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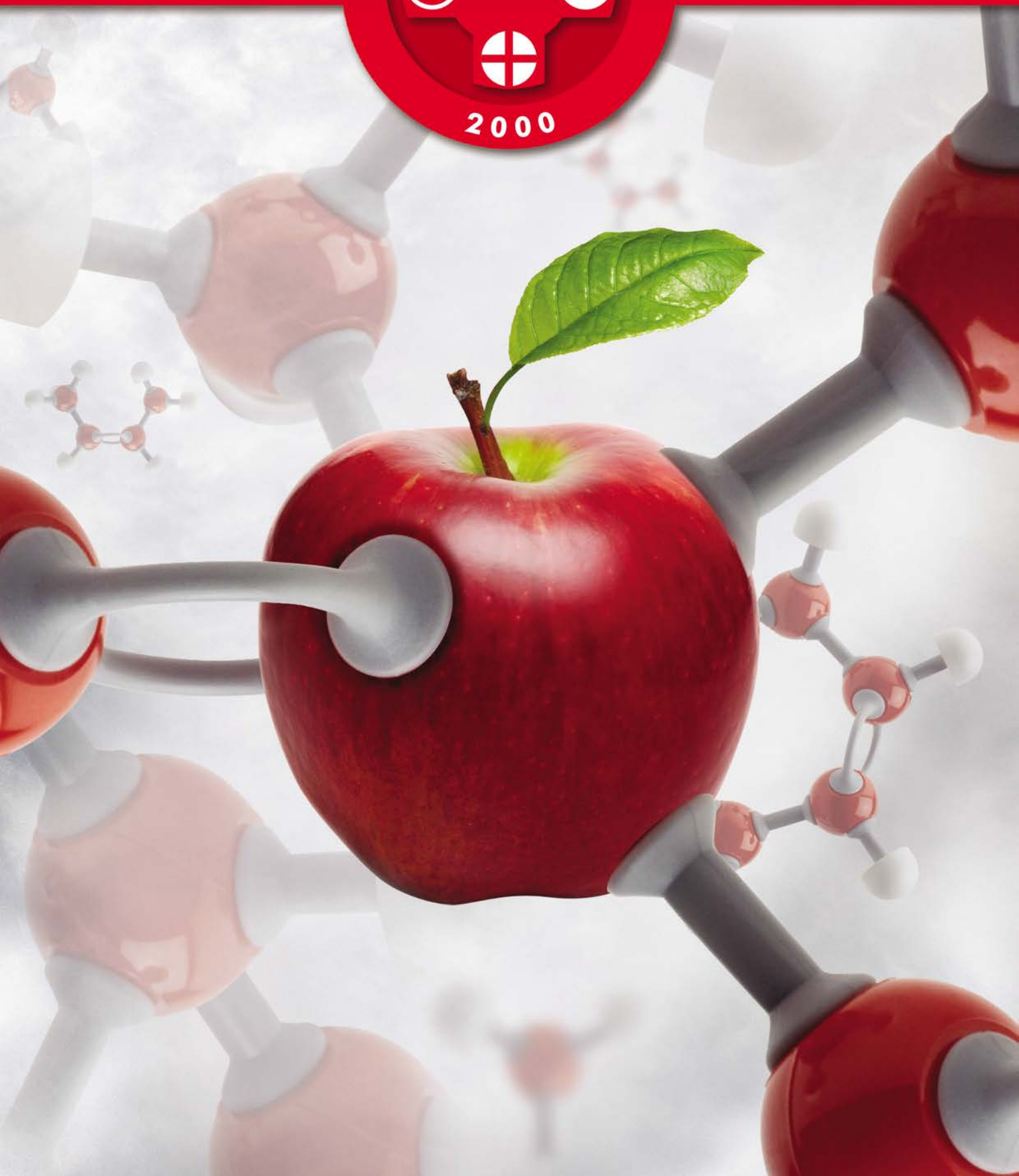

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ANTITUMORSKO DEJSTVO KOMPLEKSA PLATINE NA ĆELIJE HRONIČNE LIMFOCITNE LEUKEMIJE

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ABSTRACT

Since the discovery of the antitumor activity of cisplatin by Rosenberg and co-workers, the use of metal complexes in cancer treatment has caused a huge interest. Today, platinum-based drugs are part of standard chemotherapy in the management of a variety of cancers, germ cell tumours, sarcomas, and lymphomas. Unfortunately, toxicity and drug resistance are major obstacles to wider clinical application of these drugs. Their use is greatly limited by severe side effects such as nephrotoxicity, ototoxicity, and neurotoxicity. Although cisplatin is one of the most successful anticancer drugs to date, its biochemical mechanism of action is still unclear. Cisplatin is generally accepted as having the ability to interact with the purine bases on the DNA, causing DNA damage, interfering with DNA repair mechanisms, and subsequently inducing apoptosis in cancer cells.

Chronic lymphocytic leukaemia is a neoplastic B cell lymphoproliferative disease characterized by a highly variable clinical course. Clinical stage at the diagnosis and biological prognostic factors are the important predictors for survival. The Rai and Binet staging systems describe three major prognostic subgroups. Commonly used prognostic biomarkers in chronic lymphocytic leukaemia can be divided into genotypic, DNA-level changes and phenotypic, expression-level changes. For chronic lymphocytic leukaemia, substantial progress in therapy has not been made over the past 40 years. The main goal of future scientific research is to find new platinum complexes that have better efficacy in cancer treatment, the ability to be administered orally, without developing a cancer-drug resistance, and reduced toxic side effects.

Keywords: *Platinum-based anticancer drugs, Chronic lymphocytic leukaemia, Cisplatin mechanisms of action, Cancer treatment*

SAŽETAK

Otkad su Rosenberg i saradnici otkrili antitumorsko delovanje cisplatine, upotreba metalnih kompleksa u lečenju raka izazvala je veliki interes. Danas su lekovi bazirani na platini deo standardne hemoterapije u lečenju raznih karcinoma, tumora matičnih ćelija, sarkoma i limfoma. Nažalost, toksičnost i rezistencija glavne su prepreke za širu kliničku primenu tih lekova. Uprkos konzistentnoj brzini početnog odgovora, terapija cisplatinom često rezultira razvojem rezistencije, što dovodi do neuspeha u lečenju. Upotreba cisplatine znatno je ograničena jakim nuspojavama, kao što su nefrotoksičnost, ototoksičnosti i neurotoksičnost. Iako je cisplatina jedan od najuspešnijih lekova protiv raka do danas, njen biohemijski mehanizam delovanja još uvek je nejasan. Opšte je prihvaćeno da cisplatina ima sposobnost interakcije s purinskim bazama DNA, uzrokuje oštećenja DNA, utiče na mehanizme popravka DNA, a nakon toga indukuje apoptozu u ćelijama raka. Hronična limfocitna leukemija je neoplastična limfoproliferativna bolest B limfocita koja se karakteriše vrlo promenjivim kliničkim tokom. Klinički stadijum bolesti i biološki prognostički faktori su važni prediktori za preživljenje bolesnika. Rai i Binet klasifikacija bolesti opisuje tri glavne prognostičke podskupine. Često korišćeni prognostički biomarkeri u hroničnoj limfocitnoj leukemiji mogu se podeliti u genotipske i fenotipske promene. Za hroničnu limfocitnu leukemiju značajnog napretka u terapiji nije zabeleženo zadnjih 40 godina. Buduća naučna istraživanja imaju za cilj da pronađu nove komplekse platine efikasnije u lečenju malignih tumora, koji se mogu oralno primenjivati, ne razvijaju rezistenciju na lek i imaju manje toksične nuspojave.

Ključne reči: *Antitumorski lekovi, derivati platine, Hronična limfocitna leukemija, Cisplatina mehanizmi delovanja, Lecenje tumora*

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INTRODUCTION

Cancer is one of the most deadly diseases worldwide, and chemotherapy is a main strategy for the systemic treatment of cancer. Cancer patients are treated by repeated cycles of chemotherapy, but the clinical course in some patients is characterized by a series of relapses. Cisplatin was the first metal-based agent used in the clinics for the treatment of cancer. Platinum-based anticancer drugs are among the most potent chemotherapeutic agents for the treatment of various malignant tumours. At present, cisplatin, carboplatin, and oxaliplatin are the only metal-based anticancer agents in worldwide clinical use (1). Platinum complexes are clinically used as adjuvant therapy of cancers aiming to induce tumour cell death (2). Platinum-based drugs are used for the treatment of human and animal tumours and are currently indicated for ovarian, testicular, bladder, colorectal, non-small cell and small cell lung cancers, as well as melanomas, lymphomas, and myelomas (3). There are currently six platinum drugs with marketing approval in various regions throughout the world: cisplatin, carboplatin, oxaliplatin, nedaplatin, lobaplatin, and heptaplatin (3). Severe side effects, as well as drug resistance, have limited their clinical applications (4, 5).

The World Health Organization classification of hematopoietic malignancies describes chronic lymphocytic leukaemia/small lymphocytic lymphoma (CLL/SLL) as a neoplasm composed of monomorphic small round to slightly irregular B lymphocytes in peripheral blood, bone marrow, spleen and lymph nodes, admixed with prolymphocytes and paraimmunoblasts forming proliferation centres in tissue infiltrates (6). These two disorders are morphologically, phenotypically and genotypically indistinguishable, different only in the degree of peripheral blood lymphocytosis (7). The term SLL is used for non-leukemic cases with the tissue morphology and immunophenotype of CLL, and SLL requires lymphadenopathy, no cytopenias due to bone marrow infiltration by CLL/SLL and less than 5 x 10^9/L B lymphocytes in peripheral blood (6). Chronic lymphocytic leukaemia is the most common type of leukaemia in the Western world, accounting for approximately 30% of all leukaemias (8). Individuals over 65 years of age account for 40% of cases, but CLL is extremely rare below the age of 30 years. The overall incidence is approximately three per 100,000 per year. Studies on the racial and geographic distribution show that CLL is 20-30 times more common in white and black populations of Europe, Australasia and North America than in populations of India, China and Japan. The male/female ratio in all populations is approximately 1.5-2:1 (6).

In 70-80% cases, CLL is diagnosed as an incidental finding on a routine full blood count. A definitive diagnosis of CLL is based on the combination of a lymphocytosis and characteristic lymphocyte morphology and immunophenotype. The predominant cell is a small lymphocyte with a narrow border of scanty cytoplasm and a dense nucleus with partially aggregated chromatin and without recognizable nucleoli (perspectives). Gumprecht nuclear shadows, or smudge cells, found as cell debris, are characteristic findings in CLL (9). CLL/SLL has a distinctive immunophenotype. Using flow cytometry, the tumour cells express dim surface IgM/IgD, CD5, CD19, CD20, CD22, CD23, CD43,CD79a and CD11c (weak); however, CD10 is negative, and FMC-7 and CD79b are usually negative or weakly expressed in typical CLL (6). Unlike most other lymphoid malignancies, chromosomal translocations are rare in CLL/SLL but approximately 80% of the cases have cytogenetic abnormalities detected by FISH (10). Approximately 50% of CLL show del 13q14.3, approximately 20% trisomy 12, deletions in 10-20% of 11q22-23, and less commonly, deletions in 17p13 and 6q21 (6, 10). Somatic mutations of the Immunoglobulin Heavy Chain Variable (IGHV) genes are present in at least half of CLL patients, indicating that antigenic exposure may be relevant in the pathogenesis of CLL (11).

The clinical course of B-CLL shows a marked heterogeneity from an indolent type without need for treatment for a long period, to a rapidly progressing disease that requires immediate therapy. The decision to treat a patient is mainly based on clinical and laboratory features indicating active and advanced disease. The Rai and Binet clinical staging systems are used to define disease extent and prognosis, and this definition is based on the extent of lymphadenopathy, splenomegaly, and hepatomegaly, measured by palpation, and anaemia and thrombocytopenia measured by blood cell counts (12, 13). New biological prognostic factors have become important especially in the early stage (9). The expression of ZAP-70 and CD38 are both associated with an adverse prognosis. Deletions of 11q22-23, 17p13 and 6q21 are associated with worse outcome, and isolated del 13q14.3 is associated with a more favourable clinical course (6). Additional adverse predictive factors include a rapid lymphocyte doubling time in peripheral blood (<12 months) and serum markers of rapid cell turnover, including elevated thymidin kinase, sCD23 and β-2 microglobulin (9). Two subsets of CLL can be determined based on the presence or absence of somatic hypermutation by molecular sequencing methods (10). Patients with unmutated IGVH tend to have more advanced disease stages and a more aggressive clinical course and tend to acquire high-risk cytogenetic alterations associated with CD38 and ZAP-70 expression (10).

The standard first-line therapy includes a purine analogue-based combination (fludarabin, cladribine, and pentostatin) e.g., fludarabine, cyclophosphamide with or without rituximab or alkylating agent (chlorambucil in comorbid patients). The other protocol for CLL treatment includes steroids, vincristine, doxorubicine mitoxantrone and monoclonal antibodies (alemtuzumab) (14). Investigations on the intrinsic ability of B-CLL cells to avoid apoptosis have been mainly centred on the Bcl-2 gene product, which is the overexpressed prototype antidote to apoptosis. Targeting the apoptotic pathways may provide a new therapeutic approach. Identifying a new agent with novel

mechanism of action that complement cytotoxicity and fight the resistance will be necessary for the future therapy protocols.

Platinum - physical and chemical properties

Platinum is a transition metal located in the centre of the Periodic Table along with the other members of the group. These metals are less reactive than the typical metals (15). Because of the small energy differences between the valence shells, a number of oxidation states occur: 0, +2, +4, and +5. Cisplatin is a simple neutral inorganic compound having square planar geometry and containing a Pt (II) centre bonded to two non-labile ammine ligands and two labile chloride ligands (4).

When cisplatin enters the cell, it becomes aquated, meaning that the chloride ligands are exchanged for water. This is due to the lower concentration of NaCl in the cytoplasm compared to the blood plasma. The aquated molecule can cross-link DNA through a covalent coordinate bond to the nitrogen atom on guanine and also to a lesser extent, to adenine. This DNA damage prevents replication and transcription, which leads to apoptosis and cell death (16).

History of platinum-based therapy

Cisplatin was first synthesized in the late nineteenth century, after being described in 1845 by Michele Peyrone but its anticancer properties were accidentally discovered by Barnett Rosenberg in the 1960s while he was studying the effects of electric fields on the growth of *Escherichia coli* bacteria (3). Rosenberg and his group found that the electric current itself had no effect on cell division, but the current was causing a chemical reaction dissolving platinum from the electrodes into the medium. Cisplatin was approved for use as an anticancer drug in the 1970s. At that time, researchers focused their attention on identifying organic molecules as new anticancer agents, so cisplatin expanded the drug testing with metal-based molecules.

Carboplatin is the second-generation platinum anticancer drug. This drug was developed as a less-toxic derivative of cisplatin; it is equally effective and is used as the platinum drug of choice in the treatment of ovarian cancers (15). The main difference between carboplatin and cisplatin is the six-membered chelate ring that makes the drug less reactive and prone to hydrolase and thereby reduces some of the unwanted side reactions (15). Carboplatin was approved for clinical use in treating tumours in the 1980s.

Oxaliplatin is the third-generation platinum-based anticancer drug and it was approved in the 1990s for the treatment of colorectal carcinoma. Oxaliplatin has a more complex structure than previous platinum-based anticancer drugs and is also effective against some tumours that have become resistant to cisplatin and carboplatin (15).

Currently, there are many platinum-based drugs in various stages of clinical trials (3). Platinum (IV) complexes have greater inertness than the corresponding platinum (II) complexes. They have some advantages such as oral administration, reduced toxicity and a decrease in the amount of the complex that is lost or deactivated in the path to the target cell (17, 18).

Platinum-based drugs - mechanism of action

The target of platinum-based drugs is nuclear DNA. Once cisplatin has been intravenously administered to the patient, it rapidly diffuses into tissues and is highly bound to plasma proteins (19). The biochemical mechanism by which cisplatin crosses the cell membrane still remains unclear. Passive diffusion across the cellular membrane and active transport can both play a role in the cellular uptake of platinum-based antitumor drugs (4). Early studies noted that cellular uptake of cisplatin was linear, concentration dependent, and nonsaturable, and passively transported across the cellular membrane (20), whereas in higher concentration, endocytosis may contribute to cisplatin uptake (4, 20). In the cytoplasm, many cellular components that have soft nucleophilic sites, such as cytoskeletal microfilaments, thiol-containing peptides and proteins and RNA, may react with cisplatin (19). The most important non-DNA targets of cisplatin probably are the tripeptide glutathione and metallothioneins, and their binding has been associated with negative pharmacological properties including the development of resistance and toxicity (19). The first step in activation of cisplatin is aquation, where a chloride leaving group is replaced by water. Subsequent displacement of this water allows the platinum to coordinate to a nitrogen atom in DNA. The N7 atoms of guanine and adenine that are located in the major groove of the double helix are the most accessible and reactive nucleophilic sites for platinum coordination to DNA (19). Each platinum drug will bind two bases either through nucleosides on the same strand (intra-strand binding) or through individual bases on different strands (inter-strand binding) (19, 21). Intrastrand binding causes the DNA helix to unwind and bend, preventing DNA transcription and replication. This DNA damage initiates apoptosis.

Recently, active/facilitated transport pathways, such as copper transporter (Ctr1), have been identified. A connection between copper and platinum trafficking is bidirectional cross-residence. Cells selected for resistance to high levels of copper were found to be resistant to platinum and vice versa. Human Ctr1 is a member of a highly conserved family of copper transporters that subdivide into three regions: 1) an extracellular N-terminal domain, 2) a membrane embedded domain composed of three transmembrane helices and 3) an intracellular C-terminal domain (4). If the mechanism of cisplatin uptake involves endocytosis of the drug bound to Ctr1 and degradation of the

cisplatin-Ctr1 complex, then additional steps are needed to complete the process; that is, cisplatin needs to be released from the endocytic vesicles into the cytosol or delivered to the nucleus, mitochondria, and microsomal compartments which are known to accumulate platinum after drug exposure. Endosome and lysosome compartments of mammalian contain a Ctr2 for the release of intracellular copper stores. Ctr2 has a large effect on the accumulation of cisplatin and carboplatin (4, 22).

Platinum complexes, protein interaction and toxicity

Cisplatin and related compounds are known to bind to several classes of proteins, affecting aspects as diverse as structural, antioxidant, electron transfer, small molecule or iron transport, or DNA processing (23). These interactions should be considered among the mechanism whereby platinum-containing drugs induce toxic side effects such as nausea, vomiting, fatigue, alopecia, haematological suppression, peripheral sensory neuropathy, renal damage, and others.

More than 95% of the cisplatin that enters a cell is estimated to bind to proteins and peptides, rather than to DNA (23). Platinum-based anticancer drugs can bind to a range of proteins, especially at sulphur atoms, affecting their conformation and functions. One possible mechanism of resistance and toxicity induced by these drugs may be explained according to this interaction (23). Cytotoxicity in cancer cells should always be viewed in relation to general toxicity and not be mistaken for anticancer activity.

Clinical resistance to platinum-based therapy

The major problem with platinum-based anticancer therapy is that cancer cell exposure to the drugs causes resistance, and tumours stop responding. Increasing the dose will increase the toxicity but not gain any antitumour effect (16). The resistance mechanism is multifactorial, and several causes have been proposed. From the molecular point of view, the resistance can be caused mainly in three ways (16). First, the cell can block the influx of the drug. Second, if the drug does enter, the cells can efflux it back outside or block it from reaching the target. Third, if the drug reaches the DNA, it can be removed by a repair system (2).

The pathway controlling the copper homeostasis in the cell becomes interesting when the cisplatin-resistant cancer cells show an over-expression of the copper transporting protein (Ctr1). Ctr1 is the main copper uptake transporter in human cells. It is positioned in the cell membrane where a central pore that functions as a canal has been formed. Studies of crystallized Ctr1 with electron microscopy showed a series of rings of methionines, histidines, and cysteines lingering on the inside of the pore to facilitate copper transport (16). Later studies have discovered a second potential copper transporter, Ctr2 (24). Ctr2 is not located in the plasma membrane like Ctr1 but is located mostly in the membranes of the intracellular compartments (16). Atox 1 is a copper chaperone that transports copper from Ctr1 to ATP7A/B (16). ATP7A and ATP7B are two homologous copper ATPases in human cells located at the trans-Golgi network. They use the energy of the ATP hydrolysis to transport Cu from the cytosol across the cellular membranes. More than likely, this is an indirect process. Several studies have shown the importance of the Ctr1 in cisplatin drug uptake, regulation of the cellular accumulation, and cytotoxicity. The overexpression of Ctr1 is found to sensitize cells to the toxic effects of platinum agents (16). After Ctr1 and ATP7A/B were found to mediate cisplatin resistance, Atox 1 has gained interest in case of platinum anticancer drugs resistance. The cells resistant to cisplatin had elevated levels of ATP7B, which suggest that copper ATPases are involved in resistance to platinum anticancer drugs.

Cisplatin and carboplatin generate mutual cross-resistant cells, but oxaliplatin-resistant cells are often not cross-resistant to cisplatin, pointing to a different mechanism of action. Cisplatin resistance occurs intrinsically (e.g., colon carcinomas) or is acquired (e.g., ovarian carcinomas), and some cancers show no tendency to acquire resistance at all (e.g., testicular cancer) (25). Platinum drugs have been found to overcome resistance in cell lines but have failed in clinics (26).

Cisplatin induced cell death pathways

DNA damage and subsequent induction of apoptosis may be the primary cytotoxic mechanism of cisplatin (27). Apoptosis is a built-in cell suicide program that serves to eliminate cells in the organism that are no longer needed or have sustained severe damage (28). Apoptosis is mediated by caspases, a specialized family of aspartate-specific cysteine proteases. The caspase death effector machinery is negatively controlled by members of the Bcl-2 family. Several independent functions of Bcl-2 have been demonstrated, including the abrogation of the mitochondrial release of caspase-activating factors (such as cytochrome c), the modulation of antioxidant pathways and the regulation of calcium homeostasis (29). There are two separate mechanisms that occur in apoptosis: the extrinsic pathway, activated by pro-apoptotic receptor signals at the cellular surface, and the intrinsic pathway, activated by mitochondrial signals (28). Conventional anticancer therapy seems to stimulate apoptosis primarily via the intrinsic pathway. The Bcl-2 proteins play important roles in the intrinsic pathway, and their elimination resulted in the prolonged survival and rapid loss of leukaemia cells (29).

A critical checkpoint for the activation of the intrinsic pathway is the tumour-suppressor p53 protein, a tran-

scription factor that is considered a 'guardian of the genome'. P53 has multiple functions, including cell-cycle control in response to DNA damage, induction of apoptosis, and DNA repair (30). Recent evidence suggests that genes that regulate apoptotic cell death may play a role in determining the sensitivity of tumour cells to chemotherapy (30). Inactivation of p53 is among the most common mutations, occurring in over half of cancers, and provides a key resistance mechanism that helps cancer to avoid apoptosis (28). Several studies have shown that sensitivity to cisplatin usually correlates with the presence of wild type p53, whereas the lack of functional p53 is related to cisplatin resistance (19).

There are four basic DNA repair pathways: nucleotide excision repair, base excision repair, mismatches repair and double-strand break repairs (31). Each pathway has its own set of proteins that function entirely independently of the other pathways. ERCC1 is an excision nuclease within the nucleotide excision repair pathway. ERCC1 is essential to life, and well preserved through nature (31). Cancer cell that express higher levels of ERCC1, also show higher resistance to platinum drug exposure (31), whereas higher levels of ERCC1 in CLL cells are accompanied with resistance to alkylating agents (33).

CONCLUSION

In spite of the widespread success of cisplatin, the search continues for new platinum drugs. This has been motivated by the desire to improve upon the clinical performance of cisplatin. The side effects associated with cisplatin treatment can be severe and may include toxicity to the kidney and nervous system. The goal is to obtain a compound with better activity and less toxicity than cisplatin (32). Rosenberg`s original experiment identified both platinum (II) and platinum (IV) species as possessing anticancer activity. In spite of this fact, the vast majority of research since then has focused on platinum (II) compounds. Recognition of the enormous potential that platinum (IV) compounds possess as anticancer agents in term of high activity, low toxicity, and perhaps the ability to be effective oral agents, has revived research in this area (32). An interesting therapeutic approach is the combination of cisplatin with two or more non-platinum antitumor drugs. An alternative approach in the treatment of fludarabine-resistant CLL is the inclusion of platinum-based therapy in protocols (34). Cisplatin and oxaliplatin have a synergistic cytotoxic effect with fludarabine on CLL cells (35, 36). However, platinum-based therapy increases the risk of haematological toxicity in patients with hematopoiesis that is already compromised.

Further studies are needed for the development of new generations of more efficient platinum-based anticancer drugs that will show better clinical activity and lower toxicity than the currently used platinum-based agents, even in multidrug-resistant cancers such as CLL.

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EXPRESSION OF THE *BCL2* GENE IN CHRONIC LYMPHOCYTIC LEUKAEMIA PATIENTS

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EKSPRESIJA *BCL2* GENA KOD PACIJENATA SA HRONIČNOM LIMFOCITNOM LEUKEMIJOM

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ABSTRACT

Chronic lymphocytic leukaemia (CLL) manifests as clonal expansion of mature B lymphocytes, whose accumulation is primarily attributed to the dysregulation of apoptosis. Aberrant expression, as well as genetic alterations within various Bcl2 family members and central regulators of the intrinsic, mitochondria-mediated apoptotic pathway all hasve been observed in CLL. Here, we report the expression analysis of the anti-apoptotic Bcl2 gene in a cohort of 58 CLL patients. Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis revealed a significant overexpression of Bcl2 mRNA in CLL samples compared to control samples (p=<0.001). Receiver operating characteristic (ROC) analysis showed that the level of Bcl2 expression exerts a high discriminatory power between patients and healthy subjects (A=0.98, 95% CI=0.95-1.009, p<0.0001).

Key-words: *Chronic lymphocytic leukaemia, apoptosis, Bcl2, expression analysis*

SAŽETAK

Hronična limfocitna leukemija (HLL) se manifestuje kao klonska ekspanzija zrelih B limfocita, čija se akumulacija pripisuje prvenstveno poremećajima procesa apoptoze. U HLL su uočene genetičke promene i aberantna ekspresija različitih članova Bcl2 genske familije, koji imaju ključnu ulogu u regulaciji unutrašnjeg, mitohondrijskog puta aktivacije apoptoze. U ovom radu je analizirana ekspresija anti-apoptotskog Bcl2 gena u grupi od 58 pacijenata obolelih od HLL. Metodom kvantitativnog RT-PCRa detektovana je povišena ekspresija Bcl2 mRNA u HLL uzorcima u odnosu na kontrolne uzorke (p=<0.001). "Receiver operating characteristic" (ROC) analiza je pokazala da nivo ekspresije Bcl2 ima visoku moć diskriminacije između pacijenata i zdravih kontrola (A=0.98, 95% CI=0.95-1.009, p<0.0001).

Ključne reči: *Hronična limfocitna leukemija, apoptoza, Bcl2, analiza ekspresije*

ABBREVIATIONS

Bcl2 - B-cell lymphoma 2
CLL - Chronic lymphocytic leukaemia
mRNA – messenger ribonucleic acid
miRNA- micro ribonucleic acid
qRT-PCR - quantitative reverse-transcriptase polymerase chain reaction

INTRODUCTION

Chronic lymphocytic leukaemia (CLL) is the most frequent type of leukaemia in Western countries, affecting predominantly elderly individuals (aged 65 and over). It manifests as monoclonal expansion of small, mature $CD5^+$ $CD19^+$ $CD23^+$ $sIgM^{low}$ B lymphocytes. CLL is characterized by extremely variable clinical presentations, from indolent to rapidly progressive, with different therapy requirements and overall survival (1,2).

Circulating CLL B lymphocytes are arrested in G_0/early G_1 phase of the cell cycle (3), and their gradual accumulation in blood, bone marrow and secondary lymphoid organs is primarily a result of impaired apoptosis.

The Bcl2 family is a group of evolutionary conserved pro- and anti-apoptotic proteins coded by *Bcl2* family genes, which are involved in the regulation of the intrinsic, mitochondria-mediated pathway of apoptosis. All Bcl2 family proteins con-

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tain at least one of four highly conserved α-helical BH (Bcl2 homology) domains (BH1, BH2, BH3 and BH4). Anti-apoptotic members (Bcl2, Bcl-X_L, Mcl-1, A1, Bcl-W) contain all four BH domains and a C-terminal transmembrane domain. The pro-apoptotic members are divided into "multidomains" (Bax, Bak, Bok), containing BH1-3 and a transmembrane domain, and "BH3-only" (Bim, Bad, Bid, Bik, Bmf, Hrk, Noxa, Puma), containing only the BH3 domain (4). In the absence of apoptotic stimulus, pro-apoptotic members reside in the cytosol, whereas anti-apoptotic are anchored to the mitochondrial membrane and suppress the release of cytochrome c, which is crucial for activation of the caspase cascade and execution of apoptosis (5). Upon receiving a death stimulus, pro-apoptotic proteins translocate from the cytosol to the mitochondrial membrane and interact with anti-apoptotic proteins, ultimately leading to cytochrome c release (6).

BH domains are sites of interaction between Bcl2 family members; this interaction is essential for homo- and heterodimerization, which is one of the main mechanisms of their action. In addition to o prototypical Bcl2/Bax dimer formation, other interactions among Bcl2 family proteins can occur. In addition to dimerization, their activity is modulated through regulation at both transcriptional and post-translational levels (7).

Genetic and epigenetic alterations, as well as aberrant expression of various *Bcl2* family genes, have been observed in CLL (8).

Leukaemic B lymphocytes from the majority of CLL patients (>80%) express high levels of *Bcl2*, a typical anti-apoptotic member of the *Bcl2* family (9). The expression is higher not only in comparison to normal B lymphocytes but also when compared to cells that harbour translocation t(14;18), which places the *Bcl2* gene in close proximity to the immunoglobulin heavy chain enhancer (10). Translocation t(14;18) is common in follicular lymphoma but is a very rare event in CLL. It has been demonstrated that in a high proportion of patients, the *Bcl2* promoter is hypomethylated, leading to increased transcription (11). In addition, overexpression of Bcl2 has been linked to downregulation or loss of miR-15a and miR-16-1. These miRNAs negatively regulate Bcl2 at the post-transcriptional level and are located within the deletion of 13q14, the most frequent genomic aberration in CLL (12,13). Furthermore, Bcl2 overexpression has been reported to associate with -938C>A promoter polymorphism (14); however, this finding was not confirmed by subsequent research (15). The prognostic significance and therapeutic implications of the Bcl2 gene and protein expression level are still under investigation due to conflicting results obtained in different studies.

The aim of this study was to analyse the expression of the *Bcl2* gene in patients with chronic lymphocytic leukaemia.

PATIENTS AND METHODS

This study enrolled 58 unselected patients from the Clinic for Haematology, Clinical Centre of Serbia (Belgrade, Serbia), who were diagnosed with typical B cell CLL based on clinical criteria and laboratory features. The study was approved by the medical ethics committee of the institution.

The patient group consisted of 45 men and 13 women (male/female ratio = 3.5), with a median age of 63.5 years (range: 39 - 86) at the time of diagnosis.

The distribution of clinical Binet stages was as follows: 22 patients (42.3%) stage A, 7 patients (13.5%) stage B and 23 patients (44.2%) stage C (the staging information was unavailable for 6 patients).

The control group consisted of 10 healthy individuals, 3 men and 7 women, with a median age of 53 years (range: 44 - 84).

Peripheral blood mononuclear cells (PBMCs) of all patients contained >90% of CLL lymphocytes, as confirmed by immunophenotyping. PBMCs were isolated by Ficoll density-gradient centrifugation, and total RNA was extracted using TRI reagent (Sigma-Aldrich). The isolated RNA was reverse-transcribed using RevertAid M-MuLV Reverse Transcriptase (Fermentas) and random hexamer primers according to the manufacturer's instructions.

Bcl2 mRNA expression was analysed by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) using SYBR Green chemistry in a 7500 Real Time PCR system (Applied Biosystems). The specific primers used for qRT-PCR amplification were 5'-TCGCCCTGTGGATGACTGA-3' (forward) and 5'-CAGAGACAGCCAGGAGAAATC-3' (reverse). The amplification of Abl using the following primers: forward 5'-TGGAGATAACACTCTAAGCATAACTAAAGGT-3' and reverse 5'-GATGTAGTTGCTTGGGACCCA-3', served as an internal control. The reaction mixture contained 50 ng cDNA, 1 x *Power* SYBR® Green PCR Master Mix (Applied Biosystems) and 0.5 pmol (Bcl2) or 2 pmol (Abl) of each gene-specific primer with a final reaction volume of 10 μl. The cycling conditions were as follows: denaturation of the template at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Each qRT-PCR reaction was performed in duplicate in order to evaluate reproducibility of the results. Quantification of target gene expression was performed using the comparative ddCt method with the HL-60 cell line as the calibrator.

Statistical analyses were performed using the Mann-Whitney rank-sum test, Spearman rank order correlation and receiver operating characteristic (ROC) analysis. All statistical tests were carried out using Sigma Stat 3.5 and SigmaPlot 11.0 software (Systat Software Inc.). Statistical significance was defined as $p<0.05$.

RESULTS

In this study, we analysed the expression of the *Bcl2* gene in a cohort of 58 unselected patients with chronic lymphocytic leukaemia.

Using qRT-PCR methodology for expression analysis, we detected significantly higher levels of *Bcl2* mRNA in CLL samples compared to non-leukaemic samples ($p=<0.001$) (Fig. 1).

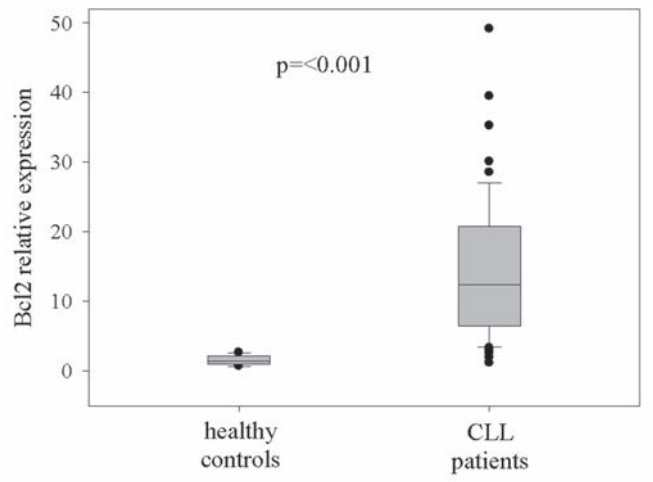

Figure 1
Relative expression of *Bcl2* mRNA in CLL and non-leukaemic samples qRT-PCR analysis showed a significantly higher expression of *Bcl2* in mononuclear cells of CLL patients in comparison to healthy controls (p=<0.001; Mann-Whitney Rank Sum Test).

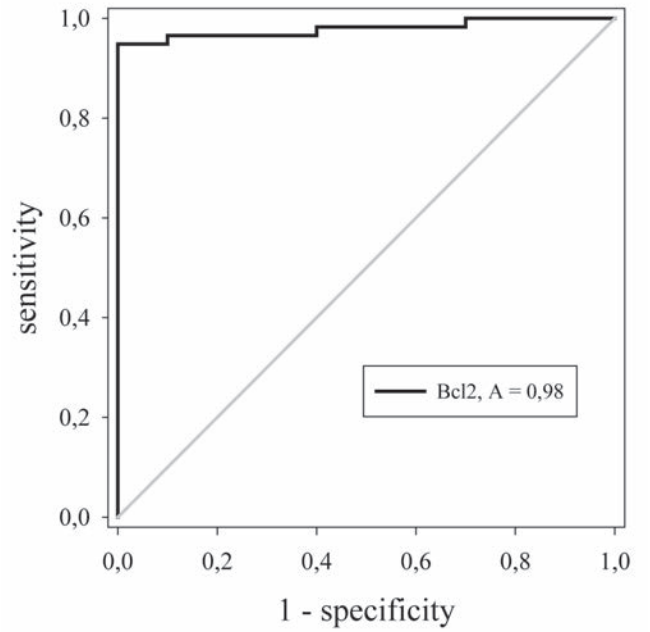

Figure 2
ROC analysis of *Bcl2* expression in CLL and non-leukaemic samples *Bcl2* mRNA expression exerts high discriminatory power between CLL patients and healthy controls.
(A=0.98, sensitivity=0.95, specificity=1, 95% CI=0.95-1.009, p<0.0001)
Abbreviations: ROC, receiver operating characteristic; A, area under the ROC curve; CI, confidence interval.

In addition, we observed a wide variability in the expression of *Bcl2* among CLL patients (1.168-49.146 relative units), in contrast to relatively homogeneous expression among healthy controls (0.685-2.629 relative units). We performed receiver operating characteristic (ROC) analysis in order to evaluate the discriminatory power of *Bcl2* expression in CLL. ROC analysis demonstrated that *Bcl2* mRNA expression efficiently distinguished CLL from control samples (**cut-off=2.76 relative units**, A=0.98, 95% CI=0.95-1.009, p<0.0001), exerting an excellent positive predictive value (PPV=1) and a slightly lower negative predictive value (NPV=0.77) (Fig. 2).

Bcl2 expression level did not show any association with either gender or Binet staging. However, a significant negative correlation with the age of patients at diagnosis (r=-0.4, p=0.004) was detected.

DISCUSSION

Dysregulation of apoptosis is considered to be a hallmark of chronic lymphocytic leukaemia. In addition to the pro-survival influence of the microenvironment-derived signals, intrinsic defects of different apoptotic pathways have been identified in CLL cells, rendering them resistant to apoptosis. For example, the function of the ATM-p53 pathway is often abrogated by 11q22-q23 and 17p13 deletions, which exert the most adverse prognostic impact among all genomic aberrations detected in CLL. Deletions, as well as mutations and aberrant expression of *p53* and *ATM,* have been associated with progressive disease and shorter overall survival (16,17,18). Impaired function of other apoptotic pathways, namely the PI3K/Akt pathway (19,20,21), NF-κB pathway (19,22) and Fas/FasL system (23,24), have also been implicated in CLL.

The role of the Bcl2 family of proteins has been extensively studied in CLL because they are key regulators of the mitochondrial apoptotic pathway. Genetic alterations and aberrant expression of various pro- and anti-apoptotic members have been observed in different studies. In addition to Bcl2, elevated expression in CLL patients vs. healthy controls of other Bcl2 family proteins and genes has been observed, namely Mcl1, $BclX_L$ and *Bcl2L12* (25,26,27). Moreover, in several studies, an upregulation of pro-apoptotic members was observed as well (28), which may seem paradoxical given the longevity of CLL cells. However, this is thought to represent a mechanism by which cells try to compensate for an excess of anti-apoptotic proteins through elevation of their functional antagonists. It should be noted though, that in other studies, pro-apoptotic Bax, Bak and $BclX_s$ were observed to be underexpressed (29). Moreover, it is generally accepted that relative expression and/or activity levels, rather than the levels of individual proteins, are critical determinants of CLL cells' susceptibility to apoptosis.

Overall, the results of different studies regarding the relationship between the expression of Bcl2 family proteins and genes and the clinical behaviour of CLL are highly discrepant, and no consistent correlation with the disease stage, clinical progression or response to treatment could be established.

In this study, we analysed the expression of *Bcl2* and anti-apoptotic members of the *Bcl2* family in CLL patients and healthy controls.

In concordance with other reports, we observed a significant overexpression of *Bcl2* in CLL samples compared to

non-leukaemic samples. According to the calculated cut-off level, 94.8% of patients in our cohort were high-expressing *Bcl2* cases. Although we detected a substantially wide range of *Bcl2* mRNA expression levels among patients, they overlapped to a very small degree with those of healthy controls; therefore, the ROC analysis showed that the *Bcl2* expression level efficiently discriminates CLL from normal samples.

In our study, no association between *Bcl2* mRNA expression and clinical stage of CLL was detected. However, the results of other studies regarding the association of Bcl2 protein expression with clinical stage remain controversial. In several cohorts, Bcl2 expression was higher in advanced stages of the disease (30,31), while in others, such a correlation could not be demonstrated (28,32). The lack of association with clinical stage reopens the question of whether elevated Bcl2 expression is acquired during leukemogenesis, or if elevated Bcl2 expression beganwas present in the originating cells of CLL (33). It should be noted that both staging systems currently in use, Rai and Binet, were applied in these studies, which may have led to the inconsistency of the results.

In summary, the findings of the present study further support the hypothesis that *Bcl2* overexpression plays a role in the pathogenesis of CLL. To what extent inter-patient variability in *Bcl2* expression contributes to the clinical heterogeneity of CLL has yet to be elucidated.

Research on the mRNA expression levels of anti-apoptotic *Bcl-2* family members in human cancer cell lines using qRT-PCR techniques and the assessment of the ability of known Bcl-2 inhibitors to induce cell death within them revealed that the effectiveness of known Bcl-2 inhibitors depends on the mRNA expression profile of tumor cells. The correlation between the cell-killing properties of known Bcl-2 inhibitors and the relative mRNA expression levels of anti-apoptotic *Bcl-2* family members has been observed in leukaemia cell lines and has provided critical insights into apoptosis-based anticancer strategies that target Bcl-2 proteins (34). Moreover, gene expression profiling emphasizes the value of the follow up of molecular markers in CLL patients (especially mRNA expression of *Bcl2* and NOXA) in order to facilitate the choice of an effective treatment for individual patients (35). Therefore, the *Bcl2* mRNA expression profile of each patient could lead to personalized treatment. Thus, the methodology used in this study represents a promising tool for individualization and optimization of therapy for CLL patients.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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EFFECTS OF THE DIRECT RENIN INHIBITOR ALISKIREN ON OXIDATIVE STRESS IN ISOLATED RAT HEART

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EFEKTI DIREKTNOG RENINSKOG INHIBITORA ALISKIRENA NA OKSIDATIVNI STRES IZOLOVANOG SRCA PACOVA

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ABSTRACT

Increased activity of the renin-angiotensin-aldosterone system (RAAS) plays a significant role in the development and progression of various cardio-metabolic diseases, such as hypertension, atherosclerosis and heart failure. Aliskiren is the newest antihypertensive drug and the first orally active direct renin inhibitor to become available for clinical use. This study investigated the acute and direct effects of Aliskiren on different parameters of oxidative stress on isolated rat heart. The hearts of male Wistar albino rats (n = 24, 8 per experimental group, age 8 weeks, body mass 180–200 g), were excised and retrogradely perfused according to the Langendorff technique at a gradually increasing perfusion pressure (40-120 cmH_2O). Markers of oxidative stress (NO_2^-, TBARS, H_2O_2 and O_2^-) were measured spectrophotometrically after perfusion with three different concentrations of Aliskiren (0.1 μM, 1 μM, and 10 μM). The results demonstrated possible dose-dependent cardioprotective properties of Aliskiren, particularly with higher CPP. Lipid peroxidation (TBARS) levels decreased with the highest dose of Aliskiren and higher CPP, and the same trend was observed in nitrite (NO_2^-) and hydrogen peroxide (H_2O_2) levels. These findings indicate that the acute effects of Aliskiren do not likely promote the production of reactive oxygen species upon higher pressure with the highest dose. Aliskiren may exert beneficial effects on oxidative stress biomarkers.

Keywords: *Aliskiren, Isolated rat heart, Langendorff technique, Oxidative stress*

SAŽETAK

Povećana aktivnost rennin-angiotenzin-aldosteron sistema (RAAS) može da ima značajnu ulogu u razvoju i progresiji različitih kardio-metaboličkih bolesti kao što su hipertenzija, ateroskleroza i srčana insuficijencija. Aliskiren se ubraja u najnovije antihipertenzivne lekove i prvi je oralni direktni inhibitor renina koji je uveden u kliničku upotrebu. Prema tome, cilj ove studije je bio da ispita akutne i direktne efekte aliskirena na različite parametre oksidativnog stresa izolovanog srca pacova. U studiji su korišćeni Wistar albino pacovi (n = 24, 8 životinja u svakoj grupi) muškog pola, starosti 8 nedelja, telesne mase 180–200g. Nakon izolovanja, srca ovih pacova su retrogradno perfundovana prema Langendorff-ovoj tehnici uz postepeno povećanje perfuzionog pritiska (40-120 cmH_2O). Markeri oksidativnog stresa (NO_2^-, TBARS, H_2O_2 i O_2^-) su mereni spektrofotometrijski nakon perfuzije sa tri različite koncentracije aliskirena (0.1μM, 1 μM i 10μM). Rezultati su pokazali da aliskiren pri najvišim vrednostima perfuzionog pritiska ostvaruje izvesna dozno-zavisna kardioprotektivna svojstva. Naime, nivoi indeksa lipidne peroksidacije (TBARS), nitrita (NO_2^-) i vodonik peroksida (H_2O_2) su bili sniženi nakon administracije najveće doze aliskirena pri višim perfuzionim pritiscima. Ova saznanja ukazuju da akutno primenjeni aliskiren (pri višim dozama i perfuzionim pritiscima) ne stimuliše proizvodnju slobodnih radikala. Aliskiren može da ima pozitivne efekte na biomarkere oksidativnog stresa.

Ključne reči: *aliskiren, izolovano srce pacova, oksidativni stres, tehnika po Langendorff-u*

ABBREVIATIONS

ACEI - Angiotensin converting enzyme inhibitors
ARB - Angiotensin receptor blockers
AT - Angiotensin
CF - Coronary flow
cGMP - Cyclic guanosine 3′,5′-monophosphate
DRI - Direct renin inhibitor
MDA – Malondialdehyde
NO - Nitric oxide
RAAS - Renin-angiotensin-aldosterone system
ROS - Reactive oxygen species

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INTRODUCTION

Increased activity of the renin-angiotensin-aldosterone system (RAAS) plays a significant role in the development and progression of various cardiometabolic diseases, such as hypertension, atherosclerosis, diabetes and heart failure (1). RAAS promotes vasoconstriction, sodium reabsorption, cardiac remodelling and other potentially detrimental effects (2, 3). Angiotensin II interaction with the AT1 receptor subtype and promotes oxidative stress, vascular smooth muscle migration, cardiomyocyte proliferation, hypertrophy and ventricular dilatation (4). Direct renin inhibitor blockade of RAAS at the rate-limiting step via reduction in plasma renin activity and levels of circulating angiotensin I and angiotensin II may be beneficial for cardiovascular risk in patients with essential hypertension and associated clinical conditions, such as diabetes and nephropathy (5, 6).

Aliskiren is the newest antihypertensive drug and the first orally active direct renin inhibitor (DRI) to become available for clinical use. The US Food and Drug Administration approved Aliskiren for clinical use in March 2007 (7). Essential hypertension is an important cause of death worldwide. Blockade of RAAS, which plays a significant role in the development of essential hypertension, is suboptimal in combination with angiotensin II (AT II)-converting enzyme inhibitors (ACEIs) or ATII type 1 receptor blockers (ARB) (8). However, whether Aliskiren is superior to conventional RAAS blockers in the prevention of heart and renal diseases is not known (9). Aliskiren is generally well tolerated, and it exhibits a placebo-like profile at doses from 75 mg to 300 mg (5). The plasma concentration of Aliskiren increases in a dose-dependent fashion, with peak concentrations after 3–6 hours. The average plasma half-life of Aliskiren is 23.7 hours, oral bioavailability is approximately 5% (95% is excreted unchanged in faeces), and plasma steady-state levels are achieved after 5–8 days of treatment (1). Aliskiren is primarily eliminated unmetabolised via biliary excretion, and less than 1% is excreted in the urine (5). Several clinical trials confirmed the efficacy of Aliskiren on blood pressure reduction as a monotherapy (10, 11) and in combination therapy (12, 13).

Oxidative stress is a well-known phenomenon that plays an important role in the pathogenesis of various diseases and syndromes. Any imbalance between pro- and anti-oxidants in which pro-oxidants prevail is known as oxidative stress (14). Existing evidence supports the view that oxidative stress may play a crucial role in cardiac and vascular abnormalities in different types of cardiovascular diseases, and antioxidant therapy may be beneficial (15). Few studies investigated the influence of Aliskiren on oxidative stress, but recent studies in different models suggested that Aliskiren monotherapy or in combination reduces blood pressure by increasing NO (nitric oxide)-cGMP (cyclic guanosine 3′,5′-monophosphate) production (16), superoxide anion production and malondialdehyde (17).

Previous data on reactive oxygen species (ROS), such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), index of lipid peroxidation and nitrites (NO_2^-), are not sufficient. Therefore, this study investigated the acute and direct effects of Aliskiren on different parameters of oxidative stress on isolated rat hearts from adult male rats.

MATERIALS AND METHODS

Isolated rat heart preparation

The hearts of male Wistar albino rats (n = 24, 8 per experimental group, age 8 weeks, body mass 180–200 g) were euthanized via cervical dislocation (Schedule 1 of the Animals/Scientific procedures, Act 1986, United Kingdom) after short ketamine/xylazine narcosis. Emergency thoracotomy and sudden cardiac arrest were performed via superfusion with ice-cold isotonic saline. Normal heart rhythm was restored, and an entrance to the left atrium of the heart was created through the damaged mitral valve. Hearts were retrogradely perfused according to the Langendorff technique at a gradually increasing perfusion pressure (40 cmH_2O – 120 cmH_2O). Hearts were perfused with a Krebs-Henseleit solution composed of: NaCl 118 mM, KCl 4.7 mM, $CaCl_2x2H_2O$ 2.5 mM, $MgSO_4x7\ H_2O$ 1.7 mM, $NaHCO_3$ 25 mM, KH_2PO_4 1.2 mM, and glucose 5.5 mM, equilibrated with 95% O_2/5% CO_2 and warmed to 37°C (pH 7.4).

Physiological assay and experimental protocol

A 30-min perfusion stabilisstabilization period was performed. CPP was lowered to 60 cmH_2O after an equilibration period (70 cmH_2O) and gradually increased to 80 cmH_2O, 100 cmH_2O, and 120 cmH_2O, then finally lowered to 40 cmH_2O. Measurements were performed at each perfusion pressure using pure a Krebs-Henseleit solution and immediately followed by perfusion with active components (different concentrations of Aliskiren) to avoid time-dependent adverse effects. Groups were assigned by the concentration of Aliskiren given in the Krebs-Henseleit perfusate: the hearts of the first group were perfused with 0.1 μM Aliskiren; the second group with 1 μM; and the third group with 10 μM. Each heart was its own control. Coronary flow (CF) was considered stable when three repeated values of CF were identical. The following markers of oxidative stress were measured spectrophotometrically in the collected samples of coronary venous effluent:

1. Nitrites (NO_2^-)
2. Index of lipid peroxidation (measured as TBARS - thiobarbituric acid-reactive substances)
3. Hydrogen peroxide (H_2O_2) and
4. Superoxide anion radical (O_2^-)

The Faculty of Medical Sciences Ethics Committee for the welfare of experimental animals, University of Kragujevac approved the experimental protocol.

Biochemical assays

Determination of nitrites (NO_2^-)

Nitric oxide decomposes rapidly to form stable metabolite nitrite/nitrate products. The nitrite level (NO_2^-) was measured and used as an index of nitric oxide (NO) production using Griess's reagent. A total of 0.5 ml of perfusate was precipitated with 200 µl of 30 % sulphosalicylic acid, vortexed for 30 min, and centrifuged at 3000 x g. Equal volumes of the supernatant and Griess's reagent, containing 1 % sulphanilamide in 5 % phosphoric acid/0.1 % naphthalene ethylenediamine dihydrochloride, was added and incubated for 10 min in the dark and measured at 543 nm. Nitrite levels were calculated using sodium nitrite as the standard (18).

TBARS determination (index of lipid peroxidation)

The degree of lipid peroxidation in the coronary venous effluent was estimated by TBARS using 1 % thiobarbituric acid in 0.05 NaOH incubated with the coronary effluent at 100°C for 15 min and measured at 530 nm. The Krebs–Henseleit solution was used as a blank probe (19).

Determination of hydrogen peroxide (H_2O_2)

Measurements of hydrogen peroxide (H_2O_2) were based on the oxidation of phenol red by hydrogen peroxide, in a reaction catalysed by horseradish peroxidase (HRPO). A volume of 200 µl of perfusate was precipitated with 800 ml of a freshly prepared phenol red solution, and 10 µl of (1:20) HRPO (made ex tempore) was added. An adequate volume of Krebs–Henseleit solution was used in blank probes (instead of coronary venous effluent). The level of H_2O_2 was measured at 610 nm (20).

Determination of superoxide anion radical (O_2^-)

Superoxide anion radical (O_2^-) levels were measured using a nitro blue tetrazolium reaction in TRIS buffer with coronary venous effluent at 550 nm. The Krebs–Henseleit solution was used as a blank probe (21).

Drug

Aliskiren was used as pure drug (Hangzhou Holypharm Biotech CO., LTD).

Statistical analysis

Experimental data are expressed as the mean value (X) ± standard deviation (SD). Paired samples t tests were used to test the statistical significance of the results and confirm the hypotheses. A database analysis of the results was performed using the software package SPSS 18th version (SPSS Inc., Chicago, IL, USA). P values lower than 0.05 ($p<0.05$) were considered significant, and p values lower than 0.01 ($p<0.01$) were considered highly significant.

RESULTS

Nitrites (NO_2^-)

The administration of 10 µM Aliskiren induced a statistically significant decrease in NO_2^- release at CPP = 120 cmH_2O. NO_2^- release did not change significantly during the administration of 0.1 µM and 1 µM Aliskiren for any CPP valu ce compared with the control conditions (Figure 1A, 1B, 1C).

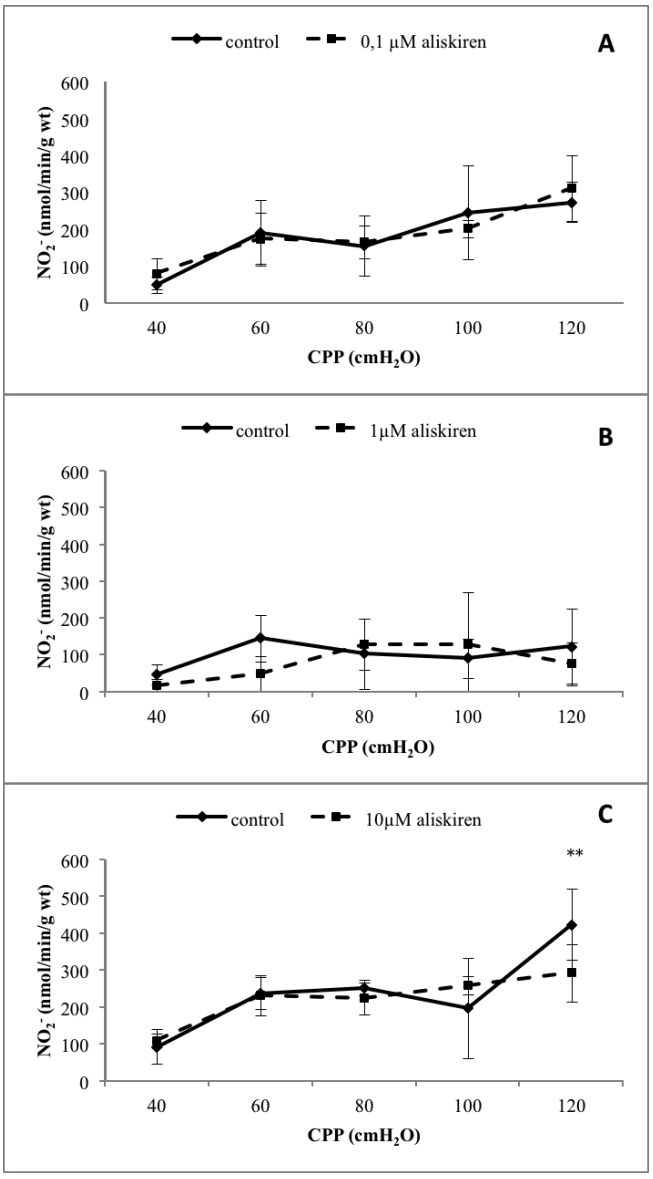

Figure 1A-C. The effects of 0.1 µM Aliskiren (1A), 1 µM Aliskiren (1B) and 10 µM Aliskiren (1C) on the oxidative stress parameter NO_2^-. Values are representedmean as the means ± SE; *p<0.05, **p<0.01.

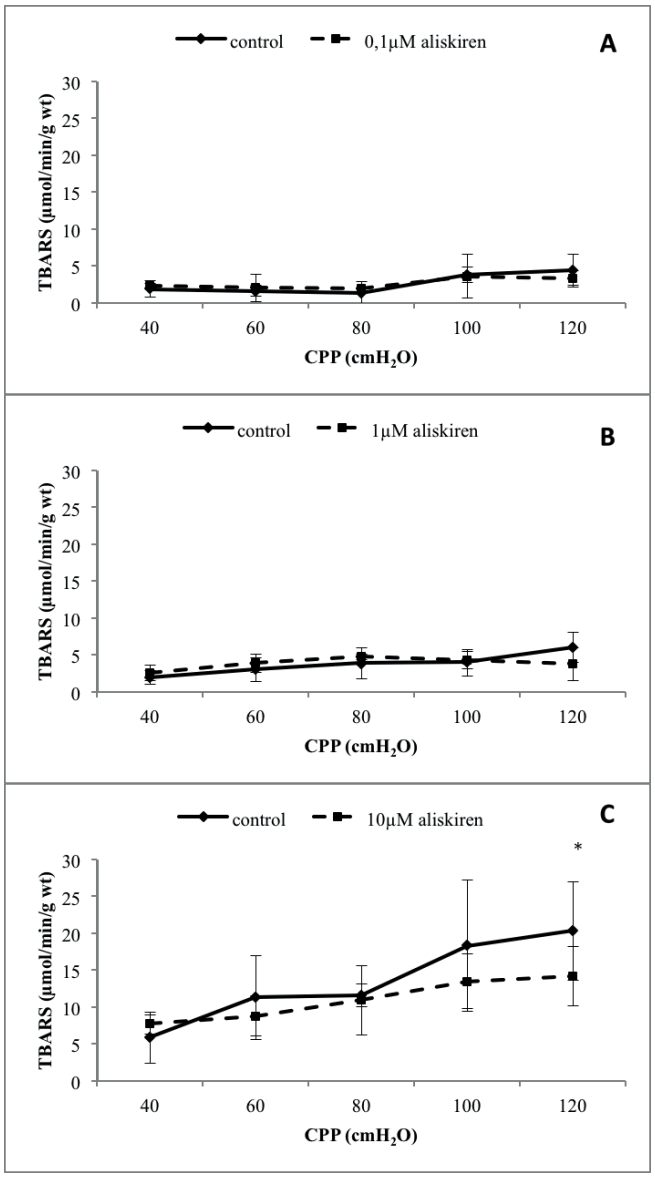

Figure 2A-C. The effects of 0.1 μM Aliskiren (2A), 1 μM Aliskiren (2B) and 10 μM Aliskiren (2C) on the oxidative stress parameter TBARS. Values represent means ± SE; *p<0.05, **p<0.01.

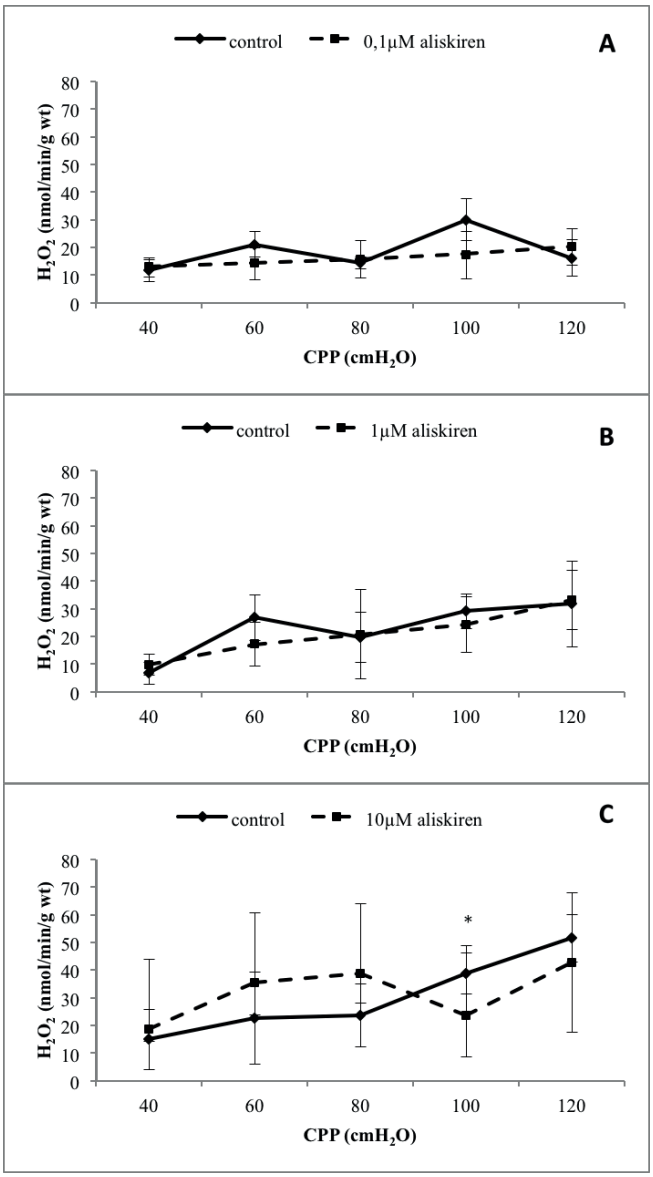

Figure 3A-C. The effects of 0.1 μM Aliskiren (3A), 1 μM Aliskiren (3B) and 10 μM Aliskiren (3C) on the oxidative stress parameter H_2O_2. Values represent means ± SE; *p<0.05, **p<0.01.

Index of lipid peroxidation (TBARS)

The administration of 10 μM Aliskiren induced a statistically significant decrease in lipid peroxidation at CPP = 120 cmH_2O. There were no statistically significant changes in TBARS values during the application of 0.1 μM Aliskiren or 1 μM Aliskiren over the entire CPP range (Figure 2A, 2B, 2C).

Hydrogen peroxide (H_2O_2)

The administration of 10 μM Aliskiren induced a statistically significant decrease in H_2O_2 release at CPP = 100 cmH_2O. There were no statistically significant changes in H_2O_2 values during the application of 0.1 μM Aliskiren or 1 μM Aliskiren over the entire CPP range (Figure 3A, 3B, 3C).

Superoxide anion radical (O_2^-)

No statistically significant changes in O_2^- release were observed after 0.1 μM, 1 μM and 10 μM Aliskiren administration over the entire CPP range (Figure 4A, 4B, 4C).

DISCUSSION

Hypertension is the disease that is most responsible for mechanical stress on the heart because of increased arterial pressure and structural and functional alterations of its target organs (22). Long-term hypertension often results in left ventricular hypertrophy, but antihypertensive drugs, including ACEI, ARB and calcium channel antagonists,

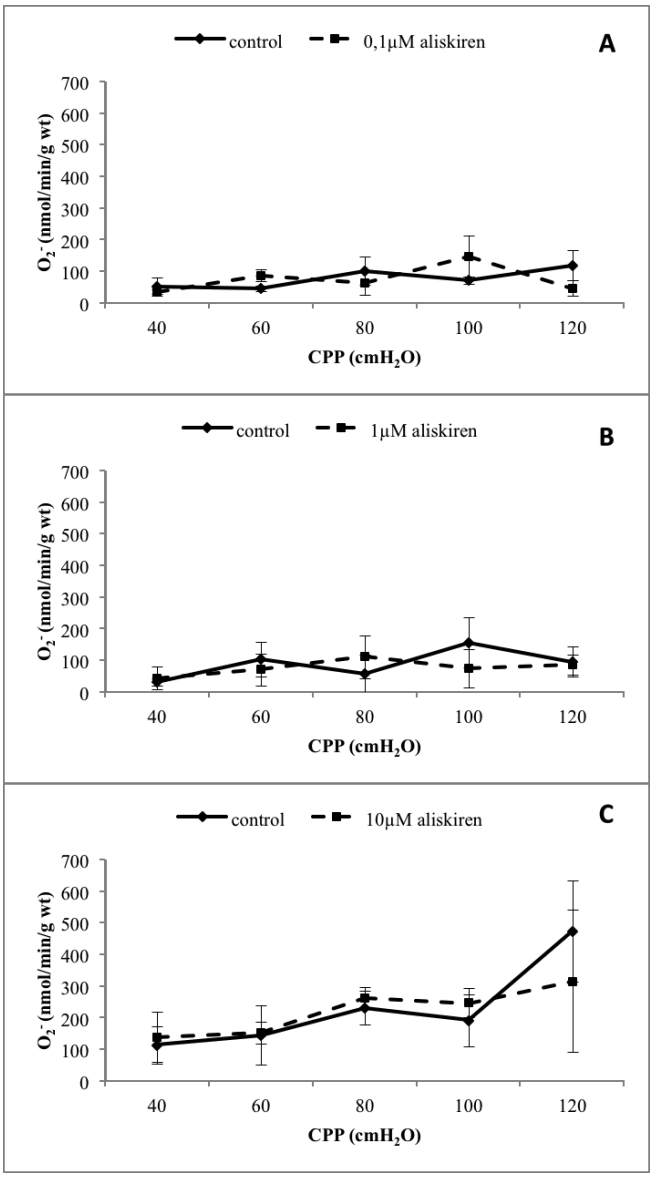

Figure 4A-C. The effects of 0.1 μM Aliskiren (4A), 1 μM Aliskiren (4B) and 10 μM Aliskiren (4C) on the oxidative stress parameter O_2^-. Values represent means ± SE; *p<0.05, **p<0.01.

exert inhibitory effects on cardiac hypertrophy. However, disease progression and subsequent cardiac dysfunction remain a significant problem in hypertensive subjects (23). Prolonged hypertension also causes structural alterations of the vascular wall, which are characterized by endothelial dysfunction, extracellular matrix deposition, medial layer thickening due to hypertrophy/hyperplasia, and vascular smooth muscle cell migration. There is increasing interest in the development of new therapeutic possibilities against hypertension. One recent addition to the family of RAAS blockers was Aliskiren, which is a DRI that is indicated for the treatment of hypertension. Several studies demonstrated that Aliskiren, as first orally active DRI, could be used as a monotherapy and in combination with other agents to lower blood pressure (24). Some studies estimated that once daily Aliskiren administration reduced hypertension compared with ACEI, ARB, and diuretics (25-29). Aliskiren is the newest antihypertensive drug, and only a few studies evaluated the influence of Aliskiren on oxidative stress markers.

Several studies investigated Aliskiren and found positive antihypertensive effects (30-32), but no studies investigated the influence of acute Aliskiren administration on the production of oxygen-free radicals in an isolated rat heart.

Yamamoto et al. used a mouse model of renal and cardiac tissue and demonstrated that Aliskiren and valsartan were associated with significant reductions in oxidative stress in these tissues and the combination of these two drugs improved cardiovascular and renal injuries in endothelial NO synthase–deficient mice, which are associated with a greater attenuation of tissue oxidative stress markers (33). Imanishi et al. noted that Aliskiren treatment exhibited protective effects on endothelial function and atherosclerotic changes and co-treatment with an angiotensin II receptor blocker exhibited additive protective effects on both conditions (34).

The present study examined the effects of acute Aliskiren administration (0.1 μM, 1 μM, and 10 μM) on oxidative stress biomarkers in isolated rat hearts.

Our study investigated NO_2^- and demonstrated statistically significant changes, especially at the highest Aliskiren concentration. NO_2^- levels were lower than control values (Fig 1C). Luis et al. investigated chronic Aliskiren administration and Aliskiren in combination with Amlodipine in diabetic rats and demonstrated that NO levels increased after Aliskiren administration individually and as a combined therapy (16).

Additionally, TBARS values decreased significantly after 10 μM Aliskiren administration compared to control (Fig 2C). Kamal investigated the effect of 4 weeks Aliskiren administration on TBARS in rat liver tissue homogenates and demonstrated increased TBARS levels, which is similar to our result (35). Lipid peroxidation was also increased in an experimental study using malondialdehyde (MDA) as an end product of polyunsaturated fatty acid oxygenation, which also correlates with our findings (15).

Administration of the highest dose of Aliskiren (10 μM) slightly increased the measured values at lower CPP, but a significant decrease in parameter values was observed at CPP = 100 cmH_2O (Fig 3C).

Superoxide anion radical levels were similar to Zang et al. who investigated chronic effects of Aliskiren on myocardial ischaemia/reperfusion injury in spontaneously hypertensive rats. This study recorded increased values of oxidative stress parameters in a control group of rats that did not receive Aliskiren. Aliskiren abolished the increased superoxide anion production in the experimental group (17). However, our results did not demonstrate any statistically significant differences, but the Figure for this parameter shows that the highest dose exhibited the lowest values (Fig 4C).

Our data may be significant because few studies examined the influence of acute Aliskiren administration on the production oxidative stress parameters, especially of H_2O_2, in isolated rat hearts.

CONCLUSIONS

The results of the present study provide important insights into the acute and direct effects of Aliskiren on oxidative stress biomarkers in isolated rat hearts. Our results demonstrated that acute Aliskiren effects do not promote the production of reactive oxygen species. Aliskiren may exert beneficial effects on the balance of pro- and antioxidants. However, further research on chronic Aliskiren administration is needed to provide a clearer explanation of this phenomenon.

ACKNOWLEDGMENT

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LACK OF *PRSS1* AND *SPINK1* POLYMORPHISMS IN SERBIAN ACUTE PANCREATITIS PATIENTS

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ODSUSTVO *PRSS1* I *SPINK1* POLIMORFIZMA KOD SRPSKIH PACIJENATA OBOLELIH OD AKUTNOG PANKREATITISA

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ABSTRACT

Acute pancreatitis represents an acute nonbacterial inflammation of the pancreas caused by a premature and ectopic activation of pancreatic digestive enzymes. Two of the most important genes in pancreatic autodigestion, PRSS1 and SPINK1, were implicated in the earliest discoveries of the genetic background of pancreatitis. However, the distribution of their variations displays interethnic variability, which could significantly affect the magnitude of their proposed effects on this disease worldwide. The aim of the present study was to investigate the distribution of the most important functional variations of PRSS1 (86A>T and 365G>A) and SPINK1 (101A>G), and their influence on the clinical course of acute pancreatitis in Serbian patients. The study enrolled 81 subjects, the severity of disease course was determined using the Atlanta Classification system, and the genotyping was conducted using a PCR-RFLP method. PRSS1 86A>T and 365G>A SNPs were not observed in the study population, while SPINK1 101A>G was present with the frequency of 0.62% (95% CI: 0.00, 3.83%). Due to extremely low frequencies or absences of examined variations, the proposed effect of these SNPs on the severity of acute pancreatitis could not be confirmed. The results do not support routine genotyping of either PRSS1 or SPINK1 in Serbs.

Keywords: *PRSS1, SPINK1, genetic polymorphism, acute pancreatitis, Serbian*

SAŽETAK

Akutni pankreatitis predstavlja akutno neinfektivno zapaljenje pankreasa, prouzrokovano prevremenom i ektopičnom aktivacijom pankreasnih digestivnih enzima. Enzimi PRSS1 and SPINK1, koji igraju neke od najvažnijih uloga u pankreasnoj autodigestiji, prvi su otkriveni faktori genetske predispozicije za nastanak pankreatitisa. Ipak, zastupljenost njihovih genetskih varijacija varira u zavisnosti od etničke pripadnosti, što u velikoj meri utiče na značaj njihovog učešća u ovoj bolesti širom sveta. Cilj ove studije bio je da ispita distribuciju najznačajnijih funkcionalnih varijacija gena PRSS1 (86A>T i 365G>A) i SPINK1 (101A>G), kao i njihov uticaj na kliničku sliku bolesti, kod Srba obolelih od akutnog pankreatitisa. Istraživanje je uključilo 81 ispitanika, težina bolesti određivana je uz korišćenje Atlanta klasifikacionog sistema, a genotipizacija je sprovedena pomoću PCR-RFLP metode. PRSS1 polimorfizmi 86A>T i 365G>A SNPs nisu detektovani u ispitivanoj populaciji, dok je učestalost SPINK1 101A>G varijacije iznosila 0,62% (95% IP: 0,00; 3,83%). Obzirom na ekstremno nisku učestalost ili potpuno odsustvo ispitivanih varijacija, njihov efekat na težinu i tok akutnog pankreatitisa nije mogao biti potvrđen. Rezultati istraživanja ne preporučuju rutinsku genotipizaciju PRSS1 i SPINK1 kod Srba.

Ključne reči: *PRSS1, SPINK1, genetski polimorfizam, akutni pankreatitis, srpski*

ABBREVIATIONS

PRSS1 - cationic trypsinogen (protease serine type 1);
SPINK1 - pancreatic secretory trypsin inhibitor (serine protease inhibitor Kazal type 1);
SNP - Single nucleotide polymorphism;
EDTA - ethylene diamine tetracetic acid;
PCR - Polymerase chain reaction;
PCR-RFLP - Polymerase chain reaction-restriction fragment length polymorphism

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INTRODUCTION

Acute pancreatitis is an acute nonbacterial inflammation of the pancreas caused by a premature and ectopic activation of pancreatic digestive enzymes (1-3). In most cases, the disease is mild and self-limiting. However, more than 25% of patients develop a severe form of acute pancreatitis. Of those, more than half die, often during the first week after admission, due to local complications, haemodynamic instability and/or multiple organ failure (2, 4-6). As the treatment outcome highly depends on both the type and timing of the management, early differentiation between mild and severe acute pancreatitis has proven to be of the utmost clinical importance (6). Ultimately, identification of biomarkers for severe disease course upon the very first admission would be extremely beneficial for improving the chance of a good response to therapy.

Genetics, and environmental factors significantly contribute to the onset, severity and outcome of acute pancreatitis (7-9). Numerous zymogens and inflammatory mediators that regulate the process are polymorphic, and their genetic variations among pancreatitis patients have often been associated with a more severe clinical course and a worse prognosis (3, 10-12). Two of the most important role players in pancreatic autodigestion, cationic trypsinogen (protease serine type 1, PRSS1) and pancreatic secretory trypsin inhibitor (PSTI, serine protease inhibitor Kazal type 1, SPINK1), were implicated in the earliest discoveries of the genetic background of pancreatitis (13, 14). PRSS1 is the major isoform of trypsinogen, the most important pancreatic zymogen, which is catalysed into trypsin by enterokinase or other trypsin molecules (3, 9). The *PRSS1* coding gene is polymorphic, and the most significant variations include 365G>A and 86A>T single nucleotide polymorphisms (SNPs) that are linked to a malfunction of the normal process of trypsin inactivation by trypsin-like molecules and other inhibitors of its enzymatic activity (7-9, 15). In contrast, SPINK1 represents the first line of defence against premature trypsinogen activation as it inhibits trypsin activity within pancreatic acinar cells (3, 7, 9, 16). SPINK1 is also encoded by a polymorphic gene, and its most important variation 101A>G has been associated with the increased risk of acute pancreatitis, especially in the presence of other significant genetic or environmental factors (7, 9, 16).

Previous research has established that both *PRSS1* and *SPINK1* polymorphisms have the potential to modulate the clinical presentation and prognosis of pancreatitis. Moreover, the distribution of these variations differs among populations (17-22), and this interethnic variability could significantly affect the magnitude of their proposed role in pancreatitis worldwide. The aim of the present study was to investigate the distribution of the most important functional *PRSS1* and *SPINK1* variations and their influence on the clinical course of acute pancreatitis Serbian patients.

MATERIALS AND METHODS

Study subjects

The study enrolled 81 Serbian patients with diagnosed acute pancreatitis, who were admitted to the Intensive Care Unit of the Clinical Centre Kragujevac in Serbia, from November 2011 until February 2014. Severity of disease course was determined using the Atlanta Classification system (23). The study was conducted in accordance with the Declaration of Helsinki and its subsequent revisions, and all patients or their legal representatives gave written informed consent. The approval for conducting the study was obtained from the ethics committee at the Clinical Centre Kragujevac.

Genotyping

Genomic DNA was isolated from EDTA blood samples using the QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany). DNA concentration was determined by a Qubit™ dsDNA HS Assay Kit on the Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA). All PCR reactions were performed on the Techne Genius PCR Thermal Cycler (Techne, Cambridge, UK). The PCR amplicons and restriction fragments were detected by gel electrophoresis on a 2.4% agarose gel stained with Sybr® safe DNA gel stain (Invitrogen, Carlsbad, CA).

A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used for genotyping *PRSS1* variation 365G>A (rs111033565, R122H) and was previously reported by Masamune et al. (24). Briefly, the PCR reaction was performed in a 16 µl mixture of 0.2 µM dNTP Mix (Thermo Scientific, Waltham, MA), 2.5 mM MgCl2, 0.2 µl of primers 5'- TGACCCACATCCCTCTGCTG -3' and 5'- TCTCCATTTGTCCTGTCTCT -3' (Invitrogen, Carlsbad, CA), 0.5 U of DreamTaqDNA Polymerase (Thermo Scientific, Waltham, MA), and ~20 ng of DNA in 1X PCR buffer (Qiagen, Hilden, Germany). The conditions included an initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 1 min, annealing at 64 °C for 1 min, extension at 72 °C for 1 min; and a final extension at 72 °C for 5 min. PCR generated 615 bp long amplicons, which remained uncut by the AflIII (NEB, Hertfordshire, UK) restriction enzyme in the presence of the 365G allele. Variant allele 365A was, on the other hand, digested to 323 bp and 292 bp fragments.

PRSS1 SNP 86A>T (rs111033566, N29I) was detected using PCR-RFLP as described by Mora et al. (25). In short, PCR was performed in a 16 µl reaction mixture, containing ~20 ng of DNA, 0.2 µM dNTP Mix (Thermo Scientific, Waltham, MA), 1.5 mM $MgCl_2$, 0.2 µl of primers 5'-CGCCACCCCTAACATGCTAT-3' and 5'-CTCTCCCAGGCAGACTGGCC-3' (Invitrogen, Carlsbad, CA) and 0.5 U of DreamTaqDNA Polymerase (Thermo Scientific, Waltham, MA) in a 1X PCR buffer (Qiagen, Hilden, Germany). The PCR conditions were an initial denaturation at 95 °C for 5 min; 40 cycles of denaturation at 95

°C for 30 sec, annealing at 64 °C for 30 sec, extension at 72 °C for 30 sec; and final extension at 72 °C for 5 min. The amplification resulted in 266 bp long PCR products, which were then exposed to the restriction enzyme TaaI (Thermo Scientific, Waltham, MA). Digestion of the wild type allele yielded three fragments of 102 bp, 79 bp and 85 bp, while variant 86T allele was cut to 181 bp and 85 bp fragments.

Genotyping for *SPINK1* 101A>G (rs17107315, N34S) was conducted using the PCR-RFLP method according to Gomez-Lira et al. (16). Briefly, an 138 bp SPINK1 region was amplified in a total PCR mixture amount of 18 µl, including ~20 ng of DNA, 0.2 µM dNTP Mix (Thermo Scientific, Waltham, MA), 1.5 mM MgCl2, 0.2 µl of primers 5'-CAATCACAGTTATTCCCCAG-3' and 5'-TGGTGCATCCATTAAGTGCA-3' (Invitrogen, Carlsbad, CA) and 0.5 U of DreamTaqDNA Polymerase (Thermo Scientific, Waltham, MA) in a 1X PCR buffer (Qiagen, Hilden, Germany). The reaction mixture was submitted to an initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 sec, annealing at 54 °C for 30 sec, extension at 72 °C for 1 min; and final extension at 72 °C for 5 min. PCR products underwent restriction digest by the Bsp1286I enzyme (Thermo Scientific, Waltham, MA), which cut only variant allele to 122 bp and 16 bp fragments, while wild type 101A remained uncut.

Statistical analysis

Genotype data were presented as haplotype and genotype frequencies, and the 95% confidence interval calculations were calculated according to the modified Wald method.

RESULTS

The study population was comprised of 54 men and 27 women aged between 23 and 86 (median age 59 years). Of them, 35 and 46 were classified as mild and severe acute pancreatitis patients, respectively. Among the 81 examined Serbian acute pancreatitis patients, *PRSS1* 86A>T and 365G>A SNPs were not observed. *SPINK1* variant 101G was present in the study population with a frequency of 0.62% (95% CI: 0.00, 3.83%), as only one study subject was a heterozygous carrier. This patient was a 48-years old overweight male (BMI: 29.3 kg/m^2), cigarette smoker (20 cigarettes per day) and long-term (10 years) alcohol consumer (3 alcohol drinks per day) with confirmed gallstone disease, who developed a severe form of acute necrotizing pancreatitis with pancreatic pseudocyst. This was his first attack of acute pancreatitis, and no one in his family suffered from this condition before. The patient was treated with analgesics, fluid and nutritional support and antibiotics and antisecretory drugs and was discharged from the hospital fully recovered.

DISCUSSION

In the present study, we investigated the distribution of the important functional *PRSS1* and *SPINK1* polymorphisms among Serbian patients diagnosed with mild or severe acute pancreatitis. To our best knowledge, this is the first study in Serbs on the genetic background of acute pancreatitis. Due to extremely low frequencies or the absence of examined variations, the proposed effect on severity of disease course could not be observed.

PRSS1 is the most abundant pancreatic precursor of trypsin, which becomes active after cleavage of a short, exposed peptide chain named trypsinogen activation peptide (26). The gene encoding *PRSS1* is located on long arm of chromosome 7 (7q35), spans approximately 3.6 kb and comprises 5 exons (27). This gene is highly polymorphic, with more than 30 genetic variations reported so far (www.uni-leipzig.de/pancreasmutation). The first described and the best studied are exonic gain-of-function G>A and A>T substitutions at positions 365 and 86, respectively (13, 28). The former leads to an arginine (R) to histidine (H) replacement at codon 122 of the trypsinogen molecule (13). Because 122R represents the initial site for trypsin autohydrolysis, R122H renders trypsin resistant to inhibition and available for excessive activation of zymogens within the pancreas (29, 30). On the other hand, 86A>T results in amino acid substitution of asparagine (N) with isoleucine (I) at codon 29 (28). This causes an alteration of the secondary structure of the protein, making 122R site inaccessible for trypsin attack, thus providing the same autolysis-preventing effect as R122H (29-31). Trypsinogen activation should take place only after leaving the pancreas, as the creation of trypsin inside acinar cells would lead to pancreatic autodigestion and inflammation (1-3, 11). Previous studies found that pancreatitis develops in approximately 80% of carriers of the 365G>A or 86A>T variation. Thus *PRSS1* is considered a causative gene in hereditary pancreatitis (8, 29, 31, 32). However, in spite of the common underlying mechanism, 365G>A and 86A>T result in a different clinical course of the disease, as patients with the N29I substitution generally have milder disease symptoms with a later age of onset (33, 34).

Hereditary pancreatitis is characterized by multiple attacks of acute pancreatic inflammation that often progresses to a chronic form (8, 29, 31, 32), confirming that all types of pancreatitis share a common pathogenetic mechanism (7, 8, 11, 32, 35, 36). Therefore, it could be expected that *PRSS1* variations contribute, at least to a certain extent, to the development and severity of sporadic pancreatitis as well. However, in cases without a strong family history, the roles of *PRSS1* 365G>A and 86A>T are less understood. In addition, there are discrepancies in observations among different populations, implying interethnic differences in *PRSS1* variation frequency (21, 25, 31, 37-45). In the present study, genotyping of Serbian acute pancreatitis patients of both mild and severe clinical course revealed no carriers of either 365G>A or

86A>T variations in the *PRSS1* gene. This corresponds well to the previous data obtained from Caucasians diagnosed with non-hereditary forms of pancreatitis (8, 17, 22, 24, 25, 32, 37, 39, 40, 43, 46), arguing against genetic testing of *PRSS1* in acute pancreatitis cases in our population (35). Most likely, environmental or other genetic risk and modifying factors are involved in pathogenesis and affect the severity of this disease in Serbs.

SPINK1 is an acute phase protein, synthesized in the acinar cells of the pancreas together with PRSS1. Being a strong protease inhibitor, its main role is to prevent premature trypsinogen activation and pancreatic autodigestion by creating a covalent bond between its lysine residue at position 41 and the catalytic serine residue of trypsin (12, 31, 47-50). The *SPINK1* coding gene is localized on chromosome 5 (5q32), and it consists of 4 exons spanning a region of approximately 7.5 kb (51). There are almost 40 genetic variations of *SPINK1* identified so far (www.uni-leipzig.de/pancreasmutation), with an A to G substitution at position 101 among the first and best described in connection with pancreatitis (14, 47). This is an exonic missense variation that leads to the replacement of asparagine (N) with serine (S) at codon 34 and was discovered in a pancreatitis family without *PRSS1* mutations (47). It has been suggested that N34S renders SPINK1 incapable of inhibiting trypsinogen by causing the conformational changes within the substrate/inhibitor binding segment of the protein (14, 31). Because control of trypsin activity largely depends on SPINK1, it has been speculated that 101A>G, as a loss-of-function variation, could be at least be partly responsible for inflammation of the pancreas (47). However, investigations yielded contradictory results (52). While some of the studies observed no association between this variation and the disease risk or severity (22, 47, 53, 54), others reported *SPINK1* 101A>G as either a cause (14, 24) or a cofactor (16, 17, 31, 32, 37, 39, 55) in pancreatitis development.

In the present study, only one out of 81 Serbian acute pancreatitis patients was found to be a carrier of *SPINK1* 101A>G. The observed frequency belongs to the lower end of the wide span of previously published data for sporadic idiopathic pancreatitis cases among different populations. These include Brazilian (22), Chinese (44), Japanese (24, 56), French (38, 43, 47), German (14, 57), Italian (16, 40), Romanian (58), British (53), Spanish (25), Danish (41), American (31), Polish (42), or Indian (37), presenting with 0.0%, 0.0%, 0.0-3.1%, 0.0-10.3%, 0.5-23.0%, 2.7-9.4%, 5.0%, 18.0%, 18.8%, 19.5%, 25.0%, 28.6%, and 32.5% of 101A>G carriers, respectively. This overall discrepancy could be a consequence of a different composition of patients involved in the studies, i.e., different forms and aetiologies of pancreatitis cases described (38, 59). However, regardless of the risk factors and clinical presentations, all types of pancreatitis have the same pathogenesis, showing premature and uninhibited pancreatic zymogens activation (7, 8, 11, 32, 35, 36). Therefore, it is more probable that the difference in observed frequencies demonstrates an interethnic variability in terms of *SPINK1* 101A>G distribution (42, 44), which could explain earlier conflicting findings on its role in pancreatitis initiation and severity. As for the Serbian *SPINK1* 101A>G carrier presented here, the acute attack of the disease in his case was severe. However, he displayed several known pancreatitis risk factors, including being overweight, alcoholism and gallstone disease (12, 15). Given the advanced age of the patient and the absence of earlier attacks or a heredity pattern, it could be speculated that this variation acted as a disease-modifying component by lowering the threshold for development of pancreatitis triggered by other causes, possibly increasing the severity of the clinical course of this disease. Nevertheless, our observation of an extremely low frequency of *SPINK1* 101A>G prevents any definite conclusion regarding its significance in the disease aetiology and course and does not support routine genotyping in sporadic cases of acute pancreatitis in Serbs.

In conclusion, in Serbian patients diagnosed with acute pancreatitis, PRSS1 86A>T and 365G>A variations were not observed, while *SPINK1* 101A>G was present with a frequency of 0.62%. The proposed effect of examined variations on pancreatitis development and severity could not be confirmed. The results do not support routine genotyping of either *PRSS1* or *SPINK1* in Serbs.

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PLASMA HOMOCYSTEINE CONCENTRATIONS IN PATIENTS WITH RHEUMATOID ARTHRITIS

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KONCENTRACIJE HOMOCISTEINA U PLAZMI KOD PACIJENATA SA REUMATOIDNIM ARTRITISOM

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ABSTRACT

In this study, we investigated the concentration of serum homocysteine (Hcy) in patients with rheumatoid arthritis (RA) compared with the control group and the connection between homocysteine and parameters of inflammation and disease activity. Sixty RA patients and 20 healthy controls were included in the study, and clinical examination and investigation were performed during which disease activity was assessed. Peripheral blood samples were used for all of the assays. Levels of Hcy were 33% higher in the RA patients than in the control subjects (mean +/- SD 11.79±3.72 μmol/L versus 8.90±1.38 μmol/L; p< 0.01). A significant correlation was found between parameters of inflammation (C-reactive protein) and homocysteine in patients (r=0.322, p=0.012). Patients with high disease activity had a significantly greater increase in homocysteine (p<0.05). An increase in plasma homocysteine in RA patients is related to the parameters of inflammation and disease activity. Elevated Hcy levels occur commonly in patients with RA and may explain some of the increased cardiovascular mortality seen in RA patients.

Keywords: *homocysteine, rheumatoid arthritis, disease activity*

SAŽETAK

U ovoj studiji, istraživali smo serumske koncentracije homocisteina (Hcy) kod pacijenata koji boluju od reumatoidnog artritisa (RA) u poređenju sa kontrolnom grupom, kao i povezanost između homocisteina sa parametrima inflamacije i stanjem bolesti. U studiju je uključeno 60 pacijenata sa RA i 20 zdravih osoba koji su podvrgnuti kliničkom istraživanju i ispitivanju na osnovu čega je izvršena procena stanja bolesti. Za sve analize korišćeni su uzorci periferne krvi. Nivoi homocisteina kod pacijenata sa RA bili su 33% viši u odnosu na ispitanike u kontrolnoj grupi (mean +/- SD 11.79±3.72μmol/L versus 8.90±1.38μmol/L; p< 0.01). Utvrđena je značajna korelacija između parametara zapaljenja (C-reaktivni protein) i homocisteina kod pacijenata (r=0.322, p=0.012). Pacijenti sa bolešću u visokom stanju aktivnosti su imali značajnije povećanje koncentracije homocisteina (p<0.05). Povećanje homocisteina u plazmi kod pacijenata sa RA je u vezi sa parametrima zapaljenja i stanjem bolesti. Povišeni nivoi Hcy obično su prisutni kod pacijenata sa RA, i mogu objasniti povećanje smrtnosti usled prisustva kardiovaskularnih oboljenja koja se sreću kod ovih pacijenata.

Ključne reči: *homocistein, reumatoidni artritis, stanje bolesti*

ABBREVIATIONS

anti-CCP - antibodies against cyclic citrullinated peptide
CIRD - chronic inflammatory rheumatic diseases
COX-2 - cyclooxygenase 2
CRP - C-reactive protein
CVD - cardiovascular disease
DAS - disease activity score
DMARD - disease-modifying antirheumatic drugs
ELISA - enzyme-linked immune sorbent assay
ESR - erythrocyte sedimentation rate
HAQ - Health Assessment Questionnaire
Hcy - homocysteine
MTHR - methylene-tetrahydrofolate reductase
NSAID - nonsteroidal anti-inflammatory drug
RA - rheumatoid arthritis
RF - rheumatoid factor
VAS - visual analogue score

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INTRODUCTION

The risk of cardiovascular morbidity and mortality is increased in rheumatoid arthritis (RA) (1). The classical cardiovascular risk factors, including smoking, hypertension, dyslipidaemia, insulin resistance and diabetes mellitus, obesity and physical inactivity, do not explain the excess cardiovascular risk observed in people with rheumatoid arthritis, although they do contribute, albeit in a different way or to a lesser extent, to rheumatoid arthritis (2). A very important link between rheumatoid arthritis and cardiovascular disease is inflammation because it plays a key role in all stages of atherosclerosis from endothelial dysfunction to plaque rupture and thrombosis. Inflammation also has an influence on and exacerbates some traditional cardiovascular risk factors. To date, the exact pathophysiologic mechanism underlying the relationship between cardiovascular disease and rheumatoid arthritis is unclear (2). Clinical research has proven that rheumatoid arthritis (RA) patients have a higher prevalence of classical risk factors than the general population. Recently, there has been an emphasis on new risk factors that can contribute to cardiovascular disease (CVD) (3).

Homocysteine (Hcy) is a sulfur amino acid which metabolism exists at the intersection of two pathways: remethylation, which requires folic acid and vitamin B12 coenzymes, and trans-sulfuration, which requires pyridoxal-5'-phosphate, the vitamin B6 coenzyme (4). Prospectively, elevated plasma Hcy is associated with increased total and cardiovascular mortality, increased incidence of stroke, increased incidence of dementia and Alzheimer's disease, increased incidence of bone fracture, and higher prevalence of chronic heart failure (4). However, Hcy is not included in current guidelines for the diagnosis of subclinical disease in high-risk asymptomatic individuals. Mean Hcy levels were consistently found to be higher in men compared with women (5).

The correlation between the plasma homocysteine levels and commonly used inflammatory markers (C-reactive protein and erythrocyte sedimentation rate) in patients with RA is unclear. To date, it has been shown that multiple rheumatic diseases including RA, scleroderma, ankylosing spondylitis, systemic lupus erythematosus and gout may be associated with hyperhomocysteinemia, which may, in turn, be associated with cardiovascular events (6-9). In many studies that examined serum Hcy levels in rheumatic disease, serum Hcy levels were higher in the case group than the control group (6-11).

In this study, we compared the concentration of plasma Hcy among patients with RA and healthy participants belonging to control group. We also examined the correlation between disease activity and inflammatory markers in RA patients.

PATIENTS AND METHODS

Study population

Sixty patients with RA (mean age 52.46 years, SD ± 7.39, min 37–max 60 years) who fulfilled the 2010 American College of Rheumatology criteria for RA were enrolled in this study (12). Patients were recruited from the outpatient unit of the rheumatology department at the Clinical Center Kragujevac, Serbia, in 2014. The median duration of illness was 7 years (range 2–11 years). The erythrocyte sedimentation rate (ESR) was determined using the Westergreen technique, and C-reactive protein (CRP) was measured using nephelometry. Serum rheumatoid factor (RF) was measured using the latex agglutination technique. Patients were considered seropositive if any determination during the study was positive. The presence of antibodies against cyclic citrullinated peptide (anti-CCP) in serum was detected using the Diastat kit (Axis-Shield Diagnostics, Dundee, UK) with a cut-off value of 17 U/mL.

Blood was obtained from fasting patients at a standardised time in the morning. The blood samples were centrifuged for 20 minutes at 2000 rpm. The serum were collected and kept at -70°C until assayed. The measurement of Hcy levels was performed using an enzyme-linked immune sorbent assay (ELISA) kit (Axis homocysteine EIA, REF FH CY 100). Increased Hcy concentration was defined as plasma Hcy concentration equal to or greater than 11.20μmol/L. We compared the homocysteine levels between the RA and control group. We also analysed the correlation between levels of homocysteine and disease characteristics.

The disease activity of RA patients was calculated using a disease activity score comprising 28 joints (DAS 28) according to the method of Prevoo et al. (13). The DAS 28 consisted of 28 joint-swelling items and 28 joint-tender items, which included the proximal interphalangeal joint, metacarpal phalangeal joint, and the wrist, elbow, shoulder, and knee joints. The DAS 28 also included the erythrocyte sedimentation rate (ESR) and the visual analogue score (VAS). The VAS uses a horizontal 100-mm line, where patients indicate their degree of pain by placing a mark on a line between "no pain" (left end, 0 mm) and "excruciating pain" (right end, 100 mm). Values of DAS 28<3.2 indicate low-disease activity, and values of DAS 28 ≥3.2 indicate moderate and high-disease activity. All patients completed Health Assessment Questionnaires (HAQ) for RA. At the time of this study, all patients were treated with one or two disease-modifying antirheumatic drugs (DMARD). Cyclooxygenase 2 (COX-2) inhibitors and nonsteroidal anti-inflammatory drugs (NSAIDs) were applied only occasionally. All 20 controls participated in this study and were matched by hospital personnel according to age sex (mean age 55.23 years ± SD 4.232, min. 43, max. 60 years).

The following exclusion criteria were used for both groups: smoking (past 5 years), alcohol intake (past 12 months), use of narcotic drugs (never), hypertension, diabetes mellitus, chronic renal diseases, deficiency of folic acid, vitamin B6, vitamin B12, and any other form of arthritis except RA. All of the patients were informed about the aims of the study and written consents were obtained from them. The local institutional ethics committee approved the study protocol.

Statistics

The statistical analysis was performed using SPSS 15.0 for Windows. The data were presented as the mean ± standard deviation (SD) and analysed using SPSS software version 10. A $P < 0.05$ was considered statistically significant. The results

Table 1. Baseline demographics and clinical characteristics of the RA patients and controls

	RA patients (n=60) (mean±SD)	Controls (n=20) (mean±SD)	P value
Age in years (range)	52.46±7.39 37-60	54.23±5.23 40-60	NS
BMI (kg/m^2)	23.59±1.54	23.30±1.09	NS
Duration of disease (years)	7.32 ± 3.18	-	-
Visual Analogue Scale (mm)	59±10	-	-
Number of swollen joints	2±2	-	-
Number of tender joints	6±2	-	-
Disease activity score (DAS 28)	4.80±0.84	-	-
Health assessment questionnaire-disability index score-HAQ	1.37±0.28	-	-

RA rheumatoid arthritis, NS not statistically significant, BMI body mass index,

are expressed as the mean ± standard error of the mean. Data distribution was checked using the Shapiro–Wilk test and depending on its results, the appropriate parametric or non-parametric test was used. The differences between the two groups were assessed using Student's T-test or the Mann-Whitney U test. However, the differences between the values of the means among more than two groups were assessed using the ANOVA or Kruskal–Wallis test. The correlation between various variables was found using bivariate correlation, i.e., Spearman's coefficient of correlation.

Table 2. Comparison of the status of inflammation parameters and homocysteine in rheumatoid arthritis patients and controls

Parameter	RA patients	Controls	p-value
ESR (mm, 1 h)	34.8±19.98	10,93±6,26	p<0.01
CRP (mg/L)	13.7±12.22	4.49±2.00	p<0.01
Hcy(μmol/L)	11.79±3.72	8.90±1.38	p<0.01

Values are represented as the mean ± SD, ESR-erythrocytes sedimentation rates, CRP- C-reactive protein, Hcy- homocysteine

Table 3. Correlation between the plasma concentrations of homocysteine and the studied factor in RA patients and healthy controls

Parameter	RA patients n=60 (r coefficient, p)	Controls n=20 (r coefficient, p)
Age	0.211(0.105)	0.129 (0.50)
BMI(kg/m^2)	–0.003 (0.97)	0.331 (0.07)
ESR (mm, 1 h)	0.173 (0.186)	0.254 (0.17)
CRP (mg/L)	**0.322 (0.012)**	0.033 (0.86)
Duration of disease (years)	-0.037 (0.780)	-
Number of swollen joints	-0.122 (0.353)	-
Number of tender joints	-0.039 (0.353)	-
HAQ	0.213(0.102)	-

RA-rheumatoid arthritis, BMI-body mass index, ESR-erythrocytes sedimentation rates, CRP- C-reactive protein, HAQ-Health assessment questionnaire-disability index score

RESULTS

The demographic and clinical characteristics of patients with RA and healthy controls are summarized in Table 1. Sixty DMARD patients received methotrexate, and 24 received a combination of methotrexate and hydroxychloroquine phosphate. Additionally, 63 % of the patients continuously received low-dose corticosteroids for at least 1 year (average dose 7.5 mg) (Table 1).

Plasma homocysteine levels in patients with RA were significantly higher than those in the control group ($p < 0.05$). The increase in the levels of ESR and CRP in the RA patient group was found to be highly significant ($p<0.01$) (Table 2).

Table 3 shows the correlation between the plasma Hcy and the studied factors in RA patients and controls. The plasma Hcy concentrations were significantly correlated with CRP ($r=0.322$, $p=0.012$) in RA patients. Our findings suggest that serum Hcy level has no significant correlation with the duration of RA, levels of ESR, scores from the HAQ, or number of swollen and painful joints.

A statistically significant increase in the levels of plasma homocysteine was also observed in the patients with more severe disease (DAS 28>5.1) compared with patients with low or moderate disease activity (DAS 28 ≤5.1) ($p<0.05$) (Figure 1).

DISCUSSION

In this study, we found that the serum Hcy levels in patients with RA were higher than the normal range and were significantly higher than the Hcy levels among participants in the control group. The results of our study confirm the findings of previous studies, which report an elevation or a significant increase in plasma Hcy concentration. Many studies have also shown that the level of Hcy is significantly elevated in patients with RA compared with the level of Hcy in healthy subjects (14- 16).

The current view is that systemic inflammation, which is specific to all chronic inflammatory rheumatic diseases (CIRD), accelerates atherogenesis (17). Immunologic and metabolic markers (anti-cyclic citrullinated peptide (CCP)

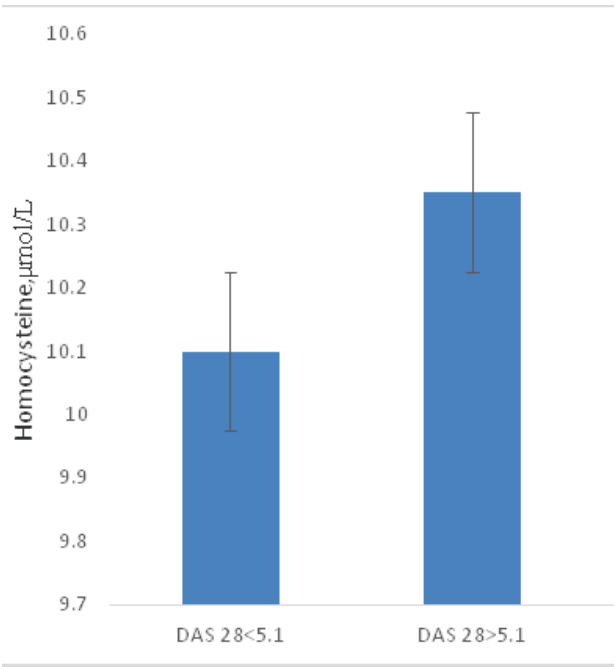

Figure 1. Comparasion of homocysteine and disease activity in rheumatoid arthritis patients

antibodies, IgM rheumatoid factor, proinflammatory cytokines and homocysteine) may play a role in the development of atherosclerotic disease in people with RA (17). This hypothesis is supported by the high cardiovascular (CV) morbidity and mortality rates and the high prevalence of all atherosclerosis stages and complications among RA patients (1).

A comprehensive evaluation and follow-up of both traditional and nontraditional CV risk factors, as well as the correct classification of risk reduction categories, are necessary in the study of RA (18). Our study shows that the values of CRP were significantly higher among RA patients than among participants in the control group. Continuous exposure to high grade systemic inflammation may be linked to accelerated atherosclerosis. Persistently elevated CRP values during the course of RA increase the risk of death due to cardiovascular disease (19). The CRP value determined using high-sensitivity assays appears to be an independent and robust prognostic factor for cardiovascular events. CRP is not only an indicator for generalized inflammatory reaction but is also a mediator involved in the pathogenesis of atherosclerosis. Increased levels of CRP have been found to correlate with the degree of subclinical atherosclerosis, measured by carotid artery intima-media wall thickness and the presence of clinically evident cardiovascular disease in adults with rheumatoid arthritis (20, 21).

The aim of this investigation was to evaluate the relationship between the duration of RA and the high activity of the inflammatory process in patients with RA (high levels of C-reactive protein, the disease activity scores, scores from the HAQ, and the number of swollen and painful joints) and hyperhomocysteinaemia. We showed a statistically significant correlation between homocysteine levels and CRP in patients with RA. Additionally, the plasma homocysteine levels were increased, and the levels of general inflammatory markers were elevated. Therefore, we conclude that homocysteine might affect the inflammatory status of patients and could be a predictive factor of hyperhomocysteinaemia in patients with RA. Regarding genetic influence, it is known that a genetic polymorphism-methylene-tetrahydrofolate reductase (MTHFR) associated with changes in the levels of homocysteine was also found to influence the development of endothelial dysfunction and the increased risk of cardiovascular disease in adults with RA (22). Based on a review, Salhab (4) concluded that there is a significant association between the subclinical atherosclerotic process and Hcy, and it shows potential as a cheap marker for risk stratification among asymptomatic patients. The results of these studies suggest the inclusion of plasma Hcy levels in future risk reduction protocols for identification of individuals at higher risk of atherosclerotic events to categorize them for more aggressive treatment with established preventive and therapeutic measures (4).

In our study, we found a correlation between plasma Hcy levels and disease activity in patients with RA. A statistically significant increase in the levels of plasma homocysteine was also observed in the patients with more severe disease when compared with those patients with low or moderate diseases activity. Recent studies have shown that RA radiological class, as an indicator of disease activity and progression, was a strong independent predictor of Hcy levels (23). In other studies, researchers found a positive correlation between homocysteine concentrations and the HAQ disability index (24). A previous study in RA patients found no association between the increase in Hcy concentration and the improvement in DAS (25) scores. A recent study has shown that myocardial ischaemia in patients with RA was associated with a high activity of the inflammatory process (high C-reactive protein levels, disease activity score, score on the HAQ disability index, and number of swollen and painful joints) and hyperhomocysteinaemia (26).

The assessment and diagnosis of traditional and nontraditional CV risk factors followed by aggressive prevention and therapy, are necessary to achieve efficient control over the inflammation, immunologic and metabolic disorders specific to RA. This finding might indicate that Hcy has a stronger role as a marker of atherosclerotic disease than as a risk factor for atherosclerotic disease. Timely identification of patients with risk factors, particularly with new risk factors, enables adequate prevention of and treatment for CVD in rheumatoid arthritis patients.

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INFLUENCE OF SMOKING HABIT ON AGE AT DIAGNOSIS OF BREAST CANCER

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UTICAJ PUŠAČKE NAVIKE NA STAROSNU DOB PACIJENATA U TRENUTKU POSTAVLJANJA DIJAGNOZE KARCINOMA DOJKE

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ABSTRACT

No studies have yet investigated the influence of smoking on age at diagnosis of breast cancer. Therefore, the present study was carried out. This study consisted of 605 females with pathologically confirmed primary adenocarcinoma of the breast and 438 healthy females matched by age. Among our participants, 86 (14.2%) patients and 62 (14.1%) control subjects, respectively, were smokers. Based on a Cox regression model, evidence suggested that smoking status influenced the age at diagnosis of breast cancer (HR=0.78, 95% CI: 0.62-0.99, P=0.040). After stratification of the patients according to their menopausal status, the same results were obtained. The present study indicated that non-smokers have a lower age at diagnosis in comparison with patients who smoke.

Keywords: *Age at diagnosis, breast cancer, smoking Habit*

SAŽETAK

Do sada nisu sprovođena istraživanja kojima se ispituje uticaj pušačke navike na starosnu dob u trenutku postavljanja dijagnoze karcinoma dojke. Iz tog razloga je sprovedena ova studija. Studija je sprovedena na 605 ispitanica sa patohistološki potvrđenim primarnim adenokarcinomom dojke, i 438 zdravih ispitanica odgovarajuće starosne dobi. 86 (14.2%) ispitanica u grupi sa potvrđenom dijagnozom, odnosno, 62 (14.1%) iz kontrolne grupe su bile pušači. Prema Cox modelu regresije dokazana je povezanost pušačkog statusa i starosne dobi u trenutku postavljanja dijagnoze karcinoma dojke (HR=0.78, 95% CI: 0.62-0.99, P=0.040). Treba napomenuti i da su isti rezultati dobijeni nakon stratifikacije ispitanica prema menopauzalnom statusu. Ova studija je pokazala da su nepušači u trenutku postavljanja dijagnoze karcinoma dojke u mlađoj životnoj dobi u odnosu na pušače.

Ključne reči: *starosna dob u trenutku postavljanja dijagnoze, karcinom dojke, pušenje*

ABBREVIATIONS

SD – standard deviation
HR – hazard ratio
OR – odds ratio
CI – confidence interval
df – Degree of freedom

INTRODUCTION

Breast cancer is a complex multifactorial disease. Thus, genetic and environmental risk factors are involved in its aetiology (1-4). Tobacco use may be one of the few modifiable risk factors for several types of cancers. Although tobacco smoke contains many potentially harmful substances that may act differently and at different stages in the development of cancers, the association between smoking and breast cancer risk remains unclear (5-11). Moderate or strong associations between smoking and breast cancer risk have been observed in some studies (6-11). Several prospective studies have focused specifically on the association between smoking and breast cancer survival rate, however, the results of these studies are not consistent (12-15).

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While a smoking habit is one of the most preventable causes of cancers, based on our knowledge, there is no study that investigates the influence of smoking on age at diagnosis of breast cancer. Therefore, the present study was carried out to study this relationship.

MATERIALS AND METHODS

A total number of 605 patients with pathologically confirmed primary breast adenocarcinoma were recruited from the chemotherapy department of the Nemazi hospital in Shiraz (Fars province, southwest Iran) from September 2008 to June 2011. The mean age at diagnosis of breast cancer (SD; Min-Max; Median) was 45.7 (10.7; 22-83; 45). Age frequency-matched control subjects (438 people) were randomly selected from healthy female blood donors. The mean age of the control group (SD; Min-Max; Median) was 46.7 (10.6; 22-80; 45).

The Iranian population is one of the most heterogeneous populations in the world (16, 17). Therefore, we selected our patients and control subjects from Persian/Muslims (Caucasian) living in the Fars province (southwest Iran).

Family medical history, specifically the incidence of breast cancer (positive, negative), and the smoking status (smoker, non-smoker) of the patients and control subjects were collected via personal interviews. A woman with at least one first-degree relative with breast cancer was considered to have a positive family history. Informed consent was obtained from each subject before being enrolled in the study, and the study was approved by the institutional review board at our department.

The association between smoking status, family history and the risk of breast cancer was assessed by calculating odds ratios (ORs) and 95% confidence intervals (CIs). To determine the effects of smoking status and family history on age at diagnosis of breast cancer, the Kaplan-Meier survival analysis and the Cox proportional hazards regression model were used. In the analysis, breast cancer was defined as the event, and the age at diagnosis was included in the analysis as time to event. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) (version 11.5). A probability of $P<0.05$ was considered statistically significant.

RESULTS

Among our participants, 86 (14.2%) patients and 62 (14.1%) control subjects, respectively, were smokers. There was no association between smoking status and risk of breast cancer (OR=1.01, 95% CI: 0.71-1.43, P=0.978). Like many studies (18), family history significantly differed between patient cases and control subjects (4.6% in cases vs 2.1% in control subjects; OR=2.31, 95%CI: 1.08-4.95, P=0.031).

Table 1 shows the distribution of age among newly diagnosed breast cancer patients and control subjects according to their smoking habit. The Kaplan-Meier survival analysis revealed that a smoking habit was associated with age at diagnosis of breast cancer (log rank statistic=4.440, df=1, P=0.035). A family history of breast cancer was also associated with age at diagnosis of breast cancer (log rank statistic=1.609, df=1, P=0.205). In the Cox proportional hazards regression model, smoking status and family history were treated as categorical variables. Hazard ratios (HR) and 95% of confidence intervals (CIs) for the categorical variables were estimated. Based on the Cox regression model, there was an association between smoking status and age at diagnosis of breast cancer (HR=0.78, 95% CI: 0.62-0.99, P=0.040). This means that non-smokers have a lower age at diagnosis in comparison to patients who smoke. After stratification of the patients according to their menopausal status, the same results were obtained.

DISCUSSION

In the present case-control study, we investigated two associations: 1) the association between smoking habit and breast cancer risk, and 2) the association between smoking habit and age at diagnosis of breast cancer. Although breast cancer has not been previously regarded as a smoking related cancer (5), recent studies haves revealed a positive association between tobacco smoke and breast cancer risk (6-11). Based on the present study, there was no significant association between a smoking habit and a risk of breast cancer. This finding is consistence with some reports (see ref. 5) and is not consistence with other reports (6-11).

We found that the association between smoking status and age at diagnosis of breast cancer was significant.

Table 1. Distribution of age among breast cancer patients and control subjects stratified by their smoking status

Smoking status	Breast cancer patients				Control subjects			
	Age at diagnosis (Years)				Current age (Years)			
	n	Mean	SD	Median	n	Mean	SD	Median
Non-Smokers	519	45.4	10.6	44.0	376	46.1	10.5	44.5
Smokers	86	47.7	11.4	47.0	62	50.5	10.1	50.0
Total	605	45.7	10.7	45.0	438	46.7	10.6	45.0

Epidemiological studies have indicated that women who smoke might experience decreased risk of breast cancer as a result of antiestrogenic effects (19). Therefore, it could be concluded that the antiestrogenic effects of tobacco smoke, at least in part, are a mechanism for explaining our present findings.

It should be mentioned that in Iran, breast cancer patients are younger than breast cancer patients in Western countries (3). We know that susceptibility to breast cancer is a multifactorial trait and its risk factors (either genetic components or environmental factors) may differ between populations. Tobacco smoke contains several thousand various compounds that are carcinogenic to humans, and metabolites of tobacco smoke have been found in the breast fluid and tissue of smokers (20, 21). Many of the carcinogenic compounds present in tobacco smoke are substrates of phase I enzymes, represented by the family of cytochrome P450 enzymes (22, 23). Certain genotypes of several genetic polymorphisms in enzymes involved in the metabolism of xenobiotics (such as P450s and antioxidant enzymes) have been suggested to alter the risk of breast cancer (24-27). The inconsistency of the findings between studies might be at attributed to differences between population gene pools, environmental factors and the interaction between the two. The main limitations of the present study are the small sample size, the lack of data on duration of smoking and/or passive smoking and possible confounding by other factors, such as diet, including phytoestrogen intake, cannot be excluded. In the future, our present finding should be confirmed by large-scale studies.

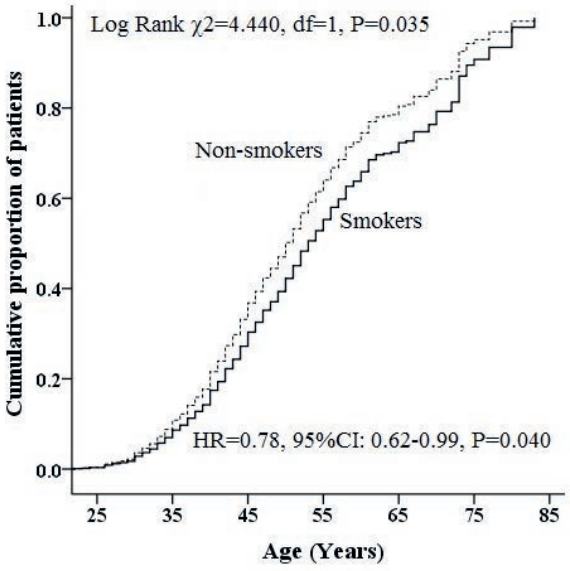

Figure 1. Comparison of age at diagnosis of breast cancer between smokers and non-smokers, after adjustment for family history

Conflict of Interest

The authors have no financial or non-financial competing interests.

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CHANGES IN QTC INTERVAL DURATION AMONG HEROIN ADDICTS ON METHADONE TREATMENT

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PROMENE U TRAJANJU QTC INTERVALA KOD HEROINSKIH ZAVISNIKA NA METADONKOM TRETMANU

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ABSTRACT

This paper aimed to collect and unite facts known about the effect of methadone treatment on QTc interval prolongation that could determine precipitating factors in the development of heart arrhythmias and their consequences (Torsade de Pointes and sudden cardiac death), and to raise the methadone treatment safety level.

Studies conducted up to now clearly demonstrate that methadone therapy evokes changes in the heart's electrical conduction, but those studies also show that QTc interval prolongation could be precipitated by other factors. The most often present risk factors in our research were dose of methadone, co-medication, and co-morbidity, but other relevant risk factors were gender, age, misuse of illicit drugs, therapy length and tobacco use.

Active participation in modern treatment processes and implementation of knowledge acquired recently into daily practice, such as setting up reutilized questionnaires and diagnostic methods to expose higher risk for complications and providing broader therapeutic range for cases of drug replacement necessity, will enhance therapy safety level and bring us to the next step of resocialization of these patients, which needs to remain the final goal of treatment.

Keywords: *methadone treatment, QTc interval prolongation, Torsade de Pointes, sudden cardiac death*

SAŽETAK

Cilj ovog preglednog članka je da prikupi i objedini do sada poznate činjenice, o efektima metadonske terapije na produženje QTc intervala, koje mogu da ukažu na predisponirajuće faktore za nastanak srčanih aritmija i njihovih posledica (Torsade de Pointes, iznenadna srčana smrt) kao i da unaprede sigurnost prilikom upotrebe ove terapije. Studije sprovedene do sada definitivno pokazuju da terapija metadonom izaziva promene u električnoj provodljivosti srca, ali i da produženje QTc intervala može da bude uslovljeno i drugim faktorima. U najčešće faktore rizika se ubrajaju doza metadona, udružena terapija, ko-morbiditet, pol, starost, zloupotreba ilegalnih droga, dužina terapije i konzumiranje duvana. Aktivno učešće u modernim terapijskim procedurama i uključivanje do sada stečenih znanja u rutinsku praksu (putem uvodjenja upitnika i dijagnostičkih metoda) radi utvrđivanja rizika za nastanak komplikacija i obezbedjivanja šireg terapeutskog spectra, će podići nivo sigurnosti prilikom upotrebe lekova i dovesti nas do sledećeg koraka – resocijalizacije pacijenata, što treba da ostane krajnji cilj terapije.

Ključne reči: *terapija metadonom, produženje QTc intervala, Torsade de Pointes, iznenadna srčana smrt*

INTRODUCTION

Methadone is the most commonly used drug in opioid maintenance treatment, and it has been on the WHO's list of essential medicines since 2005 (1); however, it was also noted by the FDA in 2006 as a drug with serious side effects, such as respiratory centre depression and cardiac dysrhythmia (2). The assumption is that methadone causes cardiac dysrhythmia by prolonging QTc interval, which is a risk for Torsade de Pointes that could evolve into sudden cardiac death.

Although methadone therapy has been used for over 40 years to treat opioid addiction, its side effects were first seriously indicated in 2006 (2), when numerous studies were published. However, there was no adequate experimental clinical study (3). A lack of controlled randomized studies with a placebo group was justified by the specific characteristics of this disease and the necessity for constant therapy. Meanwhile, in recent years, important compara-

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tive experimental studies have been published with results demonstrating new approaches towards the abovementioned problems.

The main questions on this topic are as follows: does methadone cause prolongation of QT interval, does methadone prolong QT interval enough to cause ventricular tachycardia, and is QT interval length subject to other factors present among these patients?

This review aimed to both summarize previous knowledge on this topic and to note the newest research in comparative and other clinical studies and thus consolidate knowledge in the area of methadone maintenance treatment (MMT) to improve the safety of patients receiving therapy for heroin addiction.

Methadone

Methadone, a synthetic agonist of mu receptors, is administered orally for the treatment of opioid addiction, and it is most often used as a racemic mixture of R- and S-enantiomers. Whereas R-enantiomer mostly causes an opioid effect (4), both R-and S-enantiomers function like an antagonist of N-methyl- D-aspartic acid (NMDA) (5). Oral bioavailability of methadone is 70-90%, and it is transported bound to plasma proteins (6). It is metabolized in the liver by the cytochrome P450 enzyme system - mainly by CYP3A4 and CYP2B6 - whose activities are genetically and environmentally determined (7). It is important to note the stereo selectivity of the CYP2B6 enzyme towards S-methadone, which has been shown in vitro (8) and confirmed in vivo by showing that this enzyme is a slower metabolizer of methadone and thus produces higher S-methadone serum levels in people with the CYP2B6 genotype (9). Additionally, there are certain drugs that are metabolized by or inhibit these enzymes that may increase plasma methadone levels (10).

The most effective dosage of methadone for opioid dependence treatment is 60-100 mg per day (11).

Qt Interval

The length of the QT interval, which is the time between the start of the Q wave and the end of the T wave in the heart's electrical cycle, is predictive of Torsade de Pointes, which can be fatal (12). QT interval is corrected by Bazett's formula (QTc) because it is dependent on the heart rate (13). Normal QTc interval is considered <430 ms in men and <450 ms in women, whereas a prolonged QTc interval has been defined as >450 ms for men and >470 ms for women (14). TdP will not follow every QTc interval prolongation, but QTc prolongation over 500 ms significantly increases the risk of TdP (15).

Studies on congenital long QT syndrome have provided an underlying reason for QT prolongation. Numerous mutations of the KCNH2 gene that code the human ether-a`-gogo related gene (hERG) voltage-gated potassium channel in the human heart are responsible for congenital long QT syndrome and sudden death caused by malignant ventricular arrhythmias (16). The importance of these channels among OMT patients became clearer (17) when it was shown that hERG channels might be blocked by some drugs, like methadone (18).

METHODS

We searched the PubMed database using the following key words: *Methadone maintenance treatment, and QTc prolongation with the following filters: Publication date from 1980/01/01 to 2013/01/01, Clinical Trial, Randomized Controlled Trial, Humans and English.* We obtained 4 search results.

In order to complete our research, we obtained additionally used research papers connected to results from our basic search (Related citations in PubMed) that were offered together with the results from the basic search.

Not all of the results obtained this way fit within our topic of interest, so our research team divided into four groups that independently reviewed gathered research papers to identify common parameters to select the research papers of interest. Included parameters were *Methadone maintenance treatment* and *QTc prolongation* as the topic of the research papers.

After an independent review of the research papers, all 4 groups held a meeting, excluded research papers that appeared in more than one group, and once more reviewed the rest of the research papers with the aim of determining that they all included the appropriate parameters. This method brought us a satisfactory number of research papers and increased the amount of literature to be used as source of information on this topic.

Another limitation we faced at this stage was that a great number of research papers obtained through the PubMed database contained only abstracts. We tried to overcome this by sending appeals to the authors of these papers asking for the full research paper for the review to be as complete as possible. In this way, we gained full access to the majority of the research papers.

Additionally, research papers that were not in our PubMed database search but were accessible to team members were used as information sources but will be not found in the RESULTS section because they were not the result of PubMed database search.

RESULTS

Experimental studies

In 2008, Wieneke et al. published a randomized, controlled clinical trial that compared LAAM with methadone (19). The methadone group consisted of patients on MMT for at least one month and with a stabile methadone dosage (less than 100 mg/day) for at least two

weeks. Exclusion criteria were used to form groups of patients without cardiac disease, serious psychiatric diseases or somatic disorders and with negative urine drug screen for opiates during the screening period. After 4 weeks of methadone therapy, patients were randomized into two groups (group treated with LAAM and group treated with methadone) and followed for 24 more weeks. For the purpose of this review, only the methadone group is described. The 22 patients in the methadone group had a mean age of 31 years. Twelve patients were women. The mean methadone dose before randomization was 72.0 ± 25.5 mg/day compared to 69 ± 27.6 mg/day in the next phase. This study did not show significant changes in QTc interval, which varied from 406 ms ± 29 ms at the beginning to 405 ms ± 25 ms after 24 weeks.

Another study compared different medicines in the treatment of heroin addiction (20). An advantage of the study performed by Wedam et al. was its design, a randomized, double-blinded study, but it lacked a placebo group for ethical reasons. At the beginning of the study, the mean QTc interval was 412.6 ms, and after 16 weeks, the mean QTc interval increased to 446.9 ms. Among 53 participants in the methadone group, 23 % had QTc intervals longer than 470 ms for males and 490 ms for females, and 6 participants in the study developed QTc interval values over 500 ms. It is important to mention that 12 % of participants had QTc intervals that lengthened by more than 60 ms compared with baseline values. The methadone dose used varied from 60 to 100 mg.

Observational studies

In 2008, Krantz conducted a prospective cohort study with 151 participants with the aim to evaluate the influence of methadone on QTc interval length (21). After selecting the participants, a baseline ECG was performed followed by a second ECG after 6 months. The results showed that QTc interval prolongation was present among 76% of patients on methadone, whereas the rest of the patients did not have prolongation or the QTc interval length was shortened. Specifically, QTc intervals over 450 ms were found among 7% of patients at the baseline ECG and among 19% after 6 months. A QTc interval over 470 ms was present in 3% of patients at the first ECG and in 7% at the second ECG. There was no case of a QTc interval longer than 500 ms at the baseline ECG, but this value was exceeded in 2% of patients after 6 months of study. None of the patients developed TdP during the study. The practical implications of the study were a non-significant prolongation of the QTc interval of less than 30 ms among 18% of participants, but a clinically more significant prolongation of more than 60 ms compared to the baseline ECG was present among 3% of patients.

Another prospective study performed by Krantz et al. gathered 118 newly recruited patients of an MMT program who started therapy with a 30 mg daily methadone dose that was afterwards adjusted based on information about previous heroin use, clinical assessment of withdrawal symptoms, and illicit opiate usage proved by urine analysis (22). Baseline QTc values varied from 367 to 483 ms (mean value 415.3 ms) but increased to 376 - 533 ms (mean value 429.4 ms) after 6 months. As the upper limit of normal QTc values was considered 430 ms for males and 450 ms for females, this resulted in QTc interval prolongation among 14% of patients at the beginning of the study and 31% at the end. The methadone doses used varied from 20-180 mg per day. However, Martel et al. continued the study and added an ECG performed after 12 months from the beginning of the study (23). The difference was that for this study, the upper limit of the normal QTc values was set 450 ms for men and 470 ms for women. Under these conditions, just 3% of patients had prolonged QTc values at the baseline ECG and 24% had prolonged QTc values after 6 months. At the end of the study, 22% had prolonged QTc values, and 2 participants had QTc values over 500 ms (23).

A study with 83 patients who were on MMT for longer than 6 months with a steady maintenance daily dose and with methadone as the only used medication showed 2 patients with QTc intervals longer than 500 ms, but neither patient had ever experienced symptoms of a heart disorder (24). Maremmani et al. found 69 patients (83.1%) with prolonged QTc intervals beyond the upper limits of normal QTc values of 440 ms in males and 460 ms in females. Patients did not have an electrolyte imbalance and had a negative urine test for cocaine, morphine and amphetamines. The researchers concluded that there was an absence of proof that methadone had no effect on cardiac function, but they did not find any correlation between QTc length and methadone dose. Additionally, due to the fact that there were only two cases of QTc intervals longer than 500 ms, they speculated that there was no risk of arrhythmia among MMT patients as long as methadone was administered as the only therapy.

Mayet with a group of scientists conducted a cross-sectional study on the prevalence of QT prolongation among patients on MMT and the appearance of TdP (25). The study group consisted of patients who fulfilled the criteria proposed by The Medicines and Healthcare Products Regulatory Agency (patients on methadone with heart/liver disease, electrolyte abnormalities, concomitant QT prolonging medications/CYP3A4 inhibitors or prescribed methadone >100 mg daily) (26). This research showed that the prevalence of QTc prolongation (>450 ms for males and >470 ms for females) was 18.1%, whereas there was neither QTc prolongation over 500 ms nor the occurrence of TdP.

Researchers from France followed a group of 42 outpatients by measuring the QTc interval during their visits to the centre for treatment of addiction diseases. Independently, they searched through the French pharmacovigilance medical reports database dating from 1996 to 2007 and singled out reports of cardiac symptoms connected to methadone treatment. The results of both studies were published separately (27). They recorded data on co-med-

ication, comorbidity, electrolyte status, and psychoactive substance abuse. The study started with 100 patients, although an ECG was obtained from only 42 patients, and showed QTc interval prolongation among 4 patients with a maximal prolongation of 36 ms compared to the reference values.

Another cross-sectional study followed patients on medication treatment for addiction with the aim to define the prevalence of QTc prolongation. This study collected data from a registry of all patients on medication treatment for addiction in Norway from 1997 to 2003 for the purpose of discovering mortality that was potentially caused by therapy (28). The cross-sectional study involved 173 patients treated with methadone, and among them, 4.6% (n = 8) had a QTc interval above 500 milliseconds, 15% (n = 26) had a QTc interval above 470 milliseconds, and 28.9% (n = 50) had a QTc interval above 450 milliseconds.

Another study included 138 patients who were on treatment for at least 100 days and on a stable methadone dose for at least 14 days as well as 111 (80.4%) patients who received more than 120 mg/day of methadone. This study measured methadone serum levels and QTc interval (29) and reported several comorbidities, 76 patients were positive for HCV and 11 were positive for HIV, as well as drug abuse. The results showed only 3 patients with QTc intervals longer than 500 ms (upper limit of normal and clinically relevant in this study) and found no correlation between QTc interval and methadone doses and serum levels. This study did find a significant correlation between methadone dose and QTc interval among 31 patients with urine positive for cocaine, but did not find a significant correlation among patients abusing other drugs (e.g., benzodiazepine, opiate, cannabis, amphetamine).

The cross-sectional study conducted by Eap et al. found 9% of patients with QTc prolongation among 179 patients on methadone treatment (30). All of the QTc prolongations occurred in men. Patients were on MMT for at least 6 months at the time when the study was conducted, with the exception of 9 participants with the lowest duration of treatment being 2 months. Methadone plasma concentrations were in steady state in all except one patient.

The studies that provided the most detailed data for patients on methadone treatment were clinical cases. Although these studies do not provide statistical values, they clearly show an interaction between methadone dosage, risk factors, and QTc values.

A clinical case of a 52-year-old, HIV+, female patient without previous cardiologic problems but with prolonged QTc interval and occurrence of TdP after a high dose of methadone was described by Denise D. Routhier et al. (31), suggesting the ability of methadone to induce QTc prolongation and Torsade de Pointes. Additional data showed that this patient arrived at the hospital with hypokalaemia and hypomagnesemia even though the serum K+ and Mg++ concentrations were normal at the time of the arrest. Additionally, a urine drug screen was positive for cocaine and benzodiazepines as well as other medications, among them alprazolam, which shares in CYP3A4 metabolism. The patient was diagnosed with hepatitis B and C, but the liver function was good. Daily dosage of methadone was 145 mg, and when gradually reduced to 80 mg, QTc interval narrowed from 618 ms to 503 ms on hospital day 4 and to 454 ms on day 19, at the time of discharge. Another group of scientists from the University of Pittsburgh wrote a case report on this patient, concluding that TdP occurred due to its multifactorial aetiology (32).

Another case series presented 4 patients with prolonged QTc interval and arrhythmias while on high doses of methadone (33). Here, there were also other risk factors such as hypokalaemia (among 3 patients), co-medication, abuse of illicit substances and structural heart disease. Although the authors stated that methadone treatment was continued in all 4 cases and that QTc interval was normalized and arrhythmias vanished when risk factors were suppressed, they concluded that methadone should be on the list of drugs that can produce QTc interval prolongation and TdP.

DISCUSSION

After our literature search, we need to mention couple of limitations in the research on methadone maintenance treatment programs. The biggest limitation is a lack of experimental clinical trials with placebo groups, which are impossible for ethical reasons. Other limitations are connected to the specific behaviour of patients. Almost every study faced the problem of patients ending participation in the research.

However, in recent years, progress has been made, and a couple of experimental clinical studies that compared the effect of methadone and other medications in opioid dependence treatment have been published. Additionally, new insight into the effect of methadone on QTc interval was given by in vitro studies that tested the consequences of different genotypes on QTc interval.

Does methadone therapy lead to QTc interval prolongation among MMT patients? It was impossible to systematically analyse study results using only one reference value (Table 1). The most often used reference values for QTc were the ones proposed by the Committee for Proprietary Medicinal Products (CPMP) (14). Nevertheless, many authors used different reference values (20-29), which made it impossible to produce comprehensive results using the given data. Some authors preferred to measure QTc prolongation as an absolute increase in QTc. (20, 21, 22). According to the International Conference on Harmonization, absolute increases greater than 30 ms and greater than 60 ms are important (34), which is a good way to describe the influence of methadone on QTc interval at baseline values. However, despite all the problems mentioned above, there were patients on methadone maintenance programs with prolongation in QTc interval.

Table 1: References used for interpretation of QTc values.

Name of the study	Reference	Values
QT interval prolongation: prevalence, risk factors and pharmacovigilance data among methadone-treated patients in France. *Perrin-Terrin A, Pathak A, Lapeyre-Mestre M.*	Reference not stated	3 (7.1%) >450 ms for males 0 >470 ms for females[1]
Methadone maintenance, QTc and torsade de pointes: who needs an electrocardiogram and what is the prevalence of QTc prolongation? *Mayet S, Gossop M, Lintzeris N, Markides V, Strang J.*	Moss AJ. Measurement of the QT interval and the risk associated with QTc interval prolongation: a review. Am J Cardiol 1993;72:23B–5B.	13 (15.7%) ≥450 ms for males 2 (2.4%) ≥470 ms for females
Prevalence and clinical relevance of corrected QT interval prolongation during methadone and buprenorphine treatment: a mortality assessment study. *Anchersen K, Clausen T, Gossop M, Hansteen V, Waal H.*	Krantz M. J. Heterogeneous impact of methadone on the QTc interval: what are the clinical implications? J Addict Dis 2008; 27: 5–9.	85 (49.1%) >430 ms 50 (28.9%) >450 ms 26 (15%) >470 ms 8 (4.6%) >500 ms
Heterogeneous impact of methadone on the QTc interval: what are the practical implications? *Krantz MJ*	Author set reference values	62 (41%) >430 ms 29 (19%) >450 ms 11 (7%) >470 ms 3 (2%) >500
Corrected-QT intervals as related to methadone dose and serum level in methadone maintenance treatment (MMT) patients: a cross-sectional study. *Peles E, Bodner G, Kreek MJ, Rados V, Adelson M.*	Moss A. J., Zareba W., Benhorin J., Couderc J. P., Kennedy H., Locati-Heilbron E. et al. ISHNE guidelines for electrocardiographic evaluation of drug-related QT prolongation and other alterations in ventricular repolarization: task force summary. A report of the Task Force of the International Society for Holter and Noninvasive Electrocardiology (ISHNE), Committee on Ventricular Repolarization. Ann Noninvasive Electrocardiol 2001; 6: 333–41.	12 (7.9%) ≥450 ms 7 (4.6%) 500 > QTc ≥460 ms 3 (2%) >500 ms
QTc interval prolongation in patients on long-term methadone maintenance therapy. *Maremmani I, Pacini M, Cesaroni C, Lovrecic M, Perugi G, Tagliamonte A.*	Vincent GM, Timothy K, Leppert M, Keating M: The spectrum of symptoms and QT interval in carriers of the gene for the long-QT syndrome. N Engl J Med 1992; 327:846-852.	2 (2.4%) >470 ms for males 0 >480 ms for females
Levo-alpha-acetylmethadol (LAAM) induced QTc-prolongation - results from a controlled clinical trial. Wieneke H, Conrads H, Wolstein J, Breuckmann F, Gastpar M, Erbel R, Scherbaum N.	Committee for Proprietary Medicinal Products (CPMP). Points to consider: the assessment of the potential for QT interval prolongation by non-cardiovascular medicinal products. The European Agency for the Evaluation of Medicinal Products. December 1997 [CPMP/986/96].	1 (4.5%) 431 – 450 for males and 451 – 470 for females
QT-interval effects of methadone, levomethadyl, and buprenorphine in a randomized trial. *Wedam EF, Bigelow GE, Johnson RE, Nuzzo PA, Haigney MC.*	Author's modification of thresholds for abnormal prolongation of the QTc	13 (23%) >470 ms for males >490 ms for females
Effects of methadone on QT-interval dispersion. *Krantz MJ, Lowery CM, Martell BA, Gourevitch MN, Arnsten JH*	Garson A Jr. How to measure the QT interval: what is normal? Am J Cardiol 1993;72:14B–16.	19 (16%) >430 for ms males >450 ms for females
Impact of methadone treatment on cardiac repolarization and conduction in opioid users. *Martell BA, Arnsten JH, Krantz MJ, Gourevitch MN*	Authors defined a prolonged QTc interval as >450 ms for man and >470 ms for women without stating reference.	14 (20% of man 2% of women) >450 ms for man >470 ms for females
Stereoselective Block of hERG Channel by (S)-Methadone and QT Interval Prolongation in CYP2B6 Slow Metabolizers *CB Eap, S Crettol, J-S Rougier, J Schla¨pfer, L Sintra Grilo, J-J De´glon, J Besson, M Croquette-Krokar, P-A Carrupt and H Abriel*	European Agency for the Evaluation of Medicinal Products. Committee for Proprietary Medicinal Products. The assessment of the potential for QT interval prolongation by non-cardiovascular medicinal products. CPMP/986/96. http://www.emea.eu.int/pdfs/human/swp/098696en.pdf (1997).	42 (26%) 430<QTc<450 16 (9%) >450 for males >470 for females

Does methadone prolong QTc interval enough to evoke ventricular tachycardia? The point that all the researchers agreed upon was the importance of QTc prolongation over 500 ms. Our research identified four studies that reported patients who did not have QTc intervals exceeding 500 ms (19, 22, 25, 27), but in one study, 6 of the 52 patients did have a QTc interval over 500 ms at some point in the study (20). Even though researchers have agreed that a QTc in-

terval exceeding 500 ms is an important threshold, there were no cases of TdP. A possible explanation could be the fact that for registering events as rare as TdP, much larger study groups are necessary than the ones usually formed in MMT research studies (35). The largest study group we reviewed had 179 patients (30). Studies in our research that provided any data on TdP were clinical cases (31-33). All the studies with TdP events and data on QTc length found that in these clinical cases, TdP occurred among patients with a QTc interval over 600 ms (31, 32).

Does the existence of other factors present among these patients have an effect on QTc interval length? Even though TdP appeared only among patients with QTc values significantly over the threshold known to be high risk for developing TdP, all of these patients also had 2 or more risk factors (31-33). The most often present risk factors in our research were dose of methadone (23, 25, 27, 28, 29, 30), co-medication (22, 23, 27, 30), and co-morbidity (22, 23, 27), but also present were gender (23, 24, 28), age (23, 28), misuse of illicit drugs (25, 29), therapy length (28, 29), and tobacco use (27).

The influence of methadone dose on QTc interval length was statistically demonstrated by uni- and multivariate analysis in certain studies from our research (table 2). The dose range in all of the studies from our research was 5-600 mg/day, whereas the most effective opioid dependence treatment dosage of methadone is 60-100 mg/day (11). According to the Medicines and Health Care products Regulatory Agency (MHRA), a high dosage is considered anything above 100 mg/day (26). Studies that gave detailed data on the dosage of methadone showed that all cases of QTc prolongation over 500 ms, except one (24), were receiving more than 100 mg/day of methadone (24, 28, 29).

The often-present co-morbidities among MMT patients lead to considerable co-medication. HIV and HCV infections lead to electrolyte imbalance and to acute hepatitis that reduces the efficacy of methadone-metabolizing enzymes. Concomitant use of medicines that directly affect QTc interval or indirectly by interaction with methadone-metabolizing enzymes, thus increasing serum methadone level, could affect the length of QTc prolongation. A list of medicines that interact with MMT can be found online (35).

The reviewed data undoubtedly show the presence of prolonged QTc interval among some MMT patients, but it is hard to define how much of this QTc prolongation is the result of methadone activity and how much is the effect of other risk factors. Eap et al. pointed out that the presence of co-medication was similar between groups of patients with and without QTc prolongation, although uni- and multivariate analysis in their study showed a correlation between co-medication and QTc interval prolongation (30). An explanation for this could be found in the results of the latest genetic studies that showed inter-individual variations in the methadone metabolizing process and structure of cardiac ion channels (30, 36). A group of scientists led by Prof. Eap and coworkers researched stereo selectivity of methadone-induced hERG channel blockage, and at the same time, they tested the hypothesis that CYP2B6 slow metabolizer status was connected with prolonged QTc interval (30). Out of 179 patients included in this study, 6% had the CYP2B6 slow metabolizer genotype. The results of the study showed longer QTc intervals and higher plasma S-methadone concentrations in people with the CYP2B6 slow metabolizer genotype (439+/-25 ms VS 421+/-25 ms), whereas concentrations of plasma R-methadone were the same among all the patients. Higher incidence of borderline and prolonged QTc intervals (>450 ms) among patients with the CYP2B6 slow metabolizer genotype were also found. Prolonged QTc interval was noted among 3 of the 11 patients with this genotype (27%), whereas QTc prolongation among patients with other genotypes was noted in 13 out of 168 cases (8%). If we take into account borderline and prolonged QTc intervals, both parameters were found in 8 out of 11 patients with the CYP2B6 slow metabolizer genotype (73%), and 50 out of 168 patients with other genotypes.

Another genetic study tested mutations on five genes that coded for subunits of cardiac ion channels among OMT patients identified with QTc > 500 ms (36). Out of 200 patients, 8 were recruited, and genetic testing revealed two heterozygous mutations for long QT syndrome (LQTS). The authors speculated that phenotypically silent or subclinical LQTS mutation carriers may become symptomatic in the presence of other risk factors. As LQTS carrier status is often unknown to the individual and clinician prior to the risk exposure, it could be directly manifested by syncopal and cardiac arrest episodes as had happened with these two patients from the study. During this study, 24-h ECG recordings were performed and showed diurnal fluctuations of the QTc interval in all patients; thus, the authors concluded that because of diurnal fluctuations of the QTc interval, there might have been missed cases of LQTS mutations among the rest of the patients who had a QTc < 500 ms in the assessment study.

The authors of both studies presented data on the frequency of these genetic mutations (6% of Caucasians with CYP2B6 SM status and prevalence of between 1/100 and 1/300 of heterozygous mutation carriers in the general population), stating that these genotypes are of clinical relevance for methadone treatment.

To increase the safety of patients on MMT, the authors recommend that physicians obtain a careful screen for risk factors associated with long QT syndrome (23, 24, 31, 32), cardiac evaluation and systematic ECG especially among patients with a medical history of cardiac diseases, presence of congenital long QT syndrome and sudden cardiac death in families, a methadone dosage greater than 120 mg (28) and after changes in therapy regime (27). They also mention that methadone's more favourable cost profile and efficacy will still keep it as a first line of opiate addiction treatment (22, 24, 30).

Undoubtedly, improvement in the resocialization process as a final step of opiate addiction treatment leads to a reduction in methadone dose and finally exclusion from methadone treatment, thus erasing any potential effect of methadone on QTc interval.

Table 2: Research papers that shows statistically significant risk factors.

Effects of methadone on QT-interval dispersion. *Krantz MJ, Lowery CM, Martell BA, Gourevitch MN, Arnsten JH.*	UNIVARIATE ANALYSIS	Baseline clinical variables tested for association with QT dispersion were age, sex, taking drugs that affect cardiac repolarization or methadone metabolism, self-reported cardiovascular disease, use of cocaine at baseline, and infection with human immunodeficiency virus or hepatitis C. Of these variables, the only one associated with the magnitude of QT dispersion at baseline was self-reported cardiovascular disease (42.1 vs 31.7 msec, p < 0.01). At 6 months none of these variables remained significantly associated with absolute QT dispersion. The same clinical variables were then tested for association with the magnitude of change in QT dispersion over 6 months and only concurrent antidepressant therapy was associated with a greater increase in QT dispersion (20 vs 8.5 msec, p = 0.04).
	BIVARIATE ANALYSIS	N/A
	MULTIVARIATE ANALYSIS	N/A
	STATISTICAL TEST USED	Univariate associations were tested using Pearson correlation coefficients for continuous independent variables and the Student t test for categoric independent variables. Multivariate associations with the magnitude of the change in QTc interval were tested using linear regression models.
Impact of methadone treatment on cardiac repolarization and conduction in opioid users. *Martell BA, Arnsten JH, Krantz MJ, Gourevitch MN*	UNIVARIATE ANALYSIS	Variables associated with a longer QTc interval at baseline were older age (r = 0.25, p = 0.001), female gender (women vs men, 426 vs 414 ms; p = 0.001), self-reported cardiac disease (434 vs 416 ms, p = 0.03); use of antidepressants (430 vs 418 ms, p < 0.0001) and calcium antagonists (432 vs 417 ms, p = 0.07); hepatitis C infection (423 vs 413 ms, p = 0.08) and HIV infection (423 vs 417 ms, p = 0.08). Only the methadone dose was significantly associated with the magnitude of the QTc increase from baseline to 6 months (r = 0.18, p = 0.03).
	BIVARIATE ANALYSIS	N/A
	MULTIVARIATE ANALYSIS	Controlling for all factors associated with the baseline QTc interval and use of heroin, cocaine, and/or benzodiazepines, factors that were associated with greater QTc prolongation from baseline to 6 months, included male gender, HIV infection, and methadone dose. At the 12-month follow-up only the 12-month methadone dose remained marginally associated with the magnitude of the QTc increase (p = 0.08).
	STATISTICAL TEST USED	Univariate associations were tested using Pearson's correlation coefficients for continuous variables and the Student t test for categorical variables. Multivariate associations with the magnitude of the QTc interval or change in QTc interval were tested using linear regression models with p < 0.10 as the model entry criterion.
QT interval prolongation: prevalence, risk factors and pharmacovigilance data among methadone-treated patients in France. *Perrin-Terrin A, Pathak A, Lapeyre-Mestre M.*	UNIVARIATE ANALYSIS	N/A
	BIVARIATE ANALYSIS	N/A
	MULTIVARIATE ANALYSIS	In the multiple linear regression, QTc length was associated (r2 = 0.74) with history of heart disease [b = 251.3 (SD 123.4), P = 0.04], tobacco use [b = 234.5 (SD 74.9), P = 0.003], and QT-prolonging drugs [b = 275.5 (SD 78.2), P = 0.001]. QT dispersion was associated (r2 = 0.73) with history of heart disease [b = 62.8 (SD 26.6), P = 0.02], recent increase in methadone dose [b = 68.5 (SD 21.6), P = 0.003], tobacco use [b = 50.1 (SD 16.1), P = 0.003], and QT-prolonging drugs [b = 60.8 (SD 74.9), P < 0.001].
	STATISTICAL TEST USED	Multiple linear regression
Methadone maintenance, QTc and torsade de pointes: who needs an electrocardiogram and what is the prevalence of QTc prolongation? *Mayet S, Gossop M, Lintzeris N, Markides V, Strang J.*	UNIVARIATE ANALYSIS	N/A
	BIVARIATE ANALYSIS	Bivariate analysis found no factor independently associated with QT length or QTc prolongation.
	MULTIVARIATE ANALYSIS	Multivariate analysis, via multiple linear backward regression, investigated the relationship between QTc length and the following covariates: prescribed methadone daily dose (log base 10), stimulant use (illicit and prescribed), benzodiazepine use (illicit and prescribed), alcohol use, heroin use, tobacco use, cannabis use, gender and age. Total daily methadone dose (b = 0.318, P = 0.003) and use of stimulants (cocaine or amphetamines) (b = 0.213, P = 0.043) were found to be predictive of QTc length. Binary logistic regression was conducted investigating QTc prolongation did not reveal a statistically significant association.
	STATISTICAL TEST USED	Multiple linear backward regression
Prevalence and clinical relevance of corrected QT interval prolongation during methadone and buprenorphine treatment: a mortality assessment study. *Anchersen K, Clausen T, Gossop M, Hansteen V, Waal H.*	UNIVARIATE ANALYSIS	Dose of methadone (0.373*) P =0.001 b= 0.365 Gender (0.080*) P = 0.295 b = 0.079 Age (years) (0.134*) P = 0.080 b = 0.115 Time in treatment (0.144*) P = 0.058 b = 0.027
	BIVARIATE ANALYSIS	N/A
	MULTIVARIATE ANALYSIS	Dose of methadone B 0.367** 95% CI of B 0.22 – 0.51 P = 0.001 Gender B 5.988** 95% CI of B -4.62 – 16.59 P= 0.226 Age (years) B 0.569** 95% CI of B -0.14 – 1.38 P = 0.137 Time in treatment B 0.029** 95% CI of B -0.14 – 0.20 P = 0.410
	STATISTICAL TEST USED	Pearson's correlation* Multiple linear regression** A positive correlation was found between QTc interval and dose of methadone, both in Pearson's correlation (r = 0.37, P = 0.01) and in the multiple linear regression analysis (B = 0.37, P = 0.01). No statistically significant correlation was detected between QTc interval and time in treatment, age or gender

Corrected-QT intervals as related to methadone dose and serum level in methadone maintenance treatment (MMT) patients: a cross-sectional study. *Peles E, Bodner G, Kreek MJ, Rados V, Adelson M.*	UNIVARIATE ANALYSIS	N/A
	BIVARIATE ANALYSIS	N/A
	MULTIVARIATE ANALYSIS	QTc correlated significantly with duration in MMT (r = 0.24, P = 0.005), but only as a trend with methadone daily doses (r = 0.13, P = 0.1) , and not at all with serum methadone levels (r = 0.01, P = 1). QTc intervals correlated significantly with methadone doses in cocaine abuse patients (n = 31), r = 0.4, P = 0.03), but not in the 107 'non-cocaine-abuse' patients (r = 0.04, P = 0.4).
	STATISTICAL TEST USED	The correlation between QTc interval and methadone (dose and duration) was determined using Pearson's correlation coefficients Continuous variables of differences between groups were analyzed using oneway analysis of variance (ANOVA) and results are presented as mean standard deviation. Categorical variables were presented as proportions (%).
QTc interval prolongation in patients on long-term methadone maintenance therapy. (1.14) *Maremmani I, Pacini M, Cesaroni C, Lovrecic M, Perugi G, Tagliamonte A.*	UNIVARIATE ANALYSIS	No correlation was found between QTc values and methadone doses or gender.
	BIVARIATE ANALYSIS	N/A
	MULTIVARIATE ANALYSIS	N/A
	STATISTICAL TEST USED	Pearson's correlation
Stereoselective Block of hERG Channel by (S)-Methadone and QT Interval Prolongation in CYP2B6 Slow Metabolizers *CB Eap, S Crettol, J-S Rougier, J Schla¨pfer, L Sintra Grilo, J-J De´glon, J Besson, M Croquette-Krokar, P-A Carrupt and H Abriel*	UNIVARIATE ANALYSIS	Univariate analysis between QTc interval at trough and several risk factors indicated that trough (R,S)- methadone concentrations (r = 0.31, r2 = 0.097, P = 0.00005), use of co-medications (r = 0.18, r2 = 0.034, P = 0.01), methadone daily dose (r = 0.19, r2 = 0.036, P = 0.01), CYP2B6 SM status (r = 0.18, r2 = 0.032, P = 0.02), and serum calcium (r= -0.15, r2 = 0.023, P = 0.04) , were predictive of the QTc interval duration. Other variables, such as gender (P = 0.35), age (P = 0.28), serum potassium (P = 0.42), cocaine (P = 0.45) and alcohol (P = 0.51) consumption, were not predictive of the QTc interval duration.
	BIVARIATE ANALYSIS	N/A
	MULTIVARIATE ANALYSIS	Multivariate analysis yielded a model including trough (R,S)-methadone concentrations (P < 0.0005), hypocalcemia (<2.2mmol/l; P = 0.02), use of co-medication (P = 0.03), and CYP2B6 status (P = 0.05) with a determination coefficient (r2) of 0.17 (n = 179; P = 0.00005).
	STATISTICAL TEST USED	Pearson correlation, regression, X^2

Table 3: List of all researches from Results.

QT interval prolongation: prevalence, risk factors and pharmacovigilance data among methadone-treated patients in France. *Perrin-Terrin A, Pathak A, Lapeyre-Mestre M.*	**Journal**	Fundamental & clinical pharmacology
	Type of Study	Cross-Sectional Study & Retrospective study
	Number	42-550 (in adverse drug reactions (ADRs) spontaneously reported with methadone from 1996 to 2007
	Other Medications Evaluated or Present	Amisulpride, Mianserine, Alimemazine, Cyamemazine, Fluoxetine
	Other Drugs of Abuse Evaluated	Codeine, Tobacco, Heroin, Amphetamine, Opiates, Alcohol
	Other Medical Conditions Evaluated	Depression, Insomnia, Anxiety Psychosis or personality disorders Hepatitis C HIV Cardiac arrhythmia Arterial Hypertension
	Major Findings	4 QTc prolongation in Cross Sectional Study 5 QTc prolongations (3TdP) and seven sudden cardiac deaths not correlated with Methadone overdose in retrospective study.
	Notes, Including Limitations	Small study group in Cross Sectional Study
Methadone maintenance, QTc and torsade de pointes: who needs an electrocardiogram and what is the prevalence of QTc prolongation? *Mayet S, Gossop M, Lintzeris N, Markides V, Strang J.*	**Journal**	Drug and alcohol review
	Type of Study	Cross–Sectional stady
	Number	155
	Other Medications Evaluated or Present	Citalopram, Zopiclone, Salbutamol, Quetiapine, Chlorpromazine, Temazepam, Dexamphetamine, Olanzapine
	Other Drugs of Abuse Evaluated	Cocaine, Tobacco, Heroin, Benzodiazepine, Alcohol
	Other Medical Conditions Evaluated	Hepatitis C
	Major Findings	57.4% (n = 89) fulfilled MHRA criteria for ECG monitoring, QTc prolongation prevalence was 18.1% with NO ‚clinically significant' QTc prolongation >500 ms or torsade de pointes known to be present
	Notes, Including Limitations	

Prevalence and clinical relevance of corrected QT interval prolongation during methadone and buprenorphine treatment: a mortality assessment study. *Anchersen K, Clausen T, Gossop M, Hansteen V, Waal H.*	**Journal**	Addiction (Abingdon, England)
	Type of Study	Mortality assessment study
	Number	173 patients on methadone
	Other Medications Evaluated or Present	N/A
	Other Drugs of Abuse Evaluated	N/A
	Other Medical Conditions Evaluated	N/A
	Major Findings	4.6% (n = 8) had a QTc above 500 milliseconds; 15% (n = 26) had a QTc interval above 470 milliseconds; and 28.9% (n = 50) had a QTc above 450 milliseconds.A positive dose-dependent association was identified between QTc length and dose of methadone, and all patients with a QTc above 500 milliseconds were taking methadone doses of 120 mg or more. OMT patient mortality, where QTc prolongation could not be excluded as the cause of death, was 0.06/100 patient-years. Only one death among 3850 OMT initiations occurred within the first month of treatment.
	Notes, Including Limitations	
Heterogeneous impact of methadone on the QTc interval: what are the practical implications? *Krantz MJ*	**Journal**	Journal of addictive diseases
	Type of Study	Prospective Cohort Study
	Number	151
	Other Medications Evaluated or Present	N/A
	Other Drugs of Abuse Evaluated	N/A
	Other Medical Conditions Evaluated	N/A
	Major Findings	The proportion exceeding 450 msec increased from 7% at baseline to 19% at 6 months; those exceeding 500 msec increased from 0% to 2%. Although 18% of subjects had an increase in QTc of 30 msec, only 3% had an increase exceeding 60 msec.
	Notes, Including Limitations	Critical QTc prolongation (exceeding 500 msec or increases exceeding 60 msec) occurred infrequently. This highlights the heterogeneity of QTc interval changes and measurement variability but also implies that electrocardiography screening among opioid dependent patients would only occasionally require methadone discontinuation.
Corrected-QT intervals as related to methadone dose and serum level in methadone maintenance treatment (MMT) patients: a cross-sectional study. *Peles E, Bodner G, Kreek MJ, Rados V, Adelson M.*	**Journal**	Addiction (Abingdon, England)
	Type of Study	Cross-sectional study
	Number	138
	Other Medications Evaluated or Present	Propanolol, Aspirin, Mirtazapime sodium, Valproate Acetylsalicylic Acid Simvastatin, Ramipril, Isosorbide Mononitrate Clonazepam, Fluoxetine Thyroxin sodium, Salbutamol, Enoxaparin, Haloperidol, Biperiden
	Other Drugs of Abuse Evaluated	Benzodiazepine, Opiate Cannabis Cocaine Amphetamine
	Other Medical Conditions Evaluated	HIV, HVB, HVC
	Major Findings	Mean methadone dose was 170.9 +/- 50.3 mg/day and mean serum methadone level was 708.2 +/- 363.1 ng/ml. Methadone dose and serum levels did not correlate with QTc. Three patients had QTc intervals above 500 ms (‚prolonged'). After 2 +/- 0.4 years of follow-up, two patients died; they were two of three patients with very prolonged QTc. Causes of death were not attributed to cardiac origin.
	Notes, Including Limitations	Methadone maintenance is generally safe; however, the possible toxicity of high dose (> 120 mg/day) should be monitored for QTc
QTc interval prolongation in patients on long-term methadone maintenance therapy. *Maremmani I, Pacini M, Cesaroni C, Lovrecic M, Perugi G, Tagliamonte A.*	**Journal**	European addiction research
	Type of Study	Prospective study
	Number	83
	Other Medications Evaluated or Present	N/A
	Other Drugs of Abuse Evaluated	N/A
	Other Medical Conditions Evaluated	N/A
	Major Findings	Eighty-three percent of the subjects had a more prolonged QT df (QT df is the difference between QTc and standardized values and it corresponds to the gap between an individual's QT and the expected for healthy subjects) than the reference values for persons of the same sex and age. Only 2 patients displayed a QTc interval of >500 ms. No correlation emerged between QTc values and methadone dosages.
	Notes, Including Limitations	

Levo-alpha-acetylmethadol (LAAM) induced QTc-prolongation - results from a controlled clinical trial. Wieneke H, Conrads H, Wolstein J, Breuckmann F, Gastpar M, Erbel R, Scherbaum N.	**Journal**	European Journal of Medical Research
	Type of Study	Experimental study
	Number	22 in methadone group
	Other Medications Evaluated or Present	An patient with abnormal QTc interval received tricyclic antidepressant.
	Other Drugs of Abuse Evaluated	Urine drug screening revealed continuous use of cocaine during the study in one patient with abnormal QTc intervals.
	Other Medical Conditions Evaluated	Subjects with known cardiac disease were excluded
	Major Findings	No significant change in QTc interval in methadone group (0.406 s ± 0.029 s at run-in versus 0.405 s ± 0.025 s at 24 weeks)
	Notes, Including Limitations	84 subjects were initially enrolled in the study and 53 complete ECG data sets could be obtained. It reflects the difficulties in conducting clinical studies in this patient group and has also been reported from other studies
QT-interval effects of methadone, levomethadyl, and buprenorphine in a randomized trial. *Wedam EF, Bigelow GE, Johnson RE, Nuzzo PA, Haigney MC.*	**Journal**	Archives of internal medicine
	Type of Study	Experimental study (Randomized double-blind clinical trial)
	Number	Analyses were limited to the 154 patients and among them 55 in methadone group started the study and 32 finished it
	Other Medications Evaluated or Present	One participant used Erythromicin (potentially prolonging the QT)
	Other Drugs of Abuse Evaluated	N/A
	Other Medical Conditions Evaluated	N/A
	Major Findings	Using the categorical definition for QTc prolongation of more than 470 milliseconds for males and more than 490 milliseconds for females prolongation occurred among 23% of the methadone group (P < .001). An absolute QTc value of more than 500 ms occurred among 6 of 52 individuals in the methadone group (P < .001) at some point during the trial By stringent standard of an increase in QTc from baseline of more than 60 milliseconds at any time during the study 12% of subjects treated with methadone (OR, 8.4; 95% CI, 1.9-36.4) showed this increase
	Notes, Including Limitations	Female sex has been associated with considerably higher risks for QT prolongation and TdP but in the present study there was no significant difference between men and women with respect to prolongation more than 60 milliseconds from baseline (P=.68).
Effects of methadone on QT-interval dispersion. *Krantz MJ, Lowery CM, Martell BA, Gourevitch MN, Arnsten JH.*	**Journal**	Pharmacotherapy
	Type of Study	Prospective cohort study
	Number	118 patients who were newly admitted to the facility
	Other Medications Evaluated or Present	Isoniazid Antiretroviral agents AntidepressantsDiuretics Calcium channel blockers Beta blockers Phenytoin
	Other Drugs of Abuse Evaluated	Cocain, Alcohol (> 10 drinks a week)
	Other Medical Conditions Evaluated	Hepatitis C, HIV Cardiovascular disease Hypokalemia
	Major Findings	Methadone modestly but statistically significantly increased both QTc interval and QT dispersion.
	Notes, Including Limitations	Baseline clinical variables tested for association with QT dispersion were age, sex, taking drugs that affect cardiac repolarization or methadone metabolism, self-reported cardiovascular disease, use of cocaine at baseline, and infection with human immunodeficiency virus or hepatitis C and the only one associated with the magnitude of QT dispersion at baseline was self-reported cardiovascular disease (42.1 vs 31.7 msec, p<0.01). The same clinical variables were then tested for association with the magnitude of change in QT dispersion over 6 months. Only concurrent antidepressant therapy was associated with a greater increase in QT dispersion (20 vs 8.5 msec, p=0.04).

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Impact of methadone treatment on cardiac repolarization and conduction in opioid users. *Martell BA, Arnsten JH, Krantz MJ, Gourevitch MN*	**Journal**	American Journal of Cardiology
	Type of Study	Prospective cohort study
	Number	At baseline 233 participants, ECG obtained from 149 after 6 months study period and 97 patients had all three ECG's done after 12 months; 108 participants had ECG at baseline and 12 months (without ECG recording after 6 months period)
	Other Medications Evaluated or Present	Isoniazid, Antiretroviral agents, Antidepressants, Diuretics, Calcium channel blockers, Beta blockers, Phenytoin
	Other Drugs of Abuse Evaluated	Cocain, Alcohol (>10 drinks a week), Benzodiaze-pine
	Other Medical Conditions Evaluated	Hepatitis C, HIV Cardiovascular disease, Hypokalemia
	Major Findings	The QTc interval increased significantly from baseline at both time intervals but the mean difference in QTc interval from 6 to 12 months was not significant (p = 0.4). At baseline, 5 subjects (3%) had prolonged QTc intervals, defined as >450 ms for men (n = 3) and >470 ms for women (n = 2). At 6-month follow up, 18 subjects (13% of men and 11% of women) had prolonged QTc intervals, and 2 patients had QTc intervals of >500 ms. At 12 months, 14 subjects (20% of men and 2% of women) had prolonged QTc intervals, and 2 patients had QTc intervals of >500 ms.
	Notes, Including Limitations	At the 12-month follow-up visit, a subset of 44 patients had same-day serum methadone concentrations measured at trough (immediately before daily dose) and peak (2 hours after daily dose) time intervals. In univariate analysis, only the methadone dose was significantly associated with the magnitude of the QTc increase from baseline to 6 months (r = 0.18, p = 0.03). In multivariate analysis, controlling for all factors associated with the baseline QTc interval and use of heroin, cocaine, and/or benzodiazepines, factors that were associated with greater QTc prolongation from baseline to 6 months, included male gender, HIV infection, and methadone dose. In multivariate analysis after 12 months, methadone dose remained marginally associated with the magnitude of the QTc increase (p = 0.08). The QTc interval change from baseline to 12 months was significantly correlated with the methadone trough (r = 0.37, p = 0.008) and peak levels (r = 0.32, p = 0.03).

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NON-OPIOID ANALGESICS CONSUMPTION AT THE SURGERY DEPARTMENTS OF A SECONDARY CARE HOSPITAL IN GENERAL HOSPITAL IN KRALJEVO, SERBIA

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POTROŠNJA NEOPIOIDNIH ANALGETIKA NA HIRURŠKIM ODELJENJIMA BOLNICE SEKUNDARNOG NIVOA ZDRAVSTVENE ZAŠTITE U KRALJEVU, SRBIJA

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ABSTRACT

The aim of this study was to determine the amount of non-opioid analgesics consumed at the surgical departments of a secondary care hospital in Serbia, a developing country undergoing a socioeconomic transition that thus lacks sufficient funds to finance and invest in the healthcare system.

At the departments of gynaecology, urology, otolaryngology, general surgery and orthopaedics with traumatology at the General Hospital, Kraljevo from 2010 to 2012, six different non-opioid analgesics were used: diclofenac, ketorolac, ibuprofen, metamizole sodium, paracetamol (for per os and parenteral use), and meloxicam (for parenteral use only). Drugs in the M01 Anatomical Therapeutic Chemical classification group were consumed statistically significantly more than drugs in the N02 group (U=0.000; p<0.001). With regard to the average consumption amounts of all monitored drugs, diclofenac was consumed the most, followed by ketorolac. Meloxicam was the least used drug. There were significant differences in the average annual consumption of ibuprofen between surgical departments, but this was not the case for the other non-opioid analgesics. The differences in the average consumption between the individual drugs were significant for each year of observation.

Due to the incongruity of the results of previous studies related to non-steroidal anti-inflammatory drug consumption at different surgery wards, additional research in different geographical areas of our country is necessary to enhance the quality of prescription patterns on a national level and adjust them based on the latest scientific data and European trends.

Keywords: *drug use non-steroidal anti-infl ammatory drugs DDD methodology surgery departments.*

SAŽETAK

Cilj ovog istraživanja je ispitati potrošnju neopioidnih analgetika na hirurškim odeljenjima bolnice sekundarnog nivoa zdravstvene zaštite u Srbiji, zemlji u razvoju i socioekonomskoj tranziciji, koja kao takva, nema dovoljno sredstava za finansiranje i ulaganje u zdravstveni sistem.

Na odeljenjima ginekologije, urologije, otorinolaringologije, opšte hirurgije i ortopedije sa traumatologijom u Opšoj bolnici u Kraljevu, u periodu od 2010. do 2012.godine korišćeno je šest različitih neopioidnih analgetika: diklofenak, ketorolak, ibuprofen, metamizol-natrijum, paracetamol (za oralnu i parenteralnu upotrebu) i meloksikam (za parenteralnu upotrebu). Lekovi koji pripadaju grupi M01 prema anatomsko-terapijsko-hemijskoj klacifikaciji su statistički značajno više korišćeni nego lekovi koji pripadaju NO2 grupi (U=0.000; p<0.001). Diklofenak je bio na prvom i ketorolak na drugom mestu po prosečnoj potrošnji među svim analiziranim lekovima. Meloksikam je najmanje korišćen lek. Postojala je statistički značajna razlika u prosečnoj godišnjoj potrošnji ibuprofena po odeljenju, dok za druge neopioidne analgetike ova razlika nije utvrđena. Razlika u prosečnoj potrošnji između pojedinačnih lekova bila je značajna za svaku godinu ispitivanja.

Zbog nepodudarnosti rezultata različitih studija o potrošnji nesteroidnih antiinflamatornih lekova na različitim hirurškim odeljenjima neophodna su dodatna slična istraživanja u različitim geografskim oblastima naše zemlje, u cilju poboljšanja kvaliteta propisivanja ovih lekova na nacionalnom nivou kao i u cilju prilagođavanja propisivanja sa najnovijim naučnim preporukama i evropskim trendovima.

Ključne reči: *potrošnja lekova, nesteroidni antiinfl amatorni Lekovi, DDD metodologija, hirurška odeljenja*

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ABBREVIATIONS

ADE – Adverse drug events;
ATC – Anatomical Therapeutic Chemical;
COX – cyclooxygenase;
COX-2 - cyclooxygenase-2;
DDD – defined daily dose;
GI - gastrointestinal;
MI - myocardial infarction;
NSAIDs - non-steroidal anti-inflammatory drugs;
SPSS – Service Provisioning System Software;
WHO – World Health Organization.

INTRODUCTION

The modern era is generally characterized by the increased use of medications for the treatment of various conditions and, on the other hand, by the limited financial resources of health systems. Because such widespread drug use is often associated with a higher incidence of adverse events (ADE) and high treatment costs, it is essential to monitor and analyse the consumption of medicines in order to rationalize their future use. Ensuring the appropriateness of drug use is particularly important in developing countries due to their poor financial investments in health care. (1, 2)

Non-opioid analgesics, including nonsteroidal anti-inflammatory drugs (NSAIDs) and paracetamol, are currently among the most commonly used medications in clinical practice due to their reliable effectiveness, relative ease of use, and acceptable price in most circumstances. (2) However, it is also well known that inappropriate use of these drugs generates increased risks of serious gastrointestinal and cardiovascular adverse effects as well as renal and/or hepatic impairment. (3). However, despite this fact, the majority of non-opioid analgesics are widely available, even without medical prescriptions. Furthermore, NSAIDs account for the largest portion of medicines intended for self-treatment in the home pharmacies of individuals in the community. (4)

Studies of NSAID utilization in our country and other countries in Europe have shown increasing utilization of this group of drugs. (4,5,6,7,8) In the United States of America, NSAID consumption is also high, with 34% of people older than 65 years consuming at least one dose per day and 70% consuming at least one dose per week. (9) A study in North America showed that each dollar spent on NSAIDs resulted in 0.66-1.25 cents being spent on treating gastrointestinal (GI) tract side effects. (10) NSAID utilization in hospitals depends mostly on the availability of appropriate pharmaceutical forms of these drugs for parenteral use and their pharmacological characteristics and cost. In surgery wards, the use of NSAIDs has increased in relation to the use of more toxic opioid analgesics, despite the fact that NSAIDs have less pronounced analgesic effects. (2) A study that analysed the costs associated with NSAID drugs in patients with arthritis showed that 31% of such costs are directed towards the treatment of ADE in the GI tract. (11)

The aim of this study was to determine the amount of non-opioid analgesics consumed at the surgical departments of a secondary care hospital in Serbia, a developing country undergoing socioeconomic transition that thus lacks sufficient funds to finance and invest in the healthcare system.

MATERIALS AND METHODS

This was a descriptive drug utilization study dealing with the extent of non-opioid analgesics used at the surgical units of the General Hospital in Kraljevo, Serbia, a state-owned institution with 580 beds that provides secondary health care services to approximately 250,000 inhabitants of the Raska district. The administrative centre of this hospital is in Kraljevo. In a retrospective manner, the consumption of NSAIDs and paracetamol at the Departments of: general surgery, gynaecology, urology, otolaryngology and orthopaedics with traumatology, during the 3-year period of January 1st, 2010 to December 31st 2012 was evaluated using the anatomical therapeutic chemical classification and defined daily dose (ATC/DDD) methodology adopted by the World Health Organization (WHO) Collaborating Centre for Drug Statistics Methodology (Last updated: 2013-12-19).

The data on the utilization of drugs belonging to the ATC groups M01 and N02 (i.e., NSAIDs and paracetamol, respectively) at the abovementioned surgical departments over the observed period were obtained from the annual reports of the Hospital Pharmacy, which is the department responsible for the supply and dispensing of all medicines to inpatients at the General Hospital in Kraljevo.

The Hospital's Administrative Service provided the data on the total number of patients treated at the surgical departments who were followed for non-opioid analgesic consumption. It also provided the average patient length of stay at each departments for each calendar year of observation separately. Based on these data, the total number of days patients spent at each department per year was calculated and then divided by 100 to standardize the drug consumption per 100 bad days. We did not have data regarding the sex or age of the patients to whom the therapy was administered or data about the indication for which the non-opioid analgesics were prescribed or their dosage; our data only described the total consumption of these drugs during the observation period.

Drug consumption was expressed as number of DDDs per 100 bed days, and the differences in DDDs for different drug formulations according to the routes of administration were considered. The defined daily doses for each drug formulation were obtained from the last updated version of the list of DDDs, which is available on the WHO website. (12) The number of consumed DDDs per 100 bed days per year for a particular drug was calculated separately for each surgical department.

Table 1: Number of patients treated and the average patient length of stay at each surgical department for each calendar year of the observation period

Departments	Gynaecology			Urology			Otolaryngology			Surgery			Orthopaedics		
Year	2010	2011	2012	2010	2011	2012	2010	2011	2012	2010	2011	2012	2010	2011	2012
Number of patients	1751	1834	1684	1565	1484	1451	716	775	719	4300	4516	4508	653	761	818
Average patient length of stay in days	4.98	4.48	4.58	6.70	4.83	4.36	8.24	7.29	6.84	6.76	6.09	6.19	12.65	12.89	11.39

All collected data on the utilization of non-opioid analgesics are summarized as the means and standard deviations. Considering the small sample size (n<30), a nonparametric *Kruskal-Wallis* test was used to analyse the significance of differences in average annual consumption of a particular drug between surgical departments as well as differences between individual drugs in average consumption for each department and for each year of observation. Differences were considered significant when there was a probability level of the null hypothesis being true of lower than 5% (p<0.05). All statistical analyses were performed using *SPSS* software, version 18.

RESULTS

For each surgical department, the total number of patients treated that were followed for non-opioid analgesic consumption and the average patient length of stay for each calendar year of observation is shown in Table 1. The longest average length of hospital stay was in the department of orthopaedics, and the department of general surgery had the greatest number of hospitalized patients (Table 1).

In the departments of gynaecology, urology, otolaryngology, general surgery and orthopaedics with traumatology at the General Hospital, Kraljevo from 2010 to 2012, six different non-opioid analgesics were used: diclofenac, ketorolac, ibuprofen, metamizole sodium, paracetamol (for per os and parenteral use), and meloxicam (for parenteral use only).

The consumed drugs belonged to two groups based on ATC classification: M01 (diclofenac, ketorolac, ibuprofen, and meloxicam) and N02 (paracetamol and metamizole sodium). The drugs in the M01 ATC classification group

Table 2. Consumption of non-opioid analgesics at surgical departments of the General Hospital in Kraljevo over the observed three-year period

Year	Department	Utilization of non-opioid analgesics (DDD/100 bad days)							statistical test# p value
		Diclofenac	Ketorolac	Ibuprofen	Paracetamol	Metamizole Sodium	Meloxicam	Mean ± SD	
2010	Gynaecology	20.21	2.64	2.87	0.30	1.43	0	**4.57±7.75**	χ^2=24.773 **p<0.001***
	Urology	38.9	1.72	3.81	1.66	1.82	0.05	**7.99±15.19**	
	Otolaryngology	20.08	3.05	5.9	0.26	0.42	0	**4.95±7.75**	
	Surgery	44.42	14.06	0.07	0.13	0.52	0	**9.87±17.81**	
	Orthopaedics	88.53	2.60	1.21	1.62	0	0.12	**15.68±35.70**	
	Mean ± SD	**42.43±27.99**	**4.81±5.19**	**4.16±2.32**	**0.79±0.77**	**0.84±0.76**	**0.03±0.05**	Mean ± SD	
2011	Gynaecology	23.13	3.04	1.22	0.10	0.51	0	**4.67±9.11**	χ^2=21.866 **p=0.001***
	Urology	42.11	2.50	1.95	2.27	2.43	0	**8.54±16.47**	
	Otolaryngology	19.05	1.33	4.51	0.30	0	0	**4.20±7.47**	
	Surgery	48.51	11.63	0.11	0.15	0.82	0	**10.20±19.31**	
	Orthopaedics	86.50	1.04	0.2	1.63	0	0	**14.89±35.08**	
	Mean ± SD	**43.86±26.87**	**3.91±4.39**	**1.60±1.80**	**0.89±1.00**	**0.75±1.00**	**0**	Mean ± SD	
2012	Gynaecology	26.28	2.20	0	0	0.65	0	**4.85±10.53**	χ^2=22.273 **p<0.001***
	Urology	44.93	0.95	0	0	2.90	0	**8.13±18.06**	
	Otolaryngology	28.01	0.18	1.39	0.31	0.17	0	**5.01±11.28**	
	Surgery	48.90	10.32	0	0	0.85	0	**10.01±19.48**	
	Orthopaedics	87.86	1.72	0.11	0.02	0	0	**14.95±35.72**	
	Mean ± SD	**47.20±24.83**	**3.07±4.12**	**0.30±0.61**	**0.07±0.14**	**0.91±1.16**	**0**		
statistical test§ p value		χ^2=0.740 p=0.691	χ^2=2.650 p=0.266	χ^2=7.906 **p=0.019***	χ^2=5.464 p=0.065	χ^2=0.249 p=0.883	χ^2=4.286 p=0.117		

The difference in individual consumption of NSAIDs in each year of the study period (2010, 2011, 2012);
§ The difference in annual utilization of NSAIDs
NSAIDs – non-steroidal anti-inflammatory drugs; DDD- defined daily dose.

had statistically significantly higher consumption than the drugs in the N02 group (U=0.000; p<0.001).

Regarding the average consumption of all monitored drugs, diclofenac consumed the most, followed by ketorolac. Meloxicam was the least used drug (Table 2).

The analysis of the average metamizole sodium consumption during the three-year period showed that the highest consumption of this medicine was at the urology department, and the orthopaedics department did not use metamizole sodium at all during the observation period (Table 2).

There were significant differences between the surgical departments in the average annual consumption of ibuprofen, but this was not observed for the other non-opioid analgesics. The differences in the average consumptions between the individual drugs were significant for each year of observation (Table 2).

DISCUSSION

This study showed that diclofenac, a conventional, non-selective inhibitor of cyclooxygenase (COX), was consumed the most (Table 2). Although diclofenac is listed in the category of NSAIDs with a moderate risk of causing ADE (13), according to one study, the introduction of a new analgesic generation of drugs, namely selective cyclooxygenase-2 inhibitors (COX-2), did not decrease the use of conventional NSAIDs (4, 7). However, this finding contrasts with those of other studies (6). A study of the consumption trends of NSAIDs at the departments of general and endocrine surgery at the hospitals in our country showed that diclofenac, ketorolac and ibuprofen were the most used NSAIDs; in the five-year observation period, they constituted 90% of the consumed NSAIDs (2). One of the reasons for this is the traditional prescription patterns, the low frequency of updates to pharmacotherapy guidelines and the low price of diclofenac, which is the cheapest NSAID on the market in our country. The high consumption of diclofenac is also enabled by the recommendations of family doctors and by its availability as an over-the-counter medication. (7)

Ketorolac was the second most frequently consumed non-opioid analgesic (Table 2), and its level of consumption is high considering that this drug has a powerful inhibitory effect on platelet aggregation that contraindicates its use pre-, intra- and postoperatively. (13)

Diclofenac is still the most frequently consumed medication in this group of drugs despite studies that have shown that long-term diclofenac therapy increases the risk of myocardial infarction (MI). The risk of MI remains in patients who used diclofenac for a long time but are not using it anymore. Therapeutic doses of ibuprofen do not cause an increased risk of MI, and it was the third most frequently consumed non-opioid analgesic according to the results of our research (Table 2). For both diclofenac and ibuprofen, the use of a daily dose higher than the therapeutic dose also increase the risk of MI (14).

The highest drug consumption among the investigated departments was in the department of orthopaedics. At the orthopaedics department, compared with all the departments combined, diclofenac was the most frequently consumed non-opioid analgesic (Table 2). One study of non-opioid analgesic consumption at an orthopaedics department showed that the most frequently used drug was the COX-2 selective inhibitor rofecoxib, which accounted for 30.4% of the drugs consumed; in that study, diclofenac was the second most frequently consumed drug. The same study showed a higher incidence of ADE in patients who received conventional NSAID therapy than in patients who received selective NSAIDs. (15) A study that examined the prescription of non-steroidal analgesics among general practitioners and various specialists showed that orthopaedists prescribed the highest amount of fixed doses of non-opioid analgesics (ibuprofen+paracetamol and diclofenac+paracetamol). (16)

The department of urology had the highest consumption of paracetamol in all years except 2012 (2010 – 3.46%; 2011 – 4.43%; 2012 – 0%; Table 2). A study conducted at the University Hospital in New Delhi showed that the most frequently used analgesic, paracetamol, accounted for 31.51% of total analgesic consumption, and this was greater than the percentage of total analgesic consumption accounted for by COX-2 selective inhibitors. (17)

The least used drug in the three-year observation period was meloxicam (Table 1), a COX-2 selective NSAID that causes a low incidence of ADE in the gastrointestinal tract. However, according to a recently conducted meta-analysis, COX-2 selective NSAIDs should be used with caution because of their side effects on the cardiovascular system and because they increase the risk of MI (18).

Metamizole sodium was withdrawn from the United States market in the 1970s because of the high risk of agranulocytosis (6); however, in our study, only orthopaedics with traumatology department did not use this drug during the three-year observation period (Table 2).

In the pharmaceutical market, which provides numerous choices for drugs of the same group, the quality of prescription patterns is low. In addition, prescriptions are influenced by the ability of doctors to choose a drug appropriately, the intensity of the marketing of drugs by pharmaceutical companies, and the prices of NSAIDs (19).

Due to the incongruity of the results of previous studies related to NSAID consumption in different surgery wards, additional research in different geographical areas of our country is necessary to enhance the quality of prescription patterns on a national level and adjust them based on the latest scientific data and European trends.

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BEHAVIOURS AND ATTITUDES ABOUT BODY IMAGE AND EATING DISORDERS AMONG ADOLESCENT FEMALES IN KRAGUJEVAC

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PONAŠANJE I STAVOVI O TELESNOM IZGLEDU I POREMEĆAJI ISHRANE KOD ADOLESCENTKINJA U KRAGUJEVCU

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ABSTRACT

Eating disorders are often in older teens and young women with prevalence 4-5% with increasing tendency. The aim of this study was to investigate the prevalence of eating disorders among adolescents in the city of Kragujevac, and to examine the relationship between the age and the type of eating disorder that can occur in adolescents.

This descriptive, cross sectional study involved 220 participants (16-25 years old, 105 high school students and 115 students of the Faculty of Medical Sciences in Kragujevac, Serbia). Eating Attitudes Test EAT-26 questionnaire was used as a screening instrument, which consists of three subscales related to eating disorders (dieting, bulimia, oral control diet). The frequency of disturbed attitudes and eating habits among the investigated population was 26,8% (EAT-26 score ≥20). The high school students had a significantly higher score values (30,4% of girls achieved values EAT-26 score ≥20) than the medical students. Among the respondents, 17,6% were malnourished, 72,7% normal weight, 9,3% of the overweight and 0,5% obese. Eating disorder not otherwise specified (EDNOS) was 13,7%, subclinical bulimia 4,4% and subclinical anorexia 8,8%. There was statistically significant difference (p=0,024) in the expression of behavioral disorders between students of high school and faculty education.

Our results suggest that malnutrition and anorexic syndrome are more frequently in population of medical students, but on the other hand, obesity and subclinical bulimic syndrome have a higher prevalence in high school student`s population, which can be explained by inappropriate education for adolescents.

Keywords: *eating disorders, adolescents, EAT-26.*

SAŽETAK

Poremećaji ishrane su uobičajeni kod starijih tinejdžerki i mladih žena sa prevalencom od 4-5% koja ima tendenciju rasta. Cilj ovog istraživanja je bio da se ispita učestalost poremećaja u ishrani među adolescentkinjama u gradu Kragujevcu, i da se ispita povezanost između starosti i vrste poremećaja ishrane koji se može javiti kod adolescentkinja.

Ova deskriptivna, studija preseka, je obuhvatila 220 ispitanica (starosti 16-25 godina, 105 srednjoškolki i 115 studentkinja Fakulteta medicinskih nauka u Kragujevcu, u Srbiji). Upitnik o stavovima u ishrani, EAT-26 je korišćen kao skrining instrument, koji se sastoji od tri subskale o poremećajima ishrane (dijeta, bulimija, oralna kontrola ishrane). Učestalost poremećenih stavova i navika u ishrani u ispitivanoj populaciji je bila 26,8% (EAT-26 skor ≥ 20). Učenice srednjih škola imale su statistički značajno više vrednosti EAT-26 skora od studentkinja medicine. Među ispitanicama njih 17,6% su bile pothranjene, 72,7% normalne težine, 9,3% prekomerno uhranjene i 0,5% gojazne. Poremećaj ishrane koji nije drugačije definisan je bio zastupljen u 13,7%, subklinička bulimija 4,4% i subklinička anoreksija 8,8%. Postojala je statistički značajna razlika (r=0,024) između srednjoškolki i studentkinja medicine u ispoljavanju ovih poremećaja u ishrani.

Naši rezultati pokazuju da su neuhranjenost i subklinički anoreksični sindrom češći u populaciji studentkinja medicine, a da sa druge strane gojaznost i subklinički bulimični sindrom imaju veću učestalost u populaciji srednjoškolki, što se može donekle objasniti neodgovarajućim obrazovanjem adolescenata.

Ključne reči: *poremećaji ishrane, adolescenti, EAT-26.*

ABBREVIATIONS

EDNOS-Eating disorder not otherwise specified
BED-Binge eating disorder
EAT-26-Eating Attitudes Test EAT-26
BMI-Body Mass Index
D-Dieting
B-Bulimia
O-Oral control diet

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INTRODUCTION

Proper nutrition is an essential precondition for a healthy life and is the basis for proper growth and development, physical and mental status, the defensive ability of the organism and the operation of all vital functions (1). Disordered attitudes and behaviours concerning food intake are frequent in adolescents, with a prevalence of 4-5%, and a ratio of 6:1 to 10:1 in favour of females. Research has shown an increase in the prevalence of these disorders, which are characterized by excessive concern about body shape and weight, followed by distorted attitudes and inappropriate, irregular or chaotic food intake (2).

The lifelong prevalence of anorexia in women ranges from 0.9% to 2.2%, whereas the frequency of bulimia in women is 1.5-2%. However, these eating disorders occur most often in adolescence, during which the prevalence may reach as high as 5%. The prevalence of binge eating disorders ranges from 3.5% to 10% in the general population (3,4). However, the most common eating disorders, either in clinical samples or in the general population, are other categories, which are defined as eating disorders not otherwise defined (EDNOS) (5). EDNOS is a heterogeneous group of disorders, which is not very well defined, and includes partial syndromes of anorexia, bulimia, purging and overeating - binge eating disorder (BED) (2,6). The prevalence of BED ranges from 0.7-4% in the normal population. The use of strict criteria for the diagnosis of eating disorders can hinder recognition of these disorders and their subclinical forms at an early stage. Disordered eating habits have a significant impact on health, even in the absence of complete formal criteria for eating disorders (7).

The aim of this study was to investigate the prevalence of eating disorders among adolescents in the city of Kragujevac, Serbia. Additionally, the nutritional statuses of and the relationship between age and the types of eating disorders noted among adolescents were investigated.

MATERIALS AND METHODS

This descriptive, cross sectional study was conducted in March and April, 2013. The sample consisted of 220 adolescents aged 16 to 25 years. The study was conducted in four secondary schools (gymnasium, medical, economic, and technical schools) and the Faculty of Medical Science, University of Kragujevac. The study population consisted of two groups, one of which consisted of 105 high school students, and the other of 115 medical students. Respondents were selected using simple random sampling. Inclusion criteria were as follows: female gender and age between 16 and 25 years. Exclusion criteria were as follows: pregnancy, lactation, diabetes, and previous diagnosis of an eating disorder.

The survey used the Eating Attitudes Test EAT-26 questionnaire (8). The questionnaire refers to satisfaction with body image and body weight, as well as attitudes and behaviours towards food and body. The total EAT-26 score is derived as the sum of composite items and ranges from 0 to 78. Answers were graded on a scale of six points from always to never, with 3 points awarded for a response of "always", 2 points for "usually", 1 point for "often", and 0 points for answers pertaining to "rarely", "sometimes", and "never". Results of ≥ 20 points, with at least one positive response in the behavioural part, are associated with abnormal eating attitudes and behaviour and can be identified as an eating disorder. The EAT-26 is distributed in three subscales as follows: Factor 1-D (diet), Factor 2-B (bulimia), Factor 3-O (oral control diet); this distribution allows researchers to obtain more information from the same survey. Factor 1-D determines whether respondents have a distorted image of their body, whether they are burdened with dieting and a desire to be thinner, and whether they need to constantly eat diet food. Factor 2-B determines the inability to stop overeating, which is often followed by vomiting and use of laxatives and diuretics. Factor 3-O determines the existence of self-control in eating and maintaining a low body weight, which is often associated with obsessive-compulsive behaviour. Respondents with high EAT-26 scores in this area are prone to anorexia (8).

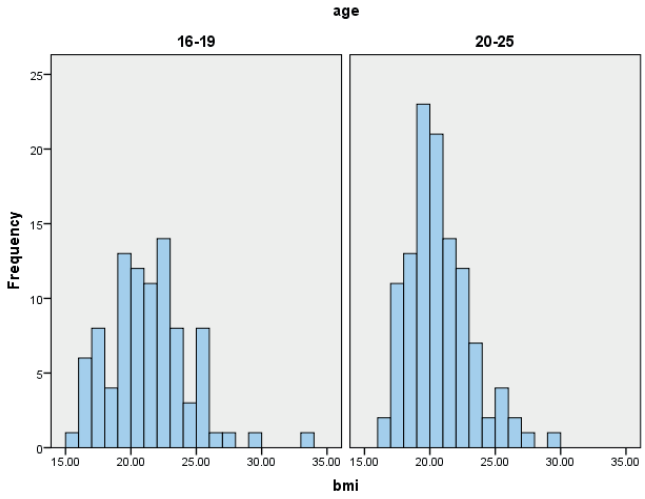

Figure 1. Frequency distribution of BMI by age group

The software package IBM SPSS Statistics 20 was used for determining the measures of central tendency and variability using descriptive statistics methods. To determine dependence between variables, the chi-square test was used. To determine differences between two groups, Student's t-test for independent samples and the Mann-Whitney U test were used. All statistical analyses were performed with confidence intervals of 95%.

RESULTS

The study comprised 205 persons aged 16 to 25 years (average age 20.67 ± 2.94 years). The mean body weight was 60.05 ± 8.557 kg, whereas the mean desired body weight was 57.8 ± 6.271 kg. The mean BMI for high school

Table 1. Percentage of respondents according to BMI and limit value EAT-26 scores

Score	Body Mass Index – BMI (kg/m²)								χ^2	p
	< 18.5		18.5-24.9		25.0-29.9		> 30			
	n	%	n	%	n	%	n	%		
0-19	21	14.0	113	75.3	15	10.0	1	0.7	5.267	0.153
20-78	15	27.3	36	65.5	4	7.3	0	0		
Total	36	17.6	149	72.7	19	9.3	1	0.5		

χ2 – value of chi-square test
p value

students was 21.29 ± 3.022 kg/m2. The mean BMI for medical students was 20.76 ± 2.409 kg/m2 (Figure 1).

Malnutrition was most common in persons with eating disorders (Table 1).

Subclinical bulimia was significantly more common in high school girls than in medical students (p=0.024), whereas subclinical anorexia was more common in medical students (p=0.024) (Table 2).

Most persons with eating disorders showed significant behaviour disorders of food intake (Table 3).

Children of divorced parents and children unsatisfied by their socioeconomic statuses more commonly exhibited signs of eating disorders (Fig. 2).

DISCUSSION

Because our research was conducted in a healthy young population, we did not expect such a large percentage of persons to meet the criteria for malnutrition. This result may be explained by the fact that our population was from an urban area, whereas studies that investigated either a larger part of our country (Vojvodina) (10) or Serbia in its entirety (11) showed much lower incidences of malnutrition. A possible explanation is that girls from more urban areas tend to have the lowest possible weights and are significantly more likely to develop an eating disorder than girls from rural areas, as shown earlier (12).

Table 2. Numerical and percentage representation of factors (D, B, O) by age group

	D		B		O		χ^2	p
	n	%	n	%	n	%		
High school girls	14	15.2	8	8,7	6	6.5	7.429	0.024
Medical students	14	12.4	1	0,9	12	10.6		
Total	28	13.7	9	4,4	18	8.8		

χ2 – value of chi-square test
p value

Table 3. Percentage of responses to questions from a group of behavioural disorders, by EAT-26 scores

Variables	Answers	Score ≤ 19	Score ≥ 20	χ^2	p
Overeating*	Never	92.0	58.2	30.163	**0.000**
	Several times a month	8.0	41.8		
Vomit	Never	97.3	87.3	6.163	**0.013**
	Several times a month	2.7	12.7		
Purgatives	Never	94.7	83.6	5.070	**0.024**
	Several times a month	5.3	16.4		
Exercise**	Never	63.8	41.8	7.056	**0.008**
	Several times a month	36.2	58.2		
Weight loss***	Yes	14.7	27.3	3.513	0.061
	No	85.3	72.7		

* Uncontrolled food intake
** Exercise lasting longer than 60 minutes a day and goal of weight loss or weight control
*** Weight loss of 9 kg or more in the last 6 months

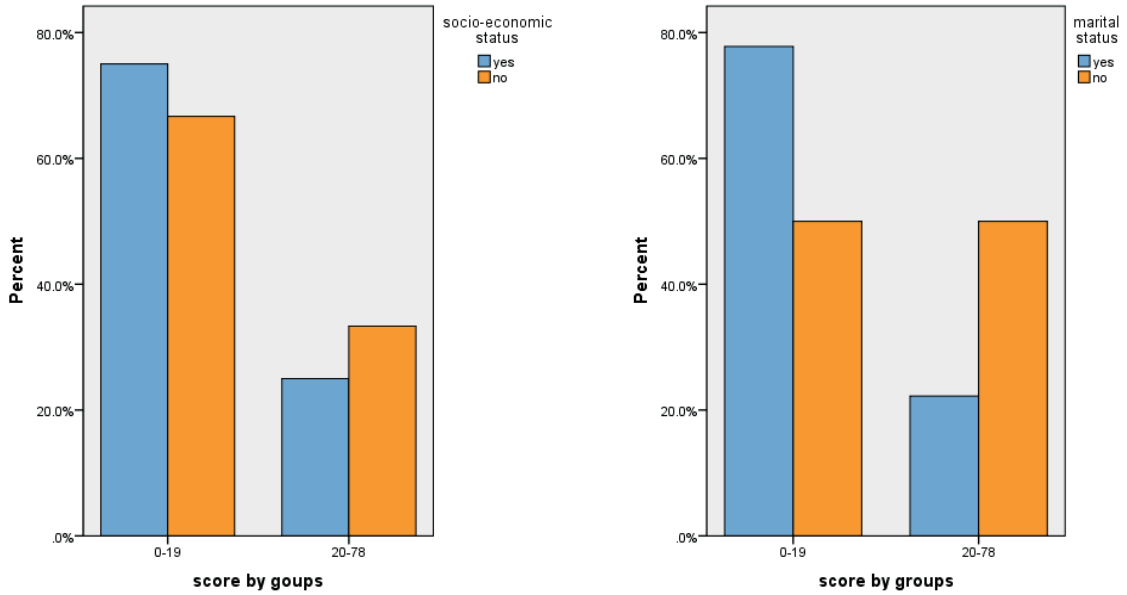

Figure 2. Satisfaction with socio-economic status or marital status of parents by EAT-26 scores

Another unexpected result was the surprisingly low representation of overweight girls in our sample compared with the numbers of girls in earlier surveys in Serbia (10, 11). It is a well-known fact that the number of overweight persons is steadily growing; therefore, the surprisingly small number of overweight girls in our study may have been due to the chronic desire of young urban girls to be thin. It should be taken into consideration that our respondents themselves gave information about their body weights and heights, and it is possible that they deliberately reduced their weights. If that were the case, it would give us important information about the desirability of thinness among young women today. More than half of the respondents indicated that they would like to lose weight, although they were not actually overweight. Research conducted on high school girls in Banja Luka showed fewer obese and underweight girls compared with our study (13), and even lower values were obtained in some European countries (14-17). The result that even 30.4% of our girls achieved EAT-26 scores over 20 is very worrisome because it puts Serbia among the countries with the highest prevalences of eating disorders (9). It is known that eating habits within our nation are significantly different from those in countries such as Japan and China, which have a significantly lower incidences of eating disorders than other parts of the world. Although the prevalences of eating disorders have grown in those countries for years, they are still far lower than the prevalence of eating disorders in our country (18-20).

Eating disorders have devastating physical, psychological and social consequences. This is reflected in high levels of mortality, morbidity and poor quality of life. The mortality rate for subclinical eating disorders is 3.31 deaths per 1,000 people per year (21). It is known that only 4-6% of individuals with eating disorders receive professional medical help (22).

We think that our results suggest the need for screening for eating disorders, which would allow for the early identification of eating disorders. Screening may be useful for general practitioners in identifying persons for earlier treatment, which has been proven to lead to more favourable outcomes.

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ANTHROPOMETRIC CHARACTERISTICS, NUTRITIONAL STATUS AND DIETARY HABITS IN A COLLEGE POPULATION

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ANTROPOMETRIJSKE KARAKTERISTIKE, NUTRITIVNI STATUS I NAVIKE U ISHRANI STUDENTSKE POPULACIJE

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ABSTRACT

The college student population is prone to irregular food intake and the excessive intake of carbohydrates and snacks. The aim of this study is to investigate the relationships among anthropometric characteristics, dietary habits and nutritional knowledge in female students attending a healthcare college.

Our investigation enrolled 100 college students at the High Health School of Professional Studies in Belgrade, 19-30 years old, who underwent anthropometric measurements and an investigation by questionnaire of their nutritional knowledge as well as recorded a 7-day food diary. The results were interpreted in relation to their location of nutritional intake.

The majority of students showed good nutritional knowledge. Of the total population, 83% were of normal weight, 11% were overweight, and 5% were underweight. The average Body Mass Index values, as well as body fat percentage, were similar regardless of the type of eating location, but all overweight and obese students were recorded in the groups that ate in the student dining facility and that prepared food and ate by themselves. Students who ate with their families ate significantly fewer fats and proteins but significantly more carbohydrates compared to students in the other two groups. Higher fat intake and snack consumption are significantly related to an increased percentage of body fat. Fruit intake is inversely related to body fat percentage.

Despite the relatively low prevalence of overweight, obesity, and underweight in the investigated population, the given results indicate that students may benefit from health promotion activities, increased knowledge and improved eating habits. This is especially important considering that they are future health professionals.

Key words: *Nutrition, students, anthropometry*

SAŽETAK

Studentska populacija je sklona nepravilnoj ishrani i prekomernom unosu ugljenih hidrata i grickalica. Cilj studije je da se ispita povezanost između antropometrijskih karakteristika, navika u ishrani i znanja o ishrani i namirnicama u populaciji studentkinja Visoke zdravstvene škole.

Ispitivanjem je obuhvaćeno 100 studentkinja Visoke zdravstvene škole strukovnih studija u Beogradu, uzrasta 19-30 godina. Nakon antropometrisjkih merenja, upitnikom je ispitano njihovo znanje o ishrani i namirnicama, a unos namirica je praćen kroz sedmodnevni dnevnik ishrane. Rezultati su tumačeni prema načinu organizacije njihove ishrane.

Većina studentkinja je pokazala dobro znanje o ishrani i namirnicama. Normalno uhranjenih je bilo 83%, sa prekomernom telesnom masom 11%, a pothranjenih 5%. Prosečne vrednosti Indeksa telesne mase, kao i procenta telesne masti bile su slične, bez obzira na način organizacije ishrane, ali su sve studenkinje sa prekomernom telesnom masom i gojaznošću pripadale grupama koje se hrane u studentskoj menzi, ili koje same organizuju ishranu. Studentkinje koje su se hranile u roditeljskom domaćinstvu unosile su značajno manje masti i belančevina, ali značajno više ugljenih hidrata u odnosu na ostale grupe. Ove studentkinje su u isto vreme unosile značajno manje obroka, manje voća i mleka i mlečnih proizvoda. Veći unos masnoća i grickalica pokazuje značajnu pozitivnu povezanost sa povećanim procentom telesne masti, a unos voća negativnu povezanost.

Uprkos relativno maloj prevalenci prekomerne telesne mase, gojaznosti i pothranjenosti u ispitivanoj populaciji, rezultati ukazuju na to da bi studentkinjama koristili preventivni programi usmereni na smanjenje učestalosti gojaznosti, kao i na poboljšanje znanja o ishrani, pogotovo što se školuju za zdravstvene radnike.

Ključne reči: *Ishrana, Studenti, Antropometrija*

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INTRODUCTION

Inadequate nutrition is often related to health problems that lower the quality of life and significantly influence morbidity and total mortality. Food is an everyday necessity. Adequate nutrition has become one of the most important issues of modern society.

Economic development, industrialization and urbanization, together with a sedentary lifestyle, have led to changes in dietary habits in most of the world population. These changes have contributed to an increase in body mass in all age groups, leading to a global crisis with significant consequences for world health (1). Nutrition is related to health, creativity, efficiency, cognitive functioning, and well-being.

Obesity rates are increasing regardless of socioeconomic status but are more pronounced in populations with lower income and lower education (2,3). This observation is expected because the cheapest foods are rich in fat and sugar but poor in nutritive value (4).

According to the World Health Organization, noncommunicable chronic diseases are the leading cause of death (60-80%) and disability (70%) in the world, while the leading risk factors are inadequate nutrition and sedentary lifestyle (6).

An analytical health study was conducted in a Serbian population in 2006 (7). The results from the study indicate that more than half of adults in Serbia (54,5%) have a high body mass—36,2% are overweight and 18,3% are obese. The average Body Mass Index (BMI) values are 26,1 kg/m^2 in men and 25,9 kg/m^2 in women. In children and adolescents (aged 7-19), there is a significant increase in the percentage of overweight and obese individuals—from 12,6% in 2000 to 18% in 2006. Regular monitoring of nutritional status in Serbia only occurs among school children and partly with older students (8).

Results from the Behavioral Monitoring of Risk Factors questionnaire indicate that the greatest increase in obesity is observed between 18 and 29 years, during the transition from adolescence to adulthood. The transition from high school to college is critical, and the risk of weight gain is significantly higher than during other significant life stages (9,10).

However, this life stage is favourable for the establishment of positive habits in nutrition and physical activity. The youth population represents a group susceptible to dietary interventions through education and the promotion of healthy behaviours and lifestyles, which may influence their future health.

Nutritional knowledge, dietary habits, nutritional status, and physical activity of the student population are the subjects of numerous studies (11-13). The results indicate that insufficient knowledge, poor food choices, increased caloric intake, stress, insufficient physical activity and sleep impairment, among other factors, may contribute to the increase in obesity among students.

During this period, some students leave the parental home and starting to live alone and maintain their own household, including food preparation, which represents a major challenge.

Obesity, other than being a disease itself, is related to cardiovascular diseases, diabetes, and metabolic impairment (14). The contemporary definition of obesity specifies an increase in fat mass, instead of in total body weight, which leads to health problems and the development of complications as a consequence of the imbalance between energy intake and energy expenditure (1,17).

In most cases, total body weight is directly related to body fat mass, but sometimes sophisticated diagnostic procedures indicate exceptions (16), i.e., individuals with normal body weight may actually have a fat mass above referent values. This type of obesity is known as *normal body weight obesity* or *sarcopenic obesity*, considering the increase in total body weight due to muscle mass reduction (17). This type of obesity is also related to numerous risk factors for the development of mild complications. Sarcopenic obesity is more often present in women and children (16). According to Gallagher, et al., this type of obesity is registered in 10% of young women (18). Obesity at a young age is related to increased risk from early onset of complications, which requires proper diagnostics and treatment (16).

The aim of this study is to investigate the relationship between anthropometric characteristics, dietary habits and nutrition knowledge in a population of female nursing students.

SUBJECTS AND METHODS

This study enrolled 100 female nursing students in Belgrade, aged 19-30 years. The students were divided in three groups according to their eating location and arrangement: the first group (35 students) ate at home with their families, the second group (33 students) ate on their own, while the third group (32 students) ate in the student dining facility. The investigation was conducted in March and April 2014.

Nutritional knowledge was investigated by questionnaire (19), consisting of four groups of questions: questions about experts' recommendations, food classification and categorization, proper combinations of foods, and relationships between diseases and inadequate nutrition. The first group of questions was weighted at 10 points each, while the three other groups were weighted at 30 points each.

The nutritive values of the students' 7-day food intake were calculated from data obtained from the students' 7-day food intake diaries (20). Students recorded every food item and the amount they took *per os* every day of the week. Obesity was categorized according to the World Health Organization Obesity Classification (21). Body composition was determined by bioelectrical impedance analysis (InBody 7200, South Korea). The observed parameters were body height, body weight, BMI, and body fat percentage.

Table1. Age structure of the examined students

Age (yrs)	Frequency table	
	Count and %	Cumulative count and %
19	37	37
20	27	64
21	10	74
22	7	81
23	7	88
24	4	92
25	1	93
27	1	94
28	3	97
29	1	98
30	2	100

Data were analysed by regression and correlation. Data were presented as the means ± standard deviation. The normality of distribution was tested by using the Kolmogorov-Smirnov test. The significance of difference between groups was tested by analysis of variance and the Kruskal-Wallis test. The correlations between parameters were tested by Spearman's correlation analysis. Statistical analyses were performed using the software package Statistica.10, and significance was accepted at $p<0,05$.

RESULTS

A total of 100 female students were randomly chosen from a larger population of nursing students. They were divided into three groups depending on their eating location and arrangement. The groups did not differ regarding body height, age, income and socio-economic characteristics. The analysis of age showed that 93% of students were 25 years old or younger, and most students were between 19 and 20 years old.

According to results from the nutritional knowledge questionnaire, we classified students into three categories by score: 4% of students were categorized as excellent (66-100 points), 91% as good (30-65 points) and 5% as unsatisfactory (less than 30 points). The average number of points in the unsatisfactory group was 21, with very large variations in the number of correct answers, as indicated by a coefficient of variation 31%. The majority of students showed good nutritional knowledge, with the average score of 47 points. The average number of correct answers in the excellent group was 69. The levels of nutritional knowledge were similar in all three investigated groups, regardless of eating location and arrangement.

The anthropometric characteristics of the three groups of students are shown in Tables 2 and 3.

Among all of the students, 83% were of normal weight (BMI between 18,5 and 24,9 kg/m^2), 11% were overweight (BMI between 25 and 29,9 kg/m^2), 5% were underweight (BMI under 18,5 kg/m^2), while one was obese, with a BMI above 30 kg/m^2.

Average values of body height, body weight, BMI and body fat percentage were similar in all of the investigated groups, regardless of eating location and arrangement ($p>0,05$). However, all overweight students were registered in the group that eats on their own, and the student classified as obese was registered in the group that eats at the student dining facility. In these two groups, we also registered underweight students. All of the students who ate with their families had normal BMIs.

Body fat percentage, as an anthropometric characteristic, showed much more variability in the investigated population (Table 4).

Table 2. Anthropometric characteristics (means ± standard deviations) in three group of students

Groups	**Body height (cm)**	**Body weight (kg)**	**BMI (kg/m^2)**	**Body fat (%)**
I	166,00 ± 6,94	60,38 ± 8,02	21,73 ± 1,87	26,60 ± 4,74
II	166,00 ± 6,14	59,50 ± 10,02	21,56 ± 3,07	28,73 ± 6,90
III	166,45 ± 7,40	58,23 ± 19,70	22,12 ± 4,22	29,04 ±15,64
Total	166,28 ± 6,77	59,40 ±13,30	21,80 ± 3,15	27,09 ± 10,08

I eating with their families
II eating on their own
III eating in student dining facilityTable 3 Statistical BMI data for all three groups

Table 2. Statistical BMI data for all three groups

Groups	**Mean (kg/m^2)**	**Interval of variation**		**SD**	**Coefficient of variation**
		Minimal	**Maximal**		
I	21,73	18,50	24,70	1,87	8,62
II	21,56	17,50	29,80	3,07	14,26
III	22,12	17,50	38,10	4,22	19,08
Total	21,80	17,10	38,10	3,15	14,23

Table 4 Statistical body fat percentage data for all three groups

Groups	Mean (%)	Interval of variation		SD	Coefficient of variation
		Minimal	Maximal		
I	26,60	16,80	35,00	4,74	17,82
II	28,73	11,40	40,00	6,90	26,83
III	29,04	11,50	49,09	15,64	53,87
Total	27,09	11,40	49,09	10,08	37,22

The average value for body fat percentage among all groups was 27,09% (ranging from 11,40% to 49,09%). The largest average value was registered in group that ate in the student dining facility (29,04%). Statistically, this parameter is the most stable in students who ate with their families, with the lowest SD (4,74) and coefficient of variation (17,82). Stability is three-fold times lower in group that ate at the student dining facility (SD=15,64, CV=53,87). However, the results from the Kruskal-Wallis test showed that the observed differences in average values of body fat percentage between the three groups were not statistically significant (H=0,4704024; p=0,7904).

The nutritive values of dietary intake were calculated based on students' self-reported 7-day diaries of food intake. Total calories, amounts of fat, carbohydrates, and proteins were calculated. The average nutritive values of each group's daily dietary intake are shown in Table 5.

The average daily intake of fats among all groups was 64,06 ±18,1 g, which represents 27,34% of total daily energy intake. However, when observed separately, the daily intake of fats in students who ate with their families was lower than in the other two groups (by 10 g and 20 g, respectively). The highest intake of fat was recorded in the group that ate at the student dining facility (73,13 ±17,0 g, or 29,99% of total daily energy intake). Similar trends were observed in daily protein intake as well as in total energy intake. Quite the opposite trend was observed for daily carbohydrate intake: the highest intake was recorded in the group that ate with their families (311,27 ±43,7 g, i.e., 62,40%), while the lowest intake was recorded in the group that ate at the student dining facility (291,77 ±30,2 g, or 52,75%). Students who ate at the student dining facility ate an average of 2206,97±277,8 kCal, which is almost 215 kCal higher than the group that ate with their families.

According to the ANOVA results, the differences between groups are statistically significant regarding fat intake (F=10,3421; p=0,000085), protein intake (F=42,48007; p=0,000000), and total energy intake (F=6,327237; p=0,002613). Post-hoc analysis, performed using Fisher's least significant difference (LSD) test, determined that students who ate with their families ate significantly less fat than the other two groups (p=0,000017), as well as protein (p=0,00000). However, although the ANOVA did not show significant differences between groups regarding carbohydrate intake, the post-hoc LSD test indicated that intake in the group that ate with their families was significantly higher than that of the other two groups (p=0,028795). Observed differences in the intake of fats, proteins and carbohydrates generated significant differences in total energy intake, with the lowest average energy intake recorded in students who ate with their families.

In addition to nutritive values, we investigated the number of meals and servings of fruit per week, and the number of days in one week when milk and other dairy products, and snacks were consumed. Average values of the investigated parameters are shown in Table 6.

Table 5. Average nutritive values of daily dietary intake in three investigated groups

Group		Nutritive values			
		Fats	Carbohydrates	Proteins	Total energy value
I	g	54,73±17,9	311,27±43,7	63,25±19,8	
	%	24,91	62,40	12,70	
	kCal	509,56±166,5	1276,21±179	259,33±81,3	1990,67±297,0
II	g	64,98±14,7	306,46±31,6	92,59±13,3	
	%	26,97	56,06	16,94	
	kCal	604,31±136,4	1256,49±129,7	379,62±54,6	2181,06±249,9
III	g	73,13±17	291,77±30,2	95,42±13,3	
	%	29,99	52,75	17,25	
	kCal	680,11±158,3	1196,26±124	391,22±55,3	2206,97±277,8
Total	g	64,06±18,1	303,44±36,5	83,23±21,6	
	%	27,34	57,06	15,65	
	kCal	595,20±168,4	1244,10±149,7	341,24±88,7	2122,71±288,3

Table 6. Average values of parameters

Groups	Meals (number per week)	Fruit servings (number per week)	Milk and dairy (number of days per week)	Snacks (number of days per week)
I	19	4	3	3
II	20	6	4	2
III	21	6	4	3
Total	20	5	4	2

The Kruskal-Wallis test indicates that the observed differences in number of meals are statistically significant (H=42,076; p=0,0000), as well as in number of fruit servings, and the number of days per week that milk and dairy products and snacks are consumed, (H=26,94012; p=0,0000, H=9,733827; p=0,0077, and H=6,514935; p=0,0385, respectively). Students who ate with their families consumed significantly fewer meals, fewer servings of fruit, and fewer milk and dairy products than the other two groups.

To investigate the relationship between percentage of body fat and intake of particular foods, Spearman's correlation was tested. The results are shown in Table 7.

The results of the correlation analysis indicate that percentage of body fat significantly correlates to fat intake and consumption of fruits and snacks. Higher fat intake leads to a significant increase in body fat percentage. Greater snack consumption is also directly related to an increase in body fat, and the relationship is even stronger than that with fat consumption. Fruit intake, however, is inversely related to body fat—the more fruit consumed, the lower the percentage of body fat.

DISCUSSION

The results obtained in our study revealed the level of knowledge about nutrition, nutritive status, and nutritional habits in a population of female nursing students.

Regarding their age, the investigated group was homogenous, with the majority (93%) of students aged 19-25 years. The majority of subjects (91%) showed a favourable amount of knowledge about nutrition. The best nutrition questionnaire results were achieved in the categorization of food as well as in recognizing the diseases related to inadequate nutrition. Slightly less knowledge was observed regarding experts' recommendations, and the worst results were achieved in the identification of the proper combinations of foods. These results were expected, considering the type of school the students attend and their previous medical education.

An analysis of the anthropometric parameters indicates that 83% of the students had normal BMI values, while 11% were overweight, 1% was obese, and 5% were underweight. These results are in accordance with the survey conducted by Simic, et al. (22). They found that population of female students in the Faculty of Sports Education had an average BMI value of 21,26 kg/m^2, which is similar to our results (21,80 kg/m^2). Like our subjects, those students were mostly of normal weight (84,85%), with 9,09% overweight, and 6% underweight. Those students were physically active and also had good knowledge regarding nutrition. A study by Lovery, et al. showed that students with normal weight also had better nutrition and achieved better results in tests investigating knowledge and opinions regarding nutrition (23). In a survey conducted at the University of Novi Sad (24), 78% of female students were of normal weight, which is less than in our investigation. The percentage of overweight and obese students was lower (7,55%), while the percentage of underweight was two-fold higher (12,42%) than in our investigations. Considering the relatively similar population investigated in both studies (student status, same age, and similar socio-economic factors), the observed differences may originate from the methodological limitations of our study.

The average nutrition status varies depending on race, ethnicity and culture. Female students in Thailand (25) have an average BMI of 19,8 kg/m^2, which is lower than in our study. However, according to our results, the nutritional status, presented as BMI, does not depend on the type of eating location or arrangement. However, body fat percentage showed much more variability and instability. The average value of body fat percentage of all investigated students was 27,09%, which is slightly above the upper normal range (15-25%). The group with the highest percentage of body fat was the group that ate at the student dining facility, with an average value was 29,04%. The only obese student belonged to this group, and her body fat percentage was 49,09%. These results are in accordance with the distribution of obesity according to BMI.

Table 7. Spearman's correlation analysis

Pair of variables	Spearman Rank Order Correlation			
	Valid N	Spearman R	T(N-2)	P
Body fat (%) / Fruit servings per week	100	-0,280779	-2,89608	0,00466
Body fat (%) / Days when snacks are consumed	100	0,685910	9,33117	0,00000
Body fat (%) / Fat intake	100	0,539575	6,34432	0,00000

The nutritive and energetic characteristics of the students' 7-day food intake results were calculated on the basis of the Nutrition and food intake plan (20) and the nutritional requirements for the participating population of students. Nutritive value was estimated based on reported content of fats, carbohydrates, proteins and total energetic value in the daily food intake. The average daily intake of fats among all students was 64 g, which is 27,34% of total energy intake. This value is within the recommended range (10-30%). However, when analysed separately for each group, the average daily intake of fats showed great differences—24,91% in the group that ate with their families, 26,97% in group that ate on their own, and 29,99% in group that ate in the student dining facility.

In students who ate at the student dining facility, we observed the greatest intake of fats (73,33 ±17 g), and the greatest intake of proteins: 95,42 ±13,5 g, i.e., 17,25%, which is more than the recommended percentage of 10-15%. The greatest average value of daily total energy intake was recorded in this group too—2206,97 kCal. The intake of fats in the first group was statistically significantly lower than that of other two groups, which ate similar amounts of fats. The daily menu of students who ate with their families contained 10 g less fat than that of the group that ate by themselves and 20 g less fat than that of the group that ate at the student dining facility.

The average intake of proteins was also lowest in students who ate with their families, with a statistically significant difference compared to the other groups ($p<0,05$). Protein intake was similar in the other two groups.

We also observed a statistically significant difference in carbohydrate intake between groups. Post-hoc analysis revealed that the difference occurred between students who ate with their families (where we recorded highest carbohydrate intake) and the group that ate at the student dining facility ($p<0,05$).

The observed differences in the intake of fats, proteins, and carbohydrates between the investigated groups lead to differences in total energy intake. The total energy intake in students who ate with their families was significantly lower than that of the other groups ($p<0,05$).

Considering the fact that all overweight and obese students were recorded in the groups that ate on their own and at the student dining facility, we may hypothesize that the nutritional style of the investigated population can be assessed by the anthropometric parameters of nutritional status—body weight, BMI, and percentage of body fat.

These results are expected, considering their busy daily schedules with lectures and practical work all day, as well as the lack of time and finances needed for organized physical activity. Leaving the parental home and adjusting to a new environment and obligations may impose substantial stress, which may reflect on eating habits, leading to an increase in body weight (11,12,13). This effect is in accordance with our results given that we recorded overweight and obese students only in groups that lived apart from their families, i.e., came from other towns.

An increase in obesity among the student population has been recorded in numerous investigations all around the world, indicating that the biggest increase is occurring between 18-29 years, during the transition from adolescence to adulthood, which coincides with the attendance of college and university (9,10).

However, the fact that students who ate with their families were not overweight/obese does not mean that they had better nutrition. For example, they tended to eat foods poor in proteins and rich in carbohydrates. Data obtained from their nutritional diaries indicate that they usually ate products from bakeries for breakfast and often for lunch as well. Together with a high intake of snacks and a low intake of fresh fruits, these habits lead to the sarcopenic type of obesity in which the percentage of body fat is increased without an increase in BMI, which is actually present in all three investigated groups of students.

Our results confirmed that insufficient knowledge, insufficient intake of foods with high nutritive value, increased energy intake, stress, and a lack of physical activity, among other factors, may be related to obesity in the student population, which is in accordance with other surveys (11,12,13).

CONCLUSION

Despite the relatively low prevalence of overweight, obesity, and underweight in the investigated population, our results indicate that students may benefit from health promotion activities directed at decreasing the incidence of obesity, as well as increasing the knowledge of and improve eating habits. During the last decade, the Republic of Serbia has been exposed to social and cultural changes related to economic transitions, which has inevitably led to major changes in nutrition, specifically the eating styles present in Western Europe. The consequences of such a nutritional transition are poor knowledge regarding nutrition and making poor food choices, leading to overweight/obesity, which was observed in this study.

Methodological limitations: Limitations in the interpretations of our results are derived from the relatively small number of subjects and the fact that all of the subjects were female.

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ACUTE URINARY TRACT OBSTRUCTION

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ABSTRACT

The kidneys are paired organs with the primary function of helping to remove toxins from the body and regulate water balance. They are vital to survival. After urine is produced in the kidneys, it must pass into the bladder, where it can be stored before being eliminated from the body through the urethra. Urinary tract obstruction is a common problem encountered by urologists, primary care physicians, and emergency medicine physicians. Urine can become obstructed at any point in this pathway. There are three groups of urinary tract obstructions: 1) obstruction of the urinary tract lumen; 2) obstruction of the urinary tract wall; and 3) extrinsic obstruction, which can press on the urinary tract lumen. An obstruction can be present from birth or develop later in life. The most common causes of obstruction include stones, strictures, tumours, and bladder dysfunction. These obstructions may result in the hydronephrosis of one or both kidneys, which, if left untreated, may lead to the deterioration of renal function. The goal of an initial treatment of urinary tract obstruction is to remove the obstruction. Later, we treat the cause that led to the obstruction. The bottom line is that all efforts should be made to preserve kidney function to avoid the need for dialysis or renal transplantation.

Keywords: *urinary tract obstruction, obstructive uropathy, urethral catheter, suprapubic catheter, ureteral stent.*

SAŽETAK

Bubrezi su parni organi, neophodni za život jer pomažu u eliminaciji toksičnih materija iz organizma i učestvuju u regulaciji vodno elektrolitnog balansa. Urin koji se stvara u bubrezima transportuje se ureterima do mokraćne bešike u kojoj se privremeno zadržava do akta mokrenja. Opstrukcije urotrakta su čest problem sa kojim se sreću urolozi, lekari opšte prakse kao i oni koji rade u urgentnoj službi. Prepreka može postojati na bilo kom delu urinarnog trakta. Obstrukcije urinarnog trakta se mogu podeliti u 3 grupe 1) obstrukcije unutar samog lumena 2) obstrukcije na zidu urinarnog trakta 3) obstrukcije nastale zbog spoljašnje kompresije urotrakta. Najčešći uzroci obstrukcija su: kalkulusi, suženja, tumori I disfunkcije mokraćne bešike. Obstrukcije dovode do formiranja hidronefroze na jednom ili oba bubrega i ukoliko se ne leče izazivaju oštećenje renalne funkcije. Cilj lečenja obstrukcije urotrakta u hitnoj ambulantnoj službi je dezopstrukcija. Kasnije se pristupa lečenju osnovne bolesti koja je dovela do obstrukcije. Sve napore treba usmeriti ka očuvanju bubrežne funkcije kako bi se izbegla eventualna dijaliza i transplantacija.

Ključne reči: *obstrukcija urinarnog trakta, obstruktivna uropatija, uretralni kateter, suprapubični kateter, ureteralni stent.*

ABBREVIATIONS

BPH- Benign prostatic hyperplasia; **MET** -medical expulsion therapy; **UPJ**- ureteropyelic junction

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Acute urinary tract obstructions are urgent conditions in urology. It is essential to recognize them quickly. This is a significant task for all medical doctors, including general practitioners or those engaged in a highly specialized medical branch (1). When patients with an obstruction reach a urologist, it can often be too late to adequately help them. Obstruction removal does not mean that a complete recovery of urinary tract function will be accomplished. Functional urinary tract recovery largely depends on the obstruction duration. Thus, a timely discovery is essential for a good final outcome, which especially emphasizes the significance of diagnosis and increases the responsibility of all medical doctors. Some of these conditions can be easily resolved in any general surgical practice. More complicated patients, as well as ones with differential diagnostic uncertainties, should be referred to a urologist.

The purpose of the urinary tract is to conduct urine from the kidneys to the urinary bladder, where it is temporarily stored and then excreted from the body. An obstacle can occur anywhere, from the renal calyces to the external urinary meatus (2).

Urinary tract obstructions can be divided into three major groups: obstructions in the urinary tract lumen, obstructions arising due to changes on the urinary tract wall and extrinsic obstructions that compress the urinary tract (3). The most frequent causes are listed in Table 1.

The urinary tract lumen is most frequently obstructed by a stone. Calculus disease can occur in all age groups. However, it is most common in middle age, and men are three times more affected. An obstruction should be considered in all patients with neurological disorders (diseases and injuries). Obstructions in elderly men are mostly caused by benign prostatic hyperplasia (BPH). Obstruction in women most commonly occur due to genitourinary prolapse, pregnancy, malignant diseases of the gynaecological region, and radiation for a malignancy. Obstructions should considered after all small pelvis surgeries, especially gynaecological ones (4).

Urethral obstruction caused by the prostate is due to changes in the urinary bladder wall. First, the fibres of the detrusor hypertrophy to make stronger contractions to overcome the pressure of the prostate on the initial part of the urethra. Hypertrophic trabeculae as well as cells and diverticula can be observed. The contractile capacity of the urinary bladder detrusor gradually decreases, leading to an increasing amount of residual urine (5).

Consequently, the intraluminal pressure in the bladder increases and transfers to the ureters and kidneys, leading to damage. The damage is caused by the direct compression of the dilated pyelocaliceal system on the renal parenchyma, as well as the compression on the arcuate arteries, which leads to the ischemic changes.

A normal renal pelvic pressure is 6 to 12 mmHg. In an acute obstruction (most commonly caused by a stone), strong contractions of the ureter occur and cause a sudden rise in pressure from 50 to 70 mmHg. Because urine is still excreted, the pressure can increase to 100 mmHg, which is 10 to 15 times higher than normal (6). The pressure leads to the distension of the wall of the ureter and renal capsule, which is rich in nerve endings. This can explain the extremely strong pain these

Table 1. Causes of urinary obstruction

CAUSES OF URINARY OBSTRUCTION	
Site of obstruction	***Possible causes***
Within the lumen	• Stones • Blood clot • Necrotic renal papillae • Tumour of renal pelvis or ureter
Within the wall	• Pinhole meatus (urethral meatus stenosis) • Congenital urethral valves • Ureteric, urethral or ureterovesicular stricture • Bladder neck stenosis • Neurogenic bladder (e.g., after a spinal trauma, multiple sclerosis, surgery in the pelvis, etc.) • Congenital megaureter • Ureteral endometriosis
Pressure from outside tract	• Phimosis • Posterior urethral injuries • Tumours and BPH • Surgery in the pelvis • Pancreatitis, appendicitis, diverticulitis • Crohn's Disease • Tumours of the uterus and ovaries, adnexal abscesses • Pregnancy • Retroperitoneal fibrosis • Retrocaval ureter • Chronic granulomatous disease • Effects of radiation • UPJ obstructed by aberrant blood vessels

patients feel. On a pain scale, pain due to renal colic is higher than any other pain. This pain is often much stronger than labour pain.

In the acute phase of the renal colic, there is the reduction of blood flow through the kidneys and glomerular filtration system. Thus, only a small quantity of new urine is produced. Therefore, a significant dilatation of the pyelocaliceal system in the initial phases of the renal colic cannot be observed. If the obstruction is not removed, the pressure in the pyelocaliceal system can gradually decrease due to pyelovenous, pyelolymphatic, pyelo-interstitial and pyelotubular reflux. New urine quantities fill and dilate the pyelocaliceal system and ureter, and they can significantly expand if the obstruction is long lasting.

The first anatomical renal changes occur after 7 days in the distal renal tubule. The first signs of the atrophy, which is not completely reversible, then occur. The papillae are flattened, and the distal tubule is expanded. After 14 days, the obstruction causes the same changes in the proximal tubule. After a month, these changes occur in the glomerulus. The medical literature suggests that there are cases of renal function recovery even a couple of months after a complete obstruction. However, this is rare, and we must rely on the fact that, due to the above-mentioned reasons, a complete obstruction must be resolved in the first seven days.

Renal colic often occurs after the intake of the large quantity of liquids or diuretics, strenuous physical activity and alcohol abuse. All of these conditions lead to stone movement.

Renal colic

Patients complain of a sudden, strong pain in the lumbar region that often expands to the iliac fossa and inguinal region (7). In men, it often spreads to the testicles and, in women, to the labia majora. It is mostly accompanied by nausea and vomiting. If the stone is near the urinary bladder, distinct dysuric problems usually occur. After the pain, macroscopic haematuria can occur. These patients are very agitated and cannot find an antalgic position; they have a need to move. Renal colic can be accompanied by signs of urinary infection and septicaemia.

Urine retention

Patients complain of impossible spontaneous micturition or urine flow in drops. Data on unintentional urine leakage may be misleading because a patient can then have so-called urine suffusion from the urinary bladder, which is overfull. Suprapubic pain is extremely strong and can sometimes present as peritoneal irritation and acute abdomen pain. Patients with acute retention and ones with neuropathy do not necessarily have a painful syndrome. Data on previous abundant haematuria and the occurrence of blood clots in the urine indicate a tamponade of the bladder by clotted blood.

Table 2. Diagnosis of acute urinary obstruction

DIAGNOSIS OF ACUTE URINARY OBSTRUCTION
ANAMNESIS
CLINICAL REVIEW
• Inspection • Palpation
LABORATORY ANALYSIS
• Urine sediment: Le, Er, nitrites, Le esterase crystals • Complete blood count • Biochemistry: urea, creatinine, Na, K, glucose
"IMAGING" METHODS
• Ultrasonography • CT scan of the abdomen and pelvis • IVU, retrograde and antegrade pyelography • MRI, scintigraphy

Diagnosis

Proper anamnesis is very significant in the diagnosis of an obstruction. Attention should be paid to the time of symptom onset and the symptom intensity, frequency and propagation. Information on a high body temperature and bleeding indicates the need for urgent diagnostic procedures. Acute inflammation of the ovaries, ectopic pregnancy, appendicitis, diverticulitis and biliary colic can sometimes provide similar symptoms and must considered during a differential diagnosis.

A physical examination is necessary in patients with a suspected urinary obstruction. An overfull bladder (globus vesicalis) can be seen and palpated. In young men with suspected renal colic, the testicles must be examined because testicular inflammation and, in particular, torsion, can cause very similar initial symptoms. At the beginning of the embryonic development, the testicles and kidneys are very near one another. They later separate and assume their normal positions. Therefore, their regions of innervation are very close. Thus, at the beginning, it is not always absolutely clear which organ is the real cause of the pain. Sometimes, an obstruction can be caused by a neglected phimosis or penile oedema in cardiac decompensation patients.

A laboratory diagnosis involves an analysis of urine sediment, a complete blood count (CBC) and a basic biochemical analysis (urea, creatinine, Na, K, glycaemia). An increased number of leukocytes in the urine, positive leukocyte esterase and the presence of nitrites imply a urinary infection. If, in addition to that, the number of leukocytes is also increased in the blood and granulocyte-dominated, the urinary infection most likely affected the organs of the upper urinary tract (ureters and kidneys). An increased number of erythrocytes in the urine occurs in renal colic, bleeding of the tumours of urinary tract organs and urinary infections. Anaemia indicates a long-lasting micro- or abundant macro-haematuria. Increased

Table 3. Indications for emergency relief of obstruction

INDICATIONS FOR EMERGENCY RELIEF OF OBSTRUCTION
• Complete urinary tract obstruction • Any type of obstruction in a solitary kidney • Obstruction with fever and/or infection • Renal failure • Any suspicion of neurological dysfunction* • Uncontrolled pain* • Nausea and vomiting sufficient to cause dehydration* *associated with urinary tract obstruction

Table 4. Placement of foley catheter

PLACEMENT OF FOLEY CATHETER
• Usually a simple procedure • Respecting the measures of asepsis • Adequate lubrication of the urethra • Contraindicated in suspicion of the pelvic ring fracture • **Not to discharge all the urine rapidly because of the possible bleeding as a result of decompression**

levels of urea and creatinine occur in the early stages of the obstruction of a solitary or simultaneous obstruction of both kidneys. (1) If only one kidney is affected, the urea and creatinine do not significantly change in the early stages of the obstruction.

Ultrasound is a simple, fast and cheap orientation method by which to evaluate the condition of the urogenital tract organs.(1) It is the method of choice in diagnosing urinary obstructions in pregnant women and young children.(1) All radiologists, and most urologists and other medical doctors with an elementary knowledge of ultrasound diagnostics, can easily assess if there is urine retention in the bladder or a dilatation of the pyelocaliceal system on kidneys. This is adequate for an initial diagnosis during the urgent admission. Unlike in this country, where ultrasound is the most frequently used method, in wealthier and more technologically developed countries in the world, a scan of the abdomen and pelvis minor is used as initial diagnosis procedure. In this way, apart from the urinary tract, the condition of its surroundings can be seen. During a diagnosis of a urinary obstruction, intravenous urography and antegrade and retrograde pyelography are very useful. However, they are mostly used to further determine any uncertainties of the initial diagnosis (8). Scintigraphy and magnetic resonance are much less frequently used and are mostly used in developed countries (9).

Acute urinary obstruction may be treated with medications or surgery.

Treatment with medication can be used if there is a partial obstruction and if there are no signs of a urinary tract infection. For the prophylaxis of urinary tract infections, broad-spectrum antibiotics, such as sulfamethoxazole-trimethoprim, nitrofurantoin, cephalosporins and fluoroquinolones, can be used in their usual therapeutic doses. Prophylaxis is essential in diabetics, patients with renal insufficiency and obstructions in a solitary kidney.

The medications of choice for the treatment of renal colic are non-steroidal anti-inflammatories, including 75 mg of diclofenac sodium intramuscularly or a suppository of 100 mg. If necessary, administration can be repeated after 30 minutes. In addition to being analgesic, this group of medications has anti-inflammatory and anti-oedematous effects, which help in stone elimination. Indomethacin, ibuprofen (Brufen), hydromorphine hydrochloride, metamizole (Novalgetol, Baralgin), pentazocine (Fortral) and tramadol (Trodon) can also be used.

Medical expulsion therapy (MET) is the administration of alpha-adrenergic alpha blockers, which leads to the relaxation of the musculature of the distal parts of the ureter and assists in the elimination of pelvic calculi. In both men and women, the most frequently administered medication is tamsulosin (Tamsol, Flosin, Omnic), at a dosage of 0.4 mg per day (10).

Patients with the acute renal colic should reduce liquid intake during the pain phase, eliminate anticholinergics and move as much as possible.

Surgical treatment

Acute urinary retention is most frequently resolved by the placement of a Foley catheter (Table 4). This is usually a simple procedure which can be performed by any medical doctor or trained nurse and a medical technician, using aseptic techniques. Before the catheter placement, mandatory lubrication of the urethra is mandatory to prevent microtrauma and the creation of a later stenosis. If the catheter cannot be placed through the urethra, a Cystofix cystostomy is performed by a urologist. Upon the placement of the catheter or Cystofix, care should be taken not rapidly to discharge all of the urine because of the possibility of bleeding as a result of decompression.

If there is a suspicion of a pelvic ring fracture and an observation of signs of a urethrorrhagia, the placement of the catheter is contraindicated. The placement of a Cystofix should be immediately performed (11).

If the cause of the acute retention is urinary phimosis, a dorsal incision or circumcision is performed.

A tamponade of the urinary bladder by blood clots must be cystoscopically resolved using a steel pump. It is important to extract all the clots because only then will the bleeding stop. In some cases, electrocauterization of the bleeding needs to be performed. In rare cases, a complete cystectomy ("salvage" cystectomy) should be performed.

An obstruction of the upper urinary tract is resolved by ureter probing in a retrograde manner by ureteral or double-J stents or in an antegrade manner by a percutaneous nephrostomy (12). Desobstruction is a primary priority. If possible, a ureterorenoscopy should be performed

with a possible lithotripsy, which will definitely resolve the obstruction cause.

The conditions that require urgent desobstruction are described in the Table 2.

Patient monitoring

After desobstruction, patients can enter a period of polyuria characterized by diuresis of more than 200 ml per hour during a minimum of 2 hours. These patients can have serious electrolyte imbalances. Therefore, they must be monitored and their intravenous infusions adjusted.

Acute urinary obstruction complications are caused by mainly urinary infections: cystitis, pyelonephritis, renal abscess and urosepsis. In rare cases, extravasation of urine with the formation of a urinoma and urinary fistula can occur. Long-lasting obstructions lead to renal insufficiency.

The outcome and prognosis of an obstruction depends on the following: the cause, location, grade and duration, as well as the presence of a urinary tract infection.

If the obstruction is removed quickly, the infection can resolve in a timely manner. If there is no renal function impairment, the prognosis and the outcome are favourable.

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MINI EXTERNAL FIXATOR ASSISTED METACARPAL LENGTHENING WITH THE DISTRACTION METHOD

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MALI SPOLJNI FIKSATOR KAO POMOĆNO SREDSTVO PRI METAKARPALNOM PRODUŽENJU DISTRAKCIONOM METODOM

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ABSTRACT

Phalangeal brachydactyly, which is caused by the abnormal development of the metacarpal, is characterized by shortness that can be seen in a single finger or in all fingers of the hands. Although brachydactyly is usually thought of as congenital, it can occur due to metabolic disorders or trauma. A twelve-year-old girl was admitted to our clinic with complaints of shortness in the fourth finger of the left hand. Osteotomy was applied with the drilling-osteotomy technique. The screws holding the upper and lower segments were adapted to the external fixator. Lengthening began one week after the osteotomy. The lengthening rate was organized as 0.25x2 mm/day. The amount of elongation was planned not to exceed 40% of the original bone length. Distraction was terminated after the planned elongation amount was reached, and the bone was allowed to heal. In patients under twenty years of age, the results from progressive distraction without bone grafting are close to perfect. Although the technique of successfully lengthening metacarpal fractures in children is simple, strict rules should not be ignored. Primarily, the external fixator and the distraction system should be sufficiently stable, lightweight, and should be appropriate for the size of a child's hand.

Keywords: *brachydactyly, osteotomy, elongation*

SAŽETAK

Falangealna brahidaktilija, koja nastaje usled abnormalnog razvoja metakarpalnih kostiju, se karakteriše skraćenjem koje može da se javi na pojedinačnom ili svim prstima. Iako je brahidaktilija obično kongenitalnog porekla, može da bude posledica metaboličkih poremećaja i trauma. Dvanaestogodišnja devojčica je primljena na kliniku žaleći se na skraćenje četvrtog prsta leve ruke. Urađena je osteotomija tehnikom bušenja. Šrafovi koji drže gornje i donje segmente su pričvršćeni za spoljni fiksator. Sa produženjem je započeto četiri nedelje nakon osteotomije. Stepen produženja je iznosio 0.25x2 mm/dan. Produženje ne bi trebalo da pređe 40% od početne dužine kosti. Distrakcija je završena nakon produženja i kost je ostavljena da se zaleči. Ukoliko se primeni pre dvadesete godine života distrakcija (bez zamene kostiju) daje gotovo savršene rezultate. Mada je ova tehnika jednostavna, striktna pravila ne bi trebalo da se ignorišu. Pre svega, spoljni fiksator i distrakcioni sistem bi trebalo da budu dovoljno stabilni, laki i veličine koja je prilagođena dečijoj ruci.

Ključne reči: *brahidaktilija, osteotomija, produženje*

INTRODUCTION

Phalangeal brachydactyly, which is caused by the abnormal development of the metacarpal, is characterized by shortness that can be seen in a single finger or in all fingers of the hands. Although brachydactyly is usually seen as congenital, it can also be due to metabolic disorders or trauma (1,2).

The exact reason for congenital shortening of the metacarpals is unknown; the deformity is generally thought to be caused by premature closure of the epiphyseal plate.

The incidence of congenital metacarpal shortening alone is approximately 0.02% to 0.05% (3). The metacarpal extension technique was first reported as a case report by Mansoor (4). In our case, we applied the callus distraction method with a mini-Orthofix type fixator for lengthening.

CASE REPORT

A twelve-year-old girl was admitted to our clinic with complaints of shortness in the fourth finger of left hand in September 2011 (Figure 1). We evaluated our patient for brachydactyly type E. She had an isolated feature and had

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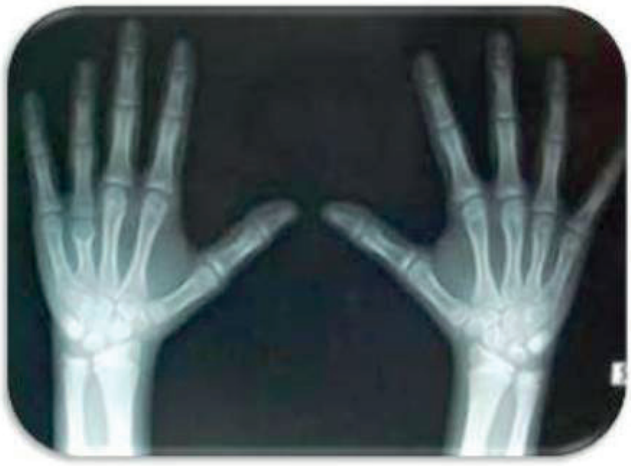

Figure 1. Short fourth metacarpal of the left hand via X-ray

no complex syndromes. The patient complained of poor cosmetic appearance and fatigue during work using her left hand. The fourth finger of the left hand was 1.4 cm shorter than the fourth finger of the right hand upon examination. There was no limitation of motion in the short finger.

An upper extremity tourniquet was applied and the osteotomy site was exposed through a one cm dorsal longitudinal incision on the dorsum of the short metacarpal under general anaesthesia. After extensor tendon exploration, the periosteum was stripped without damage and osteotomy was applied with the drilling-osteotomy technique. We used a standard dorsopalmar radiographic projection of the hand; the X-ray beam was inclined 15° from the vertical at a distance of 100 cm. After confirming by fluoroscopy that the osteotomy was complete, two pieces of 3 mm self-tapping screws were placed to the proximal and the distal segment of metacarpal along the long axis and placed on the dorsal surface; they were then adapted to the main fixator by clamps. We applied the mini-Orthofix type fixator for lengthening. Fluoroscopy was used to confirm the position of the screws and fixator. A bone graft was not used (Figure 2,3).

Lengthening began one week after the osteotomy. The amount of lengthening was determined according to the formula specified by Aydınlıoğlu (5). The lengthening rate was organized as 0.25x2 mm/day. The amount of elongation was planned not to exceed 40% of the original bone length. The time for healing and consolidation was determined based on postoperative radiographs taken once a week for 4 weeks. Sufficient length was achieved using 0.5 mm/ day elongation in four weeks. Lengthening reached 1.4 cm. The degree of lengthening ranged from 100% of the right hand fourth metacarpal length. Active finger movements began in the early period of post-operative movement. The distraction procedure was taught to family and every week the family was called for control of effective growth and to control the fixator. Pin-care with antiseptics was recommended. The parents were taught to care and dress the pin tract. Distraction was terminated after the planned elongation amount was reached and the bone was allowed to heal. The consolidation time was the time between the end of distraction osteotomy and the total consolidation or removal of the fixator. After a one month waiting period for bone consolidation, the external fixator was removed in the outpatient clinic after maturation of

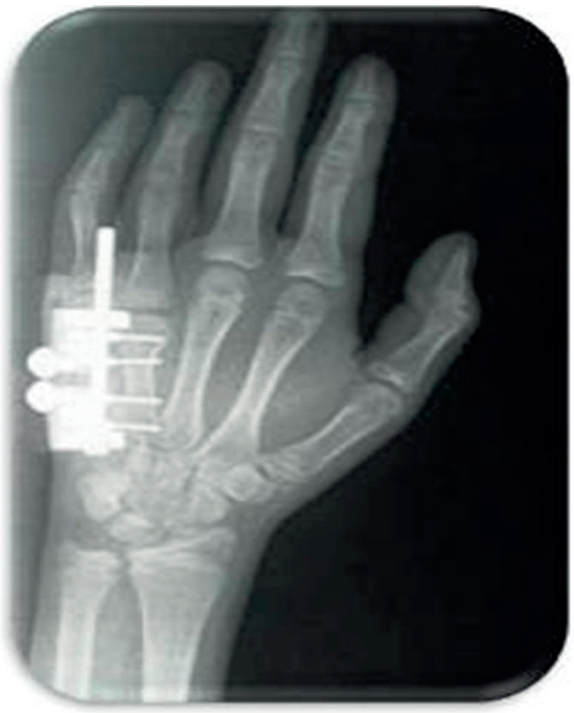

Figure 2. Post-operative X-ray

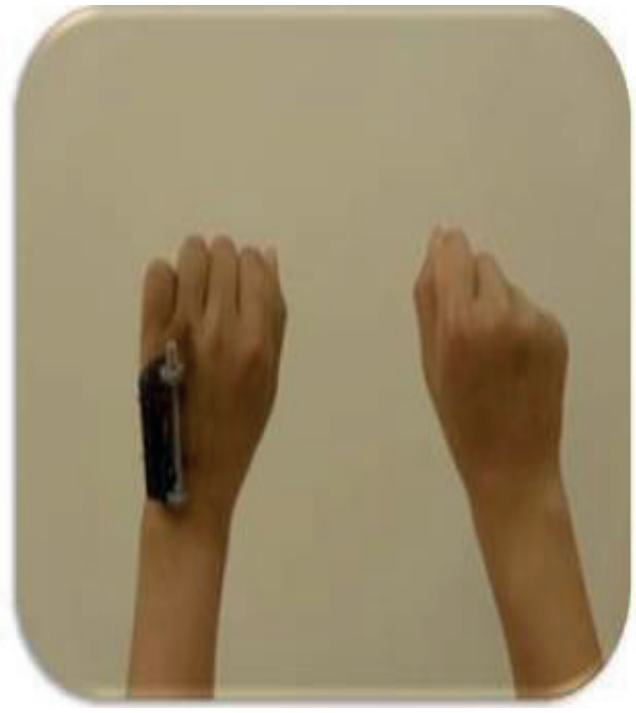

Figure 3. Post-operative picture

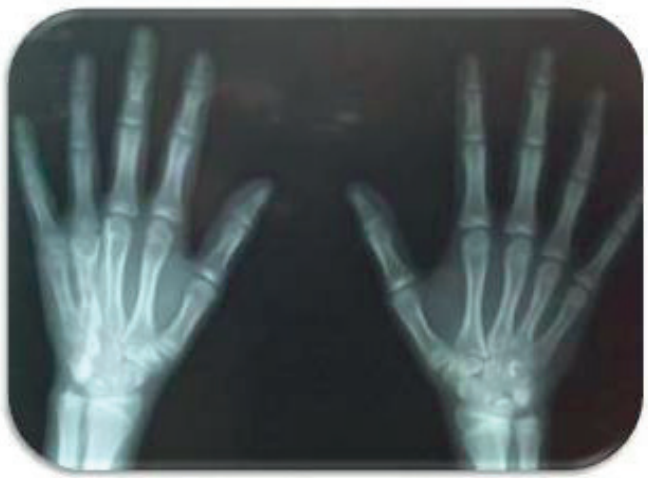

Figure 4. Fixator extracted X-ray

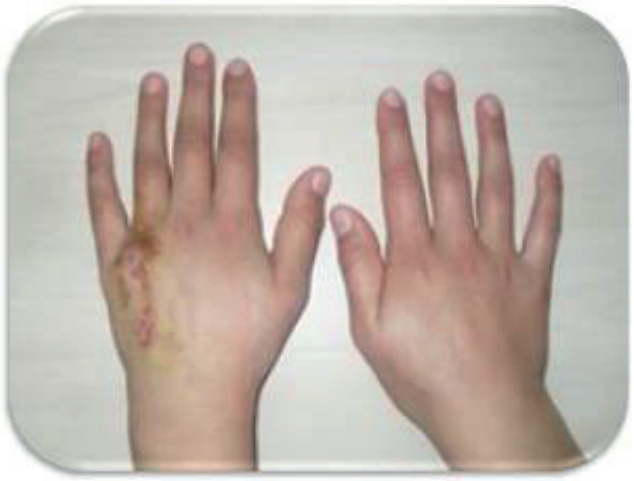

Figure 5. Fixator extracted picture

the callus. Any infection during treatment, delayed union or non-union were not found (Figure 4,5).

Considering the functional results, the patient was satisfied with the cosmetic appearance after surgery. The metacarpophalangeal range of motion was measured using a goniometer, preoperatively and 6 months postoperatively. There was no evidence of metacarpophalangeal joint stiffness. Her Quick Dash Score was eleven.

During the follow-up period, there was no re-fracture, subluxation/dislocation in the metacarpophalangeal joint, degenerative changes or neurovascular injury in the patient.

DISCUSSION

Brachymetacarpia is the congenital shortening of the metacarpals and usually affects the third, fourth, and fifth digits. Though its exact aetiology is unknown, premature closure of the epiphysis is thought to be involved. The condition may occur sporadically or occur as part of a syndrome. Brachydactyly (BD) refers to the shortening of the hands, feet or both. There are different types of BD; among them, type E (BDE) is a rare type that can present as an isolated feature or as part of more complex syndromes such as pseudohypopthyroidism, hypertension with BD, and BD with mental retardation. Each syndrome has characteristic patterns of skeletal involvement. However, brachydactyly is not a constant feature and shows a high degree of phenotypic variability. In addition, there are other syndromes that can be misdiagnosed as brachydactyly type E. Variable shortening of the metacarpals with more or less normal length of phalanges occurs in BDE. Occasionally, the metatarsals are also short. This results from hypoplastic and partially fused metacarpal epiphyses, which are visible on radiographs.

Various osteotomy and intercalary bone grafting techniques are indicated in the treatment of broken short metacarpals. It is hard to provide sufficient lengthening with these techniques, and acute lengthening increases the risk of neurovascular damage (6). The callotasis method, also known as distraction osteogenesis in metacarpal and metatarsal lengthening, is now more preferred and used by many surgeons (3,7,8,9,10,11,12,13). A gap of approximately 3 cm can be achieved within 2 to 3 months without the need for a bone graft in patients aged between 10 and 14 years. Whereas a bone graft is required in approximately 50% of patients aged between 26 and 30 years, it is still generally advised for persons older than 25 years who require metacarpal lengthening of more than 3 cm (14-15). Similarly, a bone graft is recommended in patients who need lengthening of more than 20 mm (16). We achieved a mean lengthening of 14 mm without the use of a bone graft. Limited corticotomy has been advocated when there is a compromised endosteal blood supply, but endosteal blood supply and bone marrow have no effect on consolidation and healing. The proximal metaphysis is the site with the best blood supply, and this reduces the time needed for healing and consolidation (17). The significant advantages of this method can be stated as follows: obtains further lengthening, does not require a bone graft, absence of donor site morbidity, multiple bone implementation, allows early loadings, has fewer neurovascular complications, has reduced loss of joint motion because of the better adaptation of the soft tissues to gradual lengthening and the lack of plaster (3,7,9,10,17,18). However, some disadvantages must be noted: longer consolidation time, frequent occurrence of pin tract infections, significant loss of motion and stiffness of the MP joint, MP joint subluxation/dislocation, angulation defects and delayed union or pseudoarthrosis development (3,10,19,20). In a study comparing the two methods, the functional outcome and length were not very significant, but it was clear that the consolidation time of the callotasis method was longer (10). Determining which technique should be preferred depends on the surgeon's experience. Distraction osteogenesis is an alternative method that can be used in patients who desire great lengthening with a reduced risk of complications. In patients under twenty years of age, the progressive distraction results without bone grafting are close to perfect. Although the technique of successfully lengthening metacarpal fractures in children is simple, strict rules should not be

ignored. Primarily, the external fixator and the distraction system should be sufficiently stable, lightweight, and appropriate for the size of a child's hand.

RESULTS

Bone remodelling is an excellent technique for children. For the treatment of childhood brachydactyly, one session of the metacarpal lengthening technique with mini-Orthofix type external fixator distraction osteogenesis is a good alternative to complex and technically demanding procedures. Metacarpal lengthening by callus distraction is an appropriate method that achieves adequate lengthening. Healing is faster in younger patients and, if tolerated, surgery can be performed in early childhood. It can also be performed satisfactorily in adults following traumatic amputations. To avoid additional lengthening of normal metacarpals prior to epiphyseal closure, adolescence is the most appropriate time to perform distraction lengthening in congenitally short metacarpals. Careful preoperative planning, secure intraoperative mounting of the external fixator, and sufficient postoperative patient and parental cooperation are essential to avoid complications.

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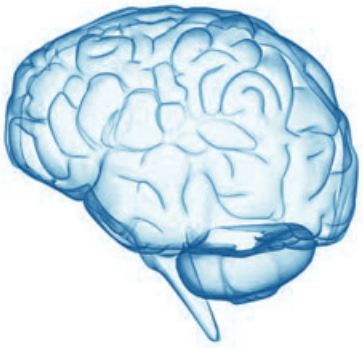

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