cancer involves thioredoxin system. This system consists of thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH and plays an important role in regulating the redox balance, redox-regulated signaling cascades, cell function and cell proliferation (23). From the literature reports so far, anticancer actions of ruthenium compounds may be exerted by reactive oxygen species (ROS)-mediated apoptosis (24, 25). Luo et al. reported that ruthenium polypyridyl complexes $[Ru(bpy)_3]^{2+}$, $[Ru(phen)_3]^{2+}$ (2), $[Ru(ip)_3]^{2+}$ (3), $[Ru(pip)_3]^{2+}$ (4) (bpy = 2,20-bipyridine, phen = 1,10-phenanthroline, ip = imidazole[4,5-f][1,10] phenanthroline, pip = 2-phenylimidazo[4,5-f][1,10] phenanthroline), and demonstrated that these compounds exhibited antiproliferative activities against A375 human melanoma cells through inhibition of TrxR (23). Costa et al. also demonstrated that piplartine-containing ruthenium complexes $[Ru(piplartine)(dppf)(bipy)](PF_6)_2$ and $[Ru(piplartine)(dppb)(bipy)](PF_6)_2$ were able to induce caspase-dependent and mitochondrial intrinsic apoptosis on human colon carcinoma HCT116 cells by ROS-mediated pathway (26). Complexes of ruthenium have been used in the deprotection of alloc/allyl-protected substrates, and to carry out olefin metathesis in cells (27, 28). Ruthenium complexes have been reported to reduce cofactor nicotinamide adenine dinucleotide (NAD^+) to NADH inside cancer cells. This way of toxic activity of ruthenium complexes aims inherent redox vulnerability of cancer cells which is consequence of their dysfunctional mitochondria (28). One emerging tendency is the identification of drug candidates that target the endoplasmic reticulum (ER) with the goal of inducing ER stress and leading to eventual cell death. Ruthenium compounds of various investigators targeted ER (29-35). ROS-mediated ER stress is involved in several novel

strategies for cancer treatment. For example, ROS-mediated ER stress is necessary to activate Type-II immunogenic cell death, a concurrent killing of cancer cells and activation of the immune system, which has been shown to significantly decrease cancer recurrence *in vivo* (29). The endoplasmic reticulum is a key participant in tumor cell apoptosis and drug resistance and, therefore, is one of the main targets in anticancer research. ER stress induced activation of protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) leads to the phosphorylation of the eucariotic initiation factor 2α (eIF2α) that inhibits the translation and subsequently triggers cell cycle arrest (35). Ruthenium complexes are also known to cause induction of oxidative stress or endoplasmic reticulum stress (ERS), and consequent apoptosis of tumor cells (30, 31). Three ruthenium(II) complexes synthesized by Xu et al., namely $[Ru(NeN)_2(HIPMP)](ClO_4)_2$ (N-N = 2,2′-bipyridine (bpy, Ru1), 1,10-phenanthroline (phen, Ru2), and 4,4′-dimethyl-2,2′-bipyridine (dmb, Ru3), were shown to induce apoptosis of human cervical carcinoma cells HeLa through endoplasmic reticulum stress and reactive oxygen species production (29). It is compelling that clinically investigated ruthenium-based metal complex, which showed promising results in solid tumors, sodium trans-[tetrachloridobis(1H-indazole) ruthenate(III)] (NKP-1339), involved both ROS- and ER-related cytotoxic effects in human colon carcinoma cell lines SW480 and HCT116 (33).

Although ruthenium complexes affect DNA and induce ROS-production, it has been discovered that these compounds accumulated in organelles predominantly (31). Mitochondria is a key target of ruthenium complexes, because ruthenium complexes can swiftly reduce mitochondrial membrane potential, leading to dysfunction of mitochondria or mitochondrial apoptosis pathways activation (24, 25, 31).

Ruthenium(II) complexes, mitochondria and cell death

Mitochondria are complex organelles found in almost all eukaryotic cells that play fundamental roles in the regulation of cellular functions. Some nonnuclear targets, and especially mitochondria, have also been reported to be targets for the anticancer activity of Ru(II) compounds (36). Under certain cellular conditions, mitochondria can release molecules that can activate the extrinsic and intrinsic apoptotic pathways (36, 37). As we already stated, ruthenium complexes may affect DNA, mitochondria, endoplasmic reticulum, protein kinases and induce ROS-generation (Figure 1) (24).

Cell death can be result of several mechanisms. Apoptosis is a process of programmed cell death and includes cell membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (38). This process is primarily induced by two basic apoptotic signaling pathways: the extrinsic and the intrinsic pathways (38, 39). Extrinsic apoptosis is activated via specific transmembrane receptors belonging to the tumour necrosis factor (TNF) receptor superfamily (40, 41). The intrinsic pathway, also known as the mitochondria-mediated pathway is activated by various intracellular stimuli, including DNA damage, growth factor deprivation, oxidative stress and endoplasmic reticulum (ER) stress (42, 43). During mitochondria-mediated pathway, cytochrome c is released from mitochondria into the cytosol of the cell and activates Apaf1 and consequently regulates other proteins involved in apoptosis (42-44). Non-apoptotic cell death includes necrosis and ER stress. Autophagy allows the orderly degradation and recycling of cellular components, and process is regulated by autophagy-related (ATG) proteins (45). The role of autophagy in cancer is complex, and this process can inhibit or promote cell death (45, 46). Necrosis is a form of cell death which results in the premature death of cells (47, 48). Cellular death due to necrosis is different than apoptotic cell death, and result is loss of cell membrane integrity and uncontrolled release of products into the extracellular space (49). Apoptosis and necrosis can be induced by increased production of reactive oxygen species (ROS), including hydrogen peroxide, superoxide anion and nitric oxide, and decreased reduced glutathione levels (50).

Cytotoxicity of RU(II) complexes

In the research for effective complexes that can be targeted against tumor cells, many groups of researchers synthesized various ruthenium(II) complexes with different ligands (51-53). The first ruthenium(II) arene complexes reported is [Ru(η 6-C_6H_6)(DMSO)C_{l2}]. Investigations into its antitumor effects showed that the complex strongly inhibited the activity of topoisomerase II which was crucial for structural organization of the mitotic chromosomal scaffold during cell replication process (54). Lazic et al. evaluated cytotoxicity of five Ru(II) terpyridine complexes against human lung carcinoma A549, human colon carcinoma HCT116 and mouse colon carcinoma CT26 cell lines by MTT assay. Complex [Ru(Cl-tpy)(en)Cl][Cl] (***1***), had IC_{50} values ranging between 32.80 and 66.30 μM and showed the highest anticancer activity compared to the other four ruthenium(II) complexes: [Ru(Cl-tpy)(dach)Cl][Cl] (***2***), [Ru(Cl-tpy)(bpy)Cl][Cl] (***3***), [Ru(tpy)Cl_3] (***4***) and [Ru(Cl-tpy)(pic)Cl] (***5***) (1). The cytotoxicity of the Ru(II) benzene complexes was screened for a panel of cancer cell lines: human hepatocyte carcinoma HepG2, human lung carcinoma A549, human breast carcinoma MCF7 and human ovary carcinoma cells SKOV3 by Jeyalakshmi et al. Results showed that the complexes [$RuCl_2$(η^6-benzene) N-(phenylcarbamothioyl)thiophene-2-carboxamide] (***6***), [$RuCl_2$(η^6-benzene) N-(o-tolylcarbamothioyl)thiophene-2-carboxamide] (***7***) and [$RuCl_2$(η^6-benzene) N-(o-tolylcarbamothioyl) thiophene-2-carboxamide] (***8***) displayed modest activity against HepG2 cells. Complex ***6*** showed moderate cytotoxicity against A549 cell lines and had IC_{50} value 95,6 μM (55). Canovic et al. explored cytotoxic properties of two new monofunctional ruthenium(II) polypyridyl complexes. The cytotoxicity of complexes [Ru(Cl-Ph-tpy)(phen)Cl]Cl (***9***) and [Ru(Cl-Ph-tpy)(o-bqdi)Cl]Cl (***10***) was evaluated against four human cancer cell lines: lung carcinoma A549, breast carcinoma MCF7, cervical carcinoma HeLa and melanoma Hs294T and against one non-cancer, fibroblast cell line MRC-5 by MTT assay. Ruthenium complex ***9*** displayed high cytotoxic activity against A549 and MCF7 cell lines with IC_{50} values of 4.6 ± 2.1 and 13.8 ± 1.8 μM.

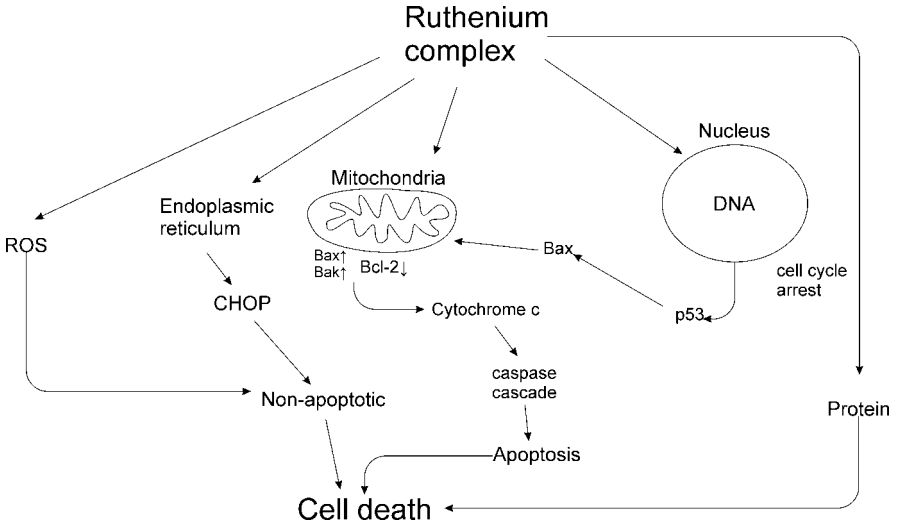

Figure 1. Principal targets and mechanisms of action of ruthenium complexes as oncotherapeutics. Reproduced from reference 24.

The cytotoxicity of complex ***9*** was about 2 times higher compared to the cytotoxicity of cisplatin under the same conditions. Ruthenium complex ***10*** significantly decreased viability of A549, MCF7 and HeLa cells, with IC_{50} values of 21.7 ± 4.3, 4.6 ± 0.9 and 6.4 ± 1.3 μM, respectively. Complex ***10*** showed higher cytotoxic activity against MCF7 and HeLa cells compared to the cytotoxicity of cisplatin under the same conditions. Also, both ruthenium complexes displayed an insignificant effect on viabilities of Hs294T cells and fibroblasts MRC-5 (9). Also, Milutinovic et al. have developed a series of six new monofunctional Ru(II) complexes (***11-16***) of the general formula mer-[Ru(Cl-Ph-tpy)(N-N)Cl]Cl (where N-N = en (***11***), dach (***12***) or bpy (***13***)) and [Ru(Cl-tpy)(N-N)Cl]Cl (where Cl-tpy = 4'-chloro 2,2':6',2''-terpyridine; N-N= en (**14**), dach (***15***) or bpy (***16***)). Only complexes ***13*** and ***14*** had IC_{50} values against non-cancer cells MRC-5 (human fibroblasts) that were less than 100 μM (56). Liao et al. reported three newly synthesized ruthenium(II) polypyridine complexes, which demonstrated potential as anticancer agents: $[Ru(bpy)_2(icip)]^{2+}$ (***17***), $[Ru(bpy)_2(pdppz)]^{2+}$ (***18***) and $[Ru(bpy)_2(tactp)]^{2+}$ (***19***). Cytotoxicity of three newly synthesized polypyridine complexes was tested against human cervical carcinoma HeLa cells and IC_{50} values for complexes ***17***, ***18*** and ***19*** were 37.45 μM, 21.37 μM and 23.85 μM, respectively (57).

Ruthenium compounds and apoptosis

Since all these results have shown that Ru(II) complexes exhibited selective cytotoxic effects against different types of tumor cells, the next step was to determine and explain mechanism of action of Ru(II) complexes. Mazuryk et al. results showed that cellular uptake of Ru(II) complexes occurs through passive diffusion and showed that Ru(II) complexes induced apoptosis of breast cancer (4T1) and human lung adenocarcinoma epithelial cells (A549). Apoptosis was induced by activation of oxidative stress by both tested Ru(II) complexes (58). Also, Cinara et al. have developed two novel Ru(II) complexes, and they discovered that complexes induced caspase-dependent and mitochondrial activated intrinsic apoptotic pathway in human colon carcinoma HCT116 cells by ROS-mediation (59). Futhermore, Martin et al. designed lipophilic Ru(II) complexes which targeted the lipid-dense endoplasmic reticulum in cells and showed the high anticancer activity against MCF-7 and HeLa cells (60). A series of Ru(II)–polypyridyl complexes synthesized by MacDonnell et al. were proven to induce apoptosis affecting both the intrinsic and extrinsic pathways (61). Recently, Canovic et al. demonstrated that ruthenium complexes decreased Bcl-2/Bax ratio causing cytochrome c mitochondrial release, the activation of caspase-3 and induction of mitochondrial apoptotic pathway (9). Three Ru(II) polypyridyl complexes were synthesized and characterized by Han et al.: $[Ru(dmb)_2(HDPIP)](ClO_4)_2$ (***20***), $[Ru(bpy)_2(HDPIP)](ClO_4)_2$ (***21***) and $[Ru(phen)_2(HDPIP)](ClO4)_2$ (***22***). Ruthenium complexes ***20***, ***21*** and ***22*** accumulated in the cell nuclei and increased the ROS levels. Complex ***22*** showed no impact on the expression of the anti-apoptotic protein Bcl-2, but increased the levels of the pro-apoptotic proteins Bax and Bid (62).

Ruthenium(II) complexes and cell cycle arrest

Both, induction of apoptosis and/or cell cycle arrest may decrease the viability of cancer cells. Lai et al. demonstrated that four ruthenium(II) polypyridyl complexes inhibited cell growth at the G0/G1 phase in A549 cells, and that the complexes could induce both autophagy and apoptosis (63). However, Mazuryk et al. showed that polyridin complexes of ruthenium arrest cell growth in the S-phase and induce apoptosis (58). Furthermore, Canovic et al. showed that ruthenium complex ***9*** induced G2/M phase arrest in A549 cells and G0/G1 phase arrest in MCF7 and Hs294T cells. Also, complex ***10*** in-

duced G2/M cell cycle arrest in A549 and HeLa cells, and G0/G1 cell cycle arrest in Hs294T cells (9).

RU(II) complexes which entered preclinical and clinical trials

The first step in discovering novel chemotherapeutics is reserved for their evaluation *in vitro*. Afterwards, characterization of the anticancer activity and toxicity *in vivo* of compounds that showed promising results *in vitro*, is fundamental to their further development (64). A number of *in vivo* studies have discovered that Ru(II) complexes exhibited anti-cancer activities comparable to, or even better than cisplatin, but with significantly less toxicity and side effects than cisplatin (65-76). Weiss et al. synthesized ruthenium complex, [Ru(h^6-p-cymene)Cl_2(pta)], where pta = 1,3,5-triaza-7-phosphaadaman tane (RAPTA-C). This complex decreased the growth of primary tumors in preclinical models for ovarian and colorectal carcinomas via anti-angiogenic mechanism. When the authors applied this complex every day at low doses (0.2 mg/kg), RAPTA-C significantly decreased the growth of the A2780 ovarian carcinoma transplanted onto the chicken chorioallantoic membrane model. Similar effect was noted in LS174T colorectal carcinoma in athymic mice, although at a higher dose (68).

Recent advances in discovering improved selectivity and cytotoxic activity of photocaged complexes led to *in vitro* testing of various ruthenium(II) complexes after their photoactivation by UVA and visible light (77-85). In general, after the excitation of the photosensitizing agent's moiety at suitable wavelength, energy or electron transfer process ultimately lead to the production of ROS which are cytotoxic to living cells and can be used for targeted singlet oxygen chemotherapy (77). Photocaged Ru complexes are usually nontoxic to nonirradiated tissues and can become toxic in tumor cells through photoactivation (78-84). Also, in other cases, light can be used to uncage toxic Ru ligands or species from the complexes for photochemotherapy (85). Therefore, Sun et al. synthesized a novel Ru-containing block copolymer (PolyRu) as a photoactivated polymetallodrug for combined photodynamic therapy and photochemotherapy *in vivo* (69).

They demonstrated *in vivo* that PolyRu accumulated at tumor tissue in mice and reduced the growth of tumors under light irradiation with minimal systemic toxicity (69). Chen et al. reported that complex (LC-003) cis-[$RuCl_2$(S-(−)-FOA)$(dmso)_2$] inhibited tumor growth in BEL-7402 xenograft mouse model via multiple mechanisms that included DNA damage, telomerase dysfunction, inhibition of p53 expression and caspase cascade activation. This complex showed higher *in vivo* safety than cisplatin (70). Other ruthenium complexes also exhibited similar effects *in vivo*; all of them inhibited growth of tumor tissue and produced less systemic toxicity in comparison to cisplatin *in vivo* (71-75). It is worth noting that complexes synthesized by Milutinovic et al., Ru(II)-tpy/ferrocene complexes [Ru(tpy) Cl_2(mtefc)] and [Ru(tpy)Cl_2(mtpfc)] (where tpy = 2,2′:6′,2″-terpyridine, mtefc = (2-(methylthio)ethyl)ferrocene, and mtpfc = (3-(methylthio)propyl)ferrocene), promoted activation of acquired and innate antitumor immunity, which led to growth reduction of mammary carcinoma *in vivo* (76).

About 50% of patients undergoing chemotherapy receive some type of a platinum medication at this time (24). But, drug resistance to platinum drugs and serious side effects limits its applications (1, 24). Four therapeutics containing ruthenium entered human clinical trials (24, 84). Sadly, the results of phase 1 and phase 2 clinical studies didn't show promising results, which consequently stopped two of these drugs (NAMI-A, and KP1019) from entering to phase III clinical trials (Figure 2) (7, 24). However, remaining two ruthenium compounds are still under consideration in clinical trials: NKP1339 and the theranostic compound TLD1433 (7, 24, 65, 74, 86, 87). We also must mention that 95% of potential oncotherapeutics entering clinical development failed, correlating with an average of 90% for compounds in all therapeutic areas (24). Nevertheless, recent results of *in vitro* and *in vivo* studies mentioned in this review give us hope that designing ruthenium compound that will selectively target tumor cells is a realistic achievement.

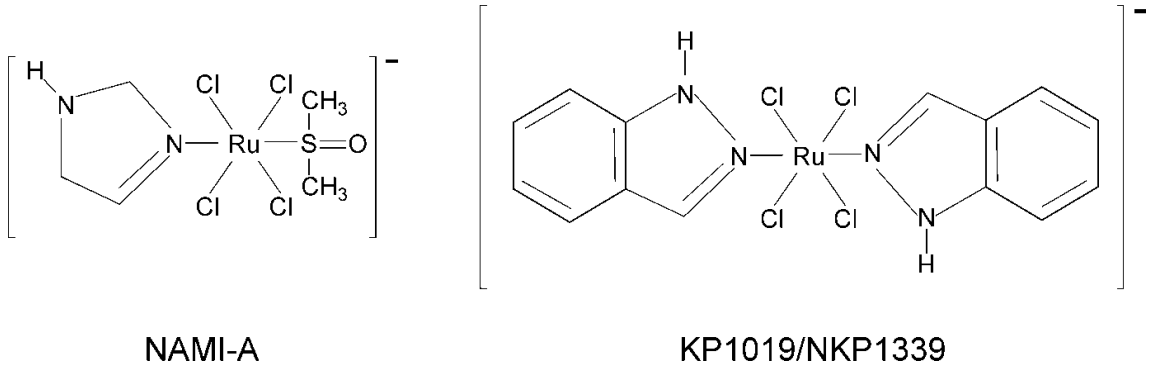

Figure 2. NAMI-A and KP1019/NKP1339 are ruthenium complexes that entered clinical trials. NAMI-A prevents metastasis and hinder neo-angiogenesis. KP1019 and its sodium salt equivalent, NKP1339, induce G2/M cell cycle arrest by ROS-generation (12).

CONCLUSIONS

After carefully reviewing the biological activity of ruthenium(II) compounds *in vitro*, it becomes clear that these complexes offer a promising approach to the advancement of new anticancer agents. These compounds exhibit remarkable features, such as low general toxicity against non-tumour cells, the ability to mimic iron binding to transferrin and albumin and exhibition of stronger affinity for tumor tissues over normal tissues. Also, newly synthesized ruthenium(II) complexes showed selective anticancer activity against different types of cancer cells. Activity of ruthenium(II) complexes in some cases is even higher than that of cisplatin against the same type of cancer cells. Precise mechanism of action of ruthenium(II) complexes is still not fully understood. The different examples mentioned in this review showed that ruthenium(II) complexes decrease viability of cancer cells by induction of apoptosis and/or by cell cycle arrest which implies their different mechanism of action against different types of cancer cells.

CONFLICT OF INTEREST

None.

FUNDING

None.

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FE ANALYSIS OF THE SYMPTOMATIC NAVICULAR - A CASE REPORT

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ABSTRACT

The accessory navicular (AN) is a bony formation located on the medial side of the foot, proximal to the navicular and continuous with the posterior tibialis tendon. It emerges as a developmental variant due to the presence of the secondary ossification center adjacent to the primary center of the navicular. It is regarded as a physiological and anatomical variant. In most instances, this is an incidental finding. Radiographic values are used to define three types of AN based on its relationship with the navicular. The symptomatic AN causes pain in the medial portion of the bone, a decreased range of motion and discomfort with shoe wearing. In case of recurrent symptoms, following an inadequate diagnosis and treatment, it can result in acquired flatfoot. Our case study examined the condition of asymptomatic AN in a young man with both flatfeet, who was initially treated conservatively, and then, after the failed response to the therapy, surgically. Following the Kidner procedure (excision with the reattachment of the insertion in the posterior tibialis tendon) and rehabilitation, the patient reports no subjective symptoms in the period of 12-month monitoring. An objective examination was conducted with the use of FE analysis during weight-bearing. We determined a reduction in total weight-bearing and the pressure distribution to the lateral side of the foot, metatarsal, and the heel region.

Keywords: *Symptomatic accessory navicular; Tibialis posterior; the Kidner procedure; FE Analysis*

INTRODUCTION

The accessory navicular (AN) is a bony formation located on the medial side of the foot, proximal to the navicular and continuous with the posterior tibialis tendon. It represents the second commonest accessory bone in the foot. The first one being the peroneal (Lat. os peroneum), and the third, the trigonal. (Lat. os trigonum) [1].

It emerges as a developmental variant due to the presence of the secondary ossification center adjacent to the primary center of the navicular. It is regarded as a physiological and anatomical variant and it occurs in 4-20% of the population [2]. In most instances, this is an incidental finding, however, usually after a trauma, intense physical activity, or in patients in certain occupations, it can cause serious symptoms, such as pain, a decreased range of motion, discomfort with shoe wearing, and flatfoot deformity. During the diagnosis, the presence of the accessory navicular needs to be considered because it is usually interpreted as the straining of the foot or the ankle joint [3]. Radiographic assessment is sufficient to define three types of the accessory navicular [4].

Tibialis posterior is a muscle of the lower leg that is responsible for plantar flexion of the foot, postural stability of the tarsals, and the formation of the medial arch. The tendon is adhered to the medial portion of the navicular [5]. Recurrent symptoms of the symptomatic navicular may cause damage to the posterior tibialis tendon. This causes abnormalities during the stance, function, and movement phase.

The symptomatic AN causes pain in the medial portion of the bone, a decreased range of motion, and discomfort with shoe wearing, however, in case of prolonged difficulties, inadequate diagnosis and treatment, it can result in acquired flatfoot. Persons who are predisposed to develop the accessory navicular are athletes, obese, or workers wearing uncomfortable shoes.

The diagnosis is commonly established after an exhaustive search of the clinical history and clinical examinations. Additional diagnoses are made by radiographs of the weight and non-weight foot, and CT or MRI visualization [6].

The treatment can be conservative or surgical. The conservative treatment is based on resting, reduced physical activity, wearing more comfortable shoes or orthotic insoles, and the use of analgesics. If the symptoms persist, corticosteroids are given locally or the foot is immobilized for six to eight weeks. The operative treatment is usually advised if nonoperative treatment is unsuccessful or in more active patients such as young athletes [7].

One of the methods for surgical treatment of AN is the Kidner procedure. Kidner's assumption is that the presence of AN modifies the position and biomechanics of the insertion of the posterior tibialis tendon. This causes weakness in the longitudinal arch of the foot and flatfoot [8]. The method of final elements (FE analysis) is included in the contemporary methods of numerical analyses. This method has been widely used in order to achieve objectivity of the success of different types of surgical treatment. Up to date, there are no cases in which FE analysis has been used to treat the symptoms of the accessory navicular bone.

The objective of this study is to show the case of the symptomatic accessory navicular bone, its treatment, and outcome after twelve-month monitoring, as well as the analysis of the stress in the period before and after the surgery. The aim is to direct attention and raise awareness of the symptoms and its early and accurate diagnosis, as well as the possibilities of its treatment. One of the proposed methods of treating these symptoms is the Kidner procedure, which includes the excision of the accessory navicular bone, after which, the insertion of the posterior tibialis tendon is removed and reattached to the lower side of the navicular bone.

CASE REPORT

A young seventeen-year-old male reported pain and swelling in the medial portion of the foot. He claims they have emerged spontaneously. The problems were present a couple of weeks ago with the increase in intensity and discomfort during long walks and physical activities. He was directed to an orthopedist for further examination and evaluation. The clinical examination determined the presence of a palpatory disease in the inner portion of the right foot, in the portion of the navicular bone, alongside the bony prominence, and the swelling in the aforementioned portion with no pain in the left foot. During the examination, the presence of pes planus was recorded. No signs provided the evidence of erythema or inflammation in the inner portion of the foot, in the zone of the bony prominence. Plantar and dorsal flexion (tested on the heel and toes) exacerbated pain. In the left foot, there were no symptoms during the examination except the pes planus deformity.

In the personal clinical history, there were no significant data related to the case.

Radiographs are sufficient to reveal the bony prominence in the affected foot. It is in a triangular shape and in the continuity of the proximal portion of the navicular (Figure 1a).

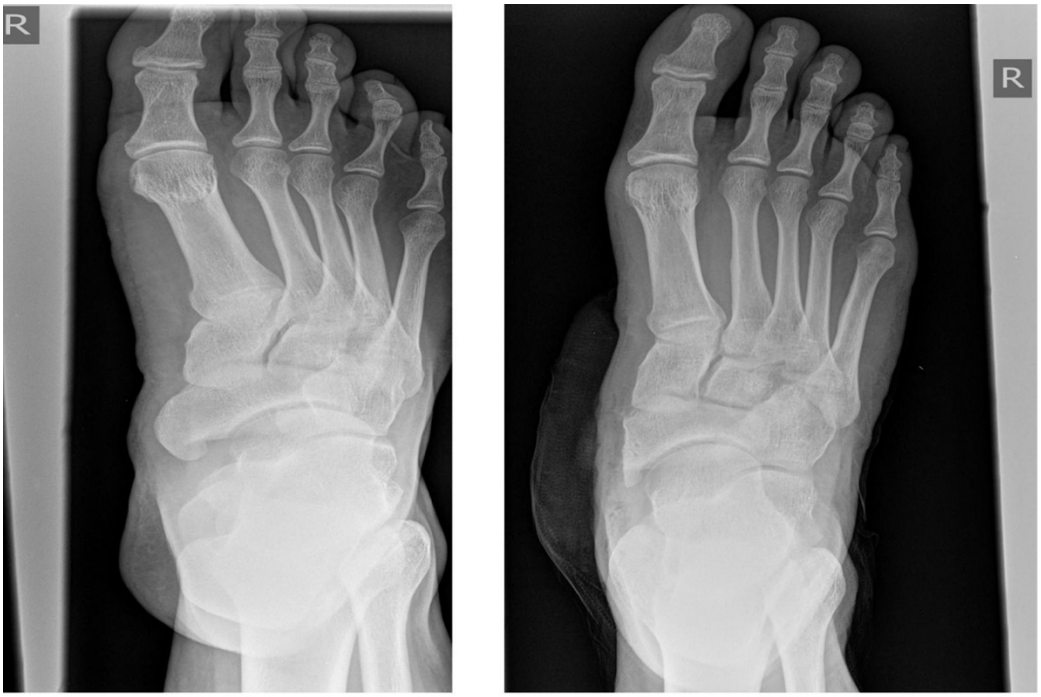
Baropodometric examinations of the weight-bearing are done before the surgery. A significant amount of stress is reported on the medial portion of the foot, i. e. on the longitudinal arch and toes.

The diagnosis of the symptomatic accessory navicular bone is established based on the findings from the clinical evaluation, radiographic images, and baropodometric examinations.

Since there are no data related to the previous symptoms in the patient's case history, the conservative treatment is advised, which included immobilization for the period of six weeks, the use of non-steroid anti-inflammatory medicine, physical therapy, resting, and wearing orthotic insoles. The response to the therapy was not positive since the pain and

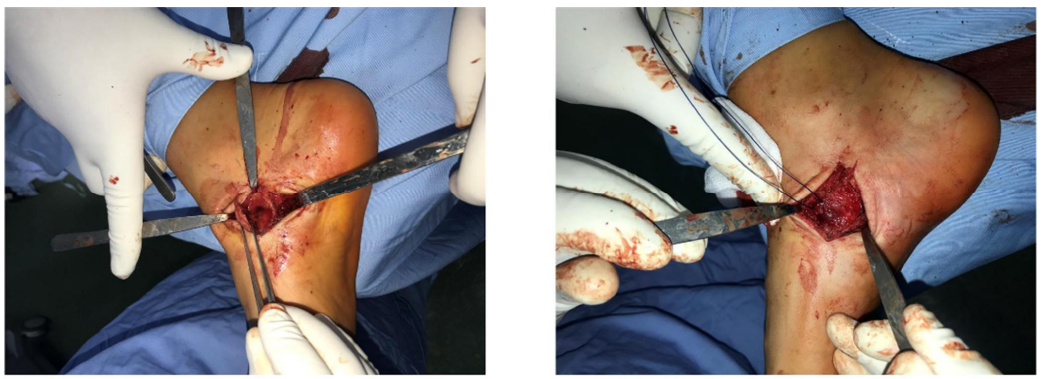
tenderness were recurrent during shoe wearing and walking. Hence, a decision was made to continue with the procedure surgically. The Kidner procedure was followed and the posterior tibial tendon was reattached (Figure 2). Afterwards, immobilization was used for the period of two weeks. After the surgery, the control radiographic images of the accessory navicular bone detected successful osteotomy (Figure 1b).

Figure 1. Radiographic images.



a) Before surgery b) Following surgery

Figure 2. The Kidner Procedure.



a) Excision of the navicular b) Reattachment of the posterior tibialis tendon

After the removal of the cast and the early rehabilitation period, baropodometric examinations of the involved foot are performed. Reduced stress in the medial portion of the foot and a more balanced pressure distribution to the lateral portion of the foot, metatarsal side, and the heel region were identified.

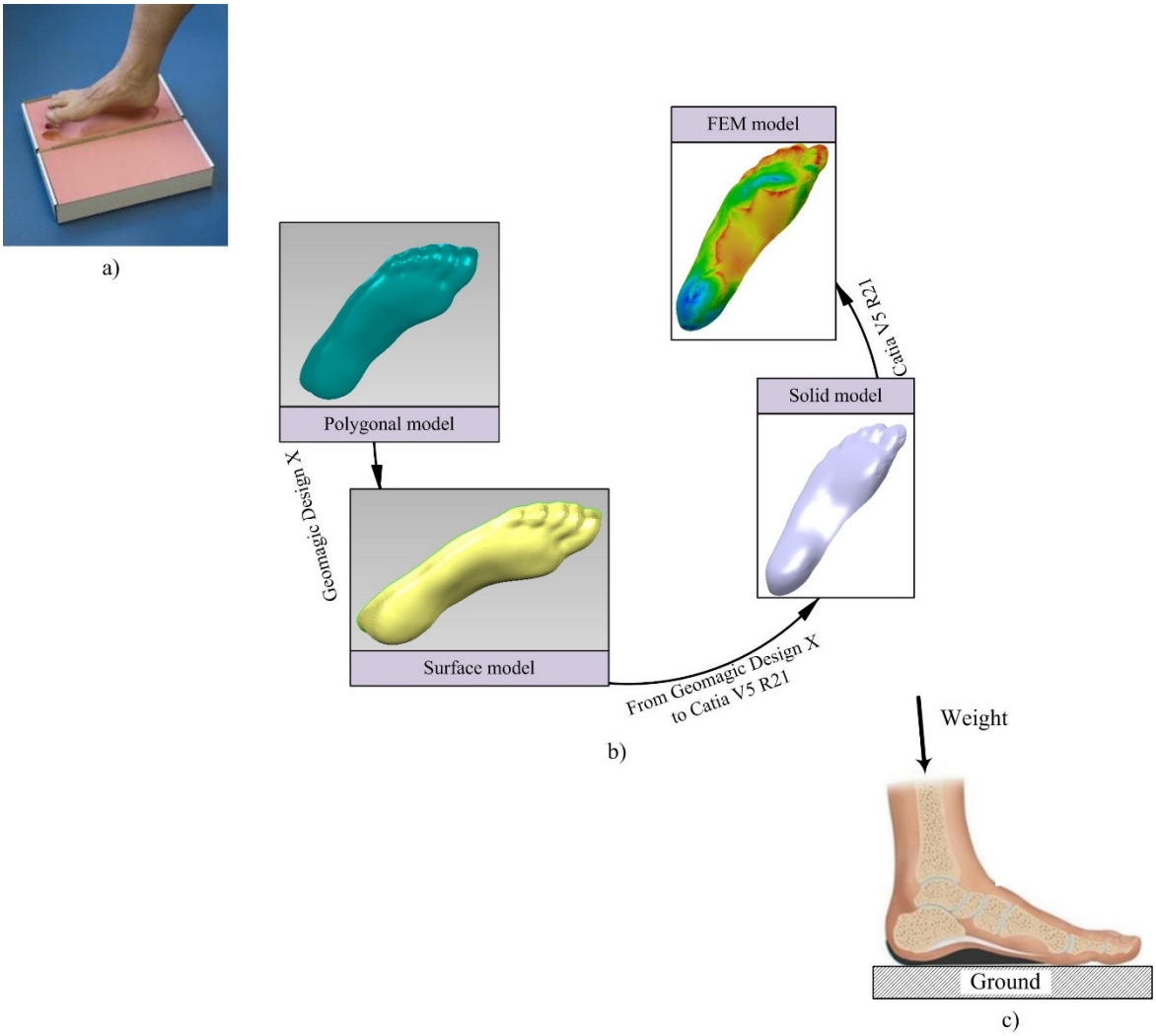
The stress in the foot was determined following the finite element (FE) analysis to the reconstructed model of the footprint before and after the surgery. The footprint was taken in the polyurethane foam. The examiner exerted a light pressure of the patient's foot onto the foam (Figure 3a).

After taking the footprint, the foot was scanned using Sense 3D scanner with the resolution of 1mm and the scanning range between 177 and 1828 mm. The scanned footprint was reconstructed, processed, and optimized using Geomagic Design X software. The footprint model was exported to Catia V5 R21 software, in which a volumetric model was created and the FE analysis performed (Figure 3b).

Following the FE analysis, the case of static pressure onto the flat floor of the footprint before and after the surgery was considered (Figure 3c). The aforementioned models correspond to the experimental trials. During the examination, tetragonal finite elements were used.

The contact was established between the surface of the foot and the ground. The stress value equated to the weight of the patient (77 kg). Young's module of the footprint was 1.15 MPa, Poisson's ratio 0.49, while Young's module of the ground was estimated 2000000 MPa, and Poisson's ratio 0.29 [9].

Figure 3. a) The footprint in the polyurethane foam
b) Process algorithm of the scanned foot
c) FE Analysis - Foot pressure onto the ground



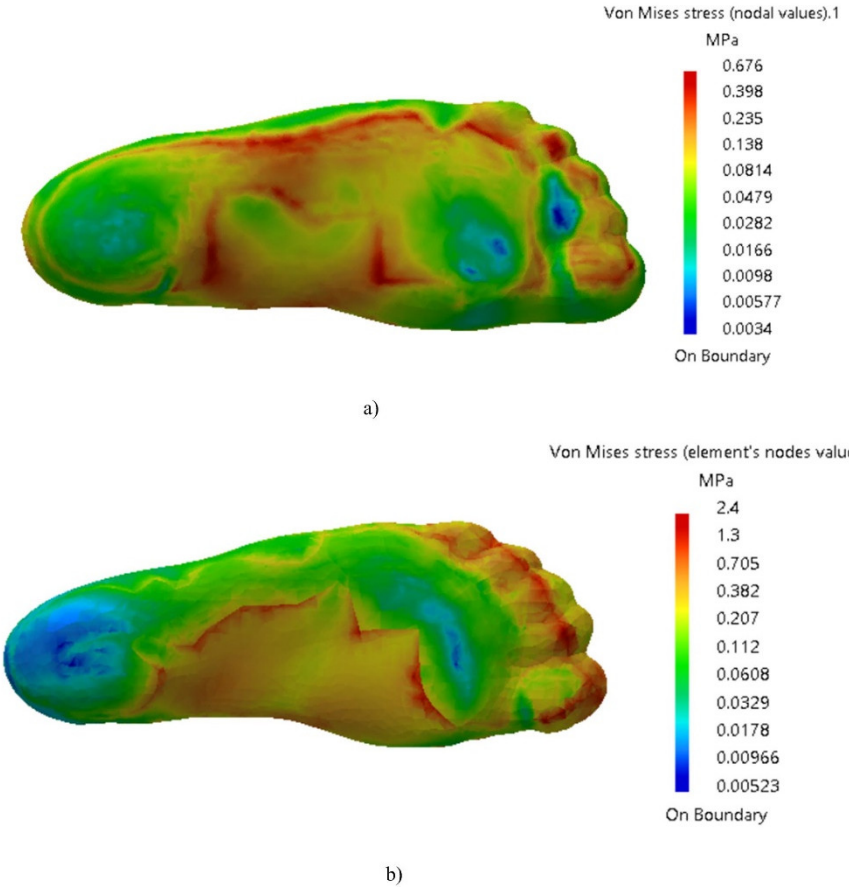
(https://www.peric-medikal.rs/kategorija/ortopedski-program/cipele-i-ulosci-za-obucu/?lang=lat)
(https://www.slideshare.net/akshayvagha/how-orthotic-insoles-can-alleviate-heel-and-foot-plantar-fasciitis)

The most significant stress value (2,4 MPa) in the patient with AN symptoms before the surgery was located on the medial side of the foot, i.e. on the longitudinal medial arch and toes. According to the values of Von Mises stresses after the FE analysis (the values were between 0,112 MPa and 0,00523 MPa). There was an insignificant amount of stress values on the heel region, metatarsal area, and the lateral portion of the foot (Figure 4a). These results point to the fact that the patient had an unequal stress distribution during standing on the affected foot, which caused the pain.

During the control examinations in the period of twelve months after the surgery, the patient reports no pain and symptoms in the portion of the involved foot, which the stress

data show after the FE analysis. In other words, following the surgery, there is a stress redistribution onto the lateral portion of the foot, metatarsal, and the heel region with the significantly reduced values of the maximal stress. Hence, the maximal stress value is 0,676 Mpa. Moreover, there is a noticeable stress relief in the portion of the navicular, which results in the pain decrease in that portion (Figure 4b).

Figure 4. Von Mises stress.



a) Before surgery b) Following surgery

DISCUSSION

The symptoms of the accessory navicular commonly emerge in front of the ankle joint and develop towards the inner side of the foot. Since the patient also had a flatfoot deformity, the stress on the foot was not equally distributed. The conservative treatment of the navicular symptoms commonly includes resting, comfortable shoe wearing, analgesics, and physical therapy, which was shown in the retrospective study conducted by the authors [10].

Many studies have been conducted related to the flatfoot classification, as well as to the analysis of the stress onto flatfoot by the use of the FE analysis or the plantar footprint analysis. In that sense, M. Costea at al. developed a methodology for classifying flatfoot based on the plantar footprint. The category of the foot is determined by the use of Chippaux-Simark Index and Hallux-Valgus Angle [11]. Contrary to the aforementioned authors, C. Cifuentes-de la Portilla et al, as well as L.Shudong et al. used the FE model for the analysis of the stress onto the foot - the former analyzed the stress on the soft tissues with the flatfoot deformity, and the latter analyzed the foot deformities during standing. In both cases, the foot reconstruction was conducted based on the CT images of the non-affected foot [12, 13]. In our study, the non-invasive methods of footprint scanning in a non-stress condition were employed. Similarly to the findings from the study by C. Cifuentes-de la Portilla et al, our study determined that the stress on the reconstructed footprint corresponded to the weight of the patient during the monopodial support [12].

The proper formats of pressure distribution in the foot are determined by examining the pressure in the foot, the analyses of plantar pressure, and the conducted FE analyses. A clinically healthy foot is considered when the stress in the metatarsal portion, heel region, and the lateral side of the foot

is equally distributed [14, 15, 16]. The patients with flat foot deformity suffer most from a stress increase in the medial portion of the foot, i.e. in toes [15]. In our case, the patient has a flat foot deformity and the stress that is created in the foot during total support corresponds to the stress distribution found by other authors [15]. However, what seems characteristic for this case is that there is virtually no support in the heel zone, i.e. the whole patient's body weight is redistributed to the medial portion and toes. After the surgery of accessory navicular syndrome, the stress is significantly reduced and leads to the stress redistribution to the portions that would correspond to a healthy foot. Due to the flatfoot deformity, certain amount of stress is still distributed to the medial portion, however, its values are significantly reduced.

CONCLUSION

The accessory navicular bone is commonly seen in everyday clinical praxis. In most instances, this is an incidental finding. The surgical treatment of the symptomatic accessory navicular is a therapy of choice in patients with an inadequate response to the conservative mode of treatment. The surgical treatment not only leads to the complete relief of symptoms, but also to the stress redistribution with the relief on the medial arch of the foot.

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CONFLICT OF INTERESTS

Authors declare no conflict of interest.

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EXCESSIVE SCREEN MEDIA EXPOSURE AND LANGUAGE DELAY IN THE DEVELOPMENT OF INFANTS AND TODDLERS - THREE CASE REPORTS

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ABSTRACT

Early exposure to digital media has become an integral part of everyday life, which is frequently being reported as one of potential risk factors for a number of developmental disorders, including language development in infants and toddlers. The current article represents introduction to a research study which will be dealing with a potential link between the risk of language delay and excessive screen media time in children. With this in mind, we will present three case reports involving young children who experienced a language delay, but did not have any health problems diagnosed, nor did they have any of the physical high-risk factors for language and other developmental disorders detected. What these three children have in common is the fact that they were all excessively exposed to digital media during the first two years of life. The existing empirical findings suggest that different forms of digital content may be overstimulating for the developing brain, which is true both for active and background screen viewing. Moreover, screen time may be seen as a distraction from other more developmentally important activities. Keeping parents informed about a potential negative influence of prolonged media exposure can prompt parents to make informed decisions about whether and to what extent they will expose their children to digital media in the earliest period of their development.

Keywords: *Infants and toddlers; language delay; screen media exposure.*

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INTRODUCTION

The first three years of life represent the critical period for the development of the child's brain, their cognitive abilities and speech. Infants are born with brains that are not fully developed yet, and research showed that the brain of a newborn triples in size during the first two years of life, and that this growth occurs in direct response to external early life experiences and stimulation (1, 2). The environment in which the child grows as well as the contents to which they are exposed may either stimulate or inhibit their development in such a sensitive period. The contents that we watch via *digital media* (television, smartphones, computers ...) have become an integral part of our everyday life, as well as our children's lives. In this context, the *screen time* concept, typically defined as "time spent with any screen, including smartphones, tablets, television, video games, computers or wearable technology" (3), holds a prominent place in research. Tablets and smartphones are particularly important because different forms of digital content have now become *accessible everywhere*, at *any time*. Although infants are capable of imitating what they see on a screen, children do not learn from television as well as they do from direct, live experiences (1). The development of language certainly holds a special place in this development.

Language development

Speech comprehension and functional communication are some of the essential functions that a child should master by the age of three. Within the period of 18 to 20 months, an infant should produce from 50 to 100 words (4). At the age of two, a normal speech development would require a vocabulary of a minimum of 50 words, along with combining shorter sentences or two-word phrases that are understandable to their environment. Typical problems that may slow down the speech development form the intricate relationship between the biological development and social environment (5). Those may be hearing impairment, complications during pregnancy or delivery (e.g., birth asphyxia), oropharyngeal malformation, or family history of speech-language difficulties, a low level of parental education, multilingual environment, and inadequate stimulation (5-7). Within this context, potential influence of digital content use, or digital media in general, has been increasingly considered in the last few decades.

When it comes to children, spending too much time in front of digital screens makes an impact on their communication with parents and social environment. The American Academy of Paediatrics has warned of the existence of numerous problems that are associated with excessive exposure to digital media, such as difficulties with sleep, speech, aggressive outbursts, and lower school achievement (8). The latest recommendation of the World Health Organisation is that children should not be exposed to digital media by the age of two, and after the age of two screen time should be no longer than one hour per day (9). These recommendations are based on the data obtained by a number of studies indicating that screen time does not only affect language acquisition, but also children's cognitive development, their physical activity, sleep patterns, attention, and ability to learn. Many studies point to the link between prolonged exposure to *digital content* with later talking times, together with language comprehension difficulties (10, 11). The majority of the studies that assessed the effects of long television exposure on language development in toddlers reported a higher risk of delays in expressive language (12). Another potential problem in language development is the fact that smartphones and tablets nowdays offer various topics in *different languages*. This exposure is expected to exert effects on children's first or foreign language development, due to *sending mixed signals* to the child (13). Language development is also connected with attention development, in such a way that the longer the children's exposure in the first three years of life, the greater the chance that they will also have attention difficulties (14). Even the *background screen media* (exposure without actively viewing or using the media, mainly to do with TV sets or computers), can have a significant effect on reduced attention during play or other children's activities, as well as the quality of their interaction with parents (1, 15).

This paper is an introduction to a research study that will be dealing with the investigation into a potential link between language delay and other problems in the early development of children, and prolonged screen time, i.e., excessive exposure to all forms of digital content. Within the current study, we will present three short case reports that have the following in common: parents sought the assistance of a psychologist due to *delayed language development* of their child in all three case reports, and it was determined that all the children were *significantly exposed* to various forms of *digital content*. What is important to underline is that none of the children had any health problems diagnosed that could influence the development of basic abilities, including the ability to speak. Keeping parents informed about a potential negative link between their children's exposure to digital media in this developmental period and some cognitive difficulties may prompt parents to make informed decisions about whether and to what extent they will expose their children to the digital media in the earliest period of their development.

CASE REPORTS

Case 1

Visit 1: A boy, aged three years and three months. Visits a psychologist due to delayed speech and language development. The firstborn child, the pregnancy was normal. Delivered at 39 weeks of gestation by natural childbirth with no complications. The Apgar score was 9 (10 being the maximum). No record of prior serious illnesses.

Early psychomotor development: Age-appropriate gross motor skills well-developed. Fine motor skills below the expected level. Sphincter control not acquired. Sleep and appetite are normal. Partly independent in basic self-care activities.

First meaningful words spoken at 12 months, with language development still being delayed. Uses 10 meaningful words actively. Does not form two-word phrases. Receptive language developed, understands commands, and performs them.

Child development was assessed by means of the Scale of Psychomotor Development of Children (the Brunet-Lézine scale), whose main purpose was to evaluate the child's motor activity (i.e., posture and hand-eye coordination) and social interactions (language and sociability) (16). The results showed a twenty-month developmental delay for this age. The delay was particularly present in the language development and hand-eye coordination domains.

Social interaction: Included in the regular peer group since the age of two. Sparse interaction with other children, does not initiate contact, but interacts with them once the interaction is established. Mainly plays independently. Symbolic play not developed. Typically arranges toys in a stereotypical manner or throws them. Hypervigilant during play.

Media exposure: Watches cartoons approximately four hours per day. The cartoons are in Serbian and English. The parents state that the boy was passively exposed to the screen *throughout the day* at the age of two.

Family status: Parents divorced. Lives with his mother, sees his father regularly. In the course of growing up, he was frequently exposed to stressful family situations. The parents disclaim any genetic disorders of significance. They had secondary-level education.

Current status: The child is five years old, with dysphasia. His sentences are short, frequently incomprehensible to the wider social environment. At this point, the boy was undergoing speech-language therapy, but with infrequent visits to the therapist. The boy is still hypervigilant. Does not initiate contact with other children. Occasionally displays aggressive outbursts towards his mother and other children in his peer group. He is still exposed to the digital media for more than three hours per day.

Case 2

Visit 1: A girl, aged two years and five months. Visits a psychologist due to delayed speech and language development. The firstborn child, the pregnancy was normal. Delivered at 40 weeks of gestation by natural childbirth with no complications. The Apgar score was 9. No record of prior serious illnesses.

Early psychomotor development: Started walking at 11 months. The walk was steady. Age-appropriate gross motor skills well-developed. Sphincter control not acquired. Did not master age-appropriate basic self-care activities. Sleep and appetite normal.

Receptive language partly developed. Understands and performs simple commands that are used every day. First meaningful words spoken at 12 months. She could produce four words that she actively used, mostly in English. According to her mother, the toddler could produce more words at one point but then she stopped using them. At times repeats a word that her mother utters, in the form of echolalia. Functional speech not developed. Communicates non-verbally, through gestures. Play below the age-appropriate level, predominantly stereotyped. The psychological assessment via the Brunet-Lézine scale suggests a thirteen-month developmental delay for this age. The delay was specifically dominant in the language development domain. Does not establish eye contact during the visits. Does not respond to her name.

Social interaction: Not included in the regular peer group. Does not have frequent contact with other children.

Media exposure: Watches cartoons almost the entire day. The cartoons are mainly in English, occasionally in German and Russian.

Family status: Lives in a three-member family, with her parents. Family relationships are adequate. The parents disclaim any genetic disorders of significance. They had secondary-level education. Financial problems present within the family.

Current status: The child is five and a half years old. Visits a special educator and a speech therapist for treatments, but irregularly due to financial family problems. Visits a psychologist for check-ups every three months. Forms shorter sentences. Expressive dysphasia. Uses Serbian, English, and occasionally German words. The sentences are ungrammatical, often incomprehensible to the social environment. The girl is hypervigilant. Has difficulty in focusing her attention and maintaining it. Increased motor activity. Emotional maturity below the age-appropriate level. Negativism still increased. Displays temper tantrums when thwarted. She is still exposed to the digital media for more than an hour a day. Included in the regular peer group, makes and initiates contact with other children, but has difficulty in adhering to the group rules.

Case 3

Visit 1: A two-year-old boy. The mother takes him to a psychologist for psychological assessment because of speech-language delay. The second-born child, the pregnancy was normal. Delivered at 40 weeks of gestation via C-section due to the previous labour complications. The Apgar score was 10. No record of prior serious illnesses.

Early psychomotor development: Started walking at 12 months. The walk was steady. Skilled motor performance. Sphincter control not acquired.

Receptive language at the basic level. Understands and performs simple commands. Does not use a single meaningful word. First meaningful words appeared at the age of one; yet the boy stopped using them. Does not establish eye contact. Understands simple commands solely. Does not imitate

animals, which he could do when he was 12 months. Does not show interest in toys, play below the age-appropriate level. Typically puts objects into his mouth or throws things. Symbolic play not developed.

The psychological assessment via the Brunet-Lézine scale suggests a fourteen-month developmental delay for this age. The delay was specifically dominant in the language development and hand-eye coordination domains.

Social interaction: Not included in the peer group. Does not have frequent contact with his peers.

Media exposure: The mother states that since 14 months old the child had been spending the majority of the day on a smartphone, tablet and in front of the TV, watching cartoons. At that time, the boy stopped using the words that he had been using before.

Family status: Lives in a four-member family, with his parents and his elder brother. Family relationships are adequate. The parents have a university degree. They disclaim any genetic disorders of significance.

Current status: The boy is three years and five months old. Forms shorter sentences. His vocabulary has significantly expanded. Play is being developed adequately. Able to focus his attention and maintain it. He was undergoing intensive treatments with a speech therapist, including a sensory room for a year. The treatments are no longer necessary. His exposure to the digital media has been completely limited. Included in the regular peer group. Adjusted adequately, accepts the company of other children, but prefers individual activities.

DISCUSSION

Regarding the case reports we have presented in this paper, it is important to underline that these children were born healthy, the pregnancies and deliveries were normal, and according to the *physical criteria they do not fall into the risk category* in terms of language development, or any other developmental disorder. This is confirmed by the fact that all the children are still healthy, with no medical history of previous major illnesses. What is also significant to note is that there were *no speech and language disorders* in any of the family, nor were there any other hereditary developmental difficulties. Finally, what is common for all the three children is that they spent a significant amount of time in front of a screen during the first two years of life.

It is important to underline that we can by no means claim any causal relationships between excessive screen media exposure and language delay in young children based on the presented case reports. However, they may serve as a basis for further empirical, scientifically rigorous investigations into the stated hypotheses. When we consider the theories and the existent empirical findings, there are two possible interpretations of the possible negative impact of screen time on these children's development. The first one would be supported with the idea that quick scene changes, flashing lights, bright colours and loud sounds may be *overstimulating for the developing brain* (1). This effect is not only relevant for active, but also for background (passive) viewing (15). This can be noticed in the boy from Case Report 1 presented in this paper, who spent four hours per day actively watching cartoons, but was passively exposed to a digital content for the rest of the day. Prolonged background TV exposure has been proven quite detrimental to language development, attention, and the overall cognitive development (17), while background viewing alone may inflict considerable harm to play development (18). This is true for all the three children from our study as well, whose play was considerably impoverished, stereotyped, with no functional use of toys. Likewise, all the three children watched cartoons in different languages. As stated in the Introduction, watching various content in different languages when the child has not yet developed their mother tongue may create obstacles to these children's language development (13), which is evident in the girl, from Case Report 2, who predominantly repeats words in the English language.

The second interpretation regarding the detrimental effect of screen time may be the fact that it creates a *distraction* from other more developmentally important activities (1). With regard to the children included in this study, the first child spent four hours a day watching cartoons actively, whereas the other two spent almost the entire day doing this. It follows from this that a larger portion of the day when a child is awake is spent on activities that may not be stimulating for their development, thereby preventing them to spend time on different content that may be so. As already stated, early-life experiences are crucial for the normal development of the child's brain, and it is of critical importance to educate parents about potentially beneficial and potentially harmful experiences to the development of their children.

The most significant difference between these three children *upon detection* of the language delay lies in the manner in which the treatments were administered. There are studies implying that untreated speech and language delay can persist in 40%–60% of the children and that these children are at a higher risk of social, emotional, behavioural, and cognitive problems in adulthood (7). It is clear that the greatest progress has been traced in the third child, whose screen time was completely restricted after the diagnosis, accompanied with *regular treatments* with a speech therapist. In the first two cases, screen time was not limited but only reduced. Inadequate family relationships, along with financial difficulties, may be disadvantageous to successful implementation of the language disorder treatment; therefore, they are commonly stated as potential risk factors (7, 13). Family stress was present with regard to one of the two children with slower progress, while with the other, those were financial problems in the family. Parents who are overly preoccupied with these problems may be less motivated to take an active part in their child's language development and are thus more likely to reach for screen time use as a babysitter (11). In a similar vein, the connection with parental educational level has also

been cited as a potential factor in the literature; thus, greater progress was detected in relation to the third child, whose parents had a higher degree. Highly educated parents are less likely to engage in screen activities because they are aware that such activities may disturb the child's development (12). They also engage their children more, use more complex words, thereby encouraging their children's language skills development (7). Consequently, certain family activities may have an ameliorating effect on the negative impact of increased screen time, such as frequent reading to children (19).

Nevertheless, it is essential to draw attention to the fact that not all forms of digital content are the same, and that media content and the context in which they are followed are often more important than the actual amount of time spent watching the media (1). Watching content aimed at older children or adults may have a negative influence on the development of infants, especially below 6 months old (20). In contrast to this, programmes with digital content designed especially for educational purposes differ from general digital programmes because they involve simple narrative structures, as well as contain pauses for children to respond (12). Similarly, it is important to state that language acquisition may only be achieved in *two-way interaction* which stimulates the expressive component of speech that is absent in prolonged screen time (12). A potentially positive impact and reduced harmfulness are achieved when screen time is spent with their parents, discussing the content with the child, connecting the screen content with everyday life (21). Finally, impoverished contact with other children may be another factor negatively influencing the communication skills development, which is noticeable in the children described in these case studies. Despite the interactivity of some of the media content, real-life social interactions are still irreplaceable in terms of the development of cognitive abilities.

CONCLUSION

Early and prolonged screen exposure may potentially cause delayed language development in children. Further research within this area of study, specifically those that will be particularly focused on *media content and the context* in which they are followed, besides screen time, may be of special importance to collecting more conclusive data based on which clear guidelines can be devised, and educational trainings for parents of infants and toddlers may be organised.

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CONFLICT OF INTERESTS

Authors declare no conflict of interest.

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