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POPULATION AGING AS A GLOBAL DEMOGRAPHIC PHENOMENON AND ITS IMPLICATIONS FOR PUBLIC HEALTH

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ABSTRACT

Population aging is defined as one of the most current phenomena and challenges facing world. The WHO defines population aging as one of greatest successes of human society, resulting from reduction of mortality among elderly and their longer life expectancy in the protection of population, pharmacology, public health and promotion of healthy lifestyles. The certainty of the continuity of population aging in future continues to capture attention of the general public and initiates a whole series of open questions that will require very specific and concrete answers from each country. The aim of this paper is to review available literature to indicate the specific determinants of the elderly population, identify the challenges faced by the elderly population and indicate implications of the phenomenon of population aging. The unstoppable process of population ageing has profound consequences for health, social and economic spheres of society due to specific and very different needs of elderly population. The role of demographics, social factors, health needs and barriers as economic resources and differences in use of health services among elderly need to be systematically addressed and monitored. A well-organized health and social care system is important for improving the health of older people. Public health policy should address the diversity of health and functional conditions experienced by older people and maximize number of people who promote a positive journey of ageing. The creation and implementation of innovative strategies to improve health and quality of life of older people would enable well-being in old age.

Keywords: *Population aging, implications, public health, ageism.*

INTRODUCTION

Population aging is defined as one of the most current phenomena and challenges facing the world. In the last decades of the last century, it was clear that the developed world expected a large increase in the proportion of elderly people, but with the entry into the 21st century, it became obvious that this is an inevitable and general tendency, which, with the tendency and tendencies it deals with. how real and expected, still represents a major challenge for modern society. The certainty of the continuity of population aging in the future also captures the attention of the general public and initiates a whole series of open questions that will require very specific and concrete (concrete) answers from each country).

The World Health Organization (WHO) defines population aging as one of the greatest successes of human society, resulting from the reduction in mortality of the elderly and their, progress in a longer life expectancy for life. pharmacology, public health and the promotion of healthy lifestyles (2). However, the unstoppable process of population aging has profound consequences for the health, social and economic spheres of society due to the specific and very different needs of the elderly population (3). As they are most often described as health needs, social needs, economic needs and many other needs of the elderly are largely intertwined, connected and essential (4). As a consequence of the biological process of aging, there is a gradual increase in the frequency of patients with chronic diseases (cardiovascular diseases, diabetes, malignant diseases, etc.), which is why it is not unusual for more people to no longer feel well. associated diseases (so-called multimorbidity) (5,6).

With aging, the number of functionally dependent people, people with some form of mental or physical impairment, and the number of cognitively impaired people progressively increases. The extensive health problems of the elderly lead to increasing pressure on the health service and increasing allocations for their health care (5). The elderly are increasingly faced with economic barriers to using health services, as a consequence of the decline in economic power due to retirement and the cessation of the active work cycle. Most elderly people experience a decline in their capacity for independent living due to limited mobility, psychological frailty, or other physical problems. Many require some form of support, assistance, and long-term care, including nursing home care, geriatric home care, home help, community care, and extended hospital stays. This means an increase in the number of dependent elderly people in economic, health and social terms and an increase in the need for reallocation of resources to long-term care (7).

All of the above has a major negative impact on the economic stability of the health system and society as a whole (8). The specificities of the aging model are primarily a consequence of the influence of a number of factors that operate in certain social circumstances and a specific social environment. Demography, a science that, among other things, monitors the dynamics of growth and development of the elderly population at the global level, has very precise and clear numerical criteria (indicators) in its definition: the share of the elderly in the total population, where both nominal age limits (60 and 65 years) are taken into account, together with the average age and the aging index. However, over time, the age limit has been moved from 60 to 65 years in response to accelerated population aging trends and the extension of life and working life in modern society (1).

Demographic Profile of the Elderly

The world's population is aging rapidly. The number of people over 65 years of age more than tripled from 131 million to 417 million between 1950 and 2000, while their share of the total population increased from 5% to 7% (1,12,13). The beginning of the 21st century indicates that demographic aging is accelerating. Of the 7.3 billion people worldwide in 2015, 8.5% of the world's population, or 617 million people, were over 65 years of age. It is predicted that the number of older people will increase by more than 60% in just 15 years - so that by 2030, those over 65 will make up 12% of the total population. The proportion of older people will continue to grow over the next 20 years – by 2050 there will be 1.6 billion older people, representing 17% of the world's total population. Globally, the projected rate of ageing in the future predicts that the number of people aged 65 and over will increase by around 27.1 million per year (11). The increase will be greatest and fastest in developing countries, where the elderly population is expected to quadruple in the next 50 years. It is clear that the world is entering a new demographic period and that the 21st century is the century of population ageing (13).

Regions of the world vary in the stages of demographic transition and also in the rate of ageing. In more developed countries, especially those in Western and Eastern Europe, the demographic transition began more than a century ago and most have taken decades to complete the process. However, Asia and Latin America are rapidly advancing towards the demographic transition and are ageing more rapidly. Less than 8% of Asians were aged 65 or over in 2015, but this regional average masks the huge variations that exist within Asia itself. While about half of Asian countries currently have less than 5% of their population aged, some Asian countries are already among the oldest in the world. East Asia is one of the oldest subregions and includes the oldest major country in the world, Japan (26.6%). The proportion of older people in Asia is expected to reach 12.1% in 2030 and 18.8% in 2050 (11). Comparing continents, it is evident that Africa has the smallest elderly population (3.5%), while Europe is inhabited by the oldest population (17.4%).

Unlike other regions, Africa is still in the early stages of its demographic transition, with high birth rates and a young population structure, particularly in western, central, and some eastern countries. Researchers have identified several possible explanations for the delayed decline in fertility in some parts of Africa, including slow economic growth, insufficient progress in improving women's access to education, and increased mortality due to the HIV epidemic

(14,15). Most African countries now have less than 5% of their population aged 65 years or older, while 21 countries have populations of 3% or less (e.g., Ethiopia, 2.9%, and Uganda, 2%). While Africa is a young continent, some African countries already have large populations of older people. In 2015, the number of older people in the population exceeded one million in 11 countries, including Nigeria (5.6 million), Egypt (4.6 million) and South Africa (3.1 million). By 2050, more than half of African countries are expected to have more than one million older people, including three countries with more than 10 million (Nigeria -18.8 million, Egypt - 18.1 million and Ethiopia - 11.5 million) and another six countries with more than 5 million (11). The percentage of the population aged 65 and over ranges from a high of over 26.6% in Japan to a low of around 1% in Qatar and the United Arab Emirates. Of the 25 oldest countries, 22 are in Europe, with Germany and Italy having held the top spot for several years (16).

Slovenia and Bulgaria are projected to be the oldest European countries in 2050. Japan, however, is currently the oldest country in the world and is expected to retain this position until at least 2050. With the rapid ageing of Asia, South Korea, Hong Kong and Taiwan will join Japan as the oldest countries and regions by 2050, when more than one third of the population in Asia will be16 (more than one third of the population of Asia). It is estimated that China's elderly population will exceed the number of Europeans aged 65 and over by 2025 (1). A special aspect of demographic ageing is the intensive ageing within the older population itself, i.e. the ageing of the elderly, which is characteristic of most countries in the world, regardless of geographical location or economic development (17).

The WHO estimates that the number of "oldest old" will quadruple in the first half of the 21st century, reaching 395 million by 2050, with one in five older people aged 80 years or older (1.18). The oldest old differ from the rest of the older population in many sociodemographic characteristics and are more likely to have multiple chronic conditions requiring long-term care, and may therefore consume public resources disproportionately and place a greater burden on informal care, often provided by families themselves (19,20).

Another important indicator of the degree of aging of the population is the average age of the population, which ranges from 15 to almost 50 years. Demographically, the youngest continent is Africa, with an average age of 19.7 years, while Europe is the oldest continent, with an average age of 41.6 years. For example, Niger, Uganda and Mali have average values of this indicator around 15-16 years, since more than half of the population in these countries is under 18 years of age. At the other end of the spectrum are Japan and Germany with an average age of 47 years. According to projections, the average age in Japan will be 53 years in 2030, and in 2050 the average age will reach 56 years (11).

In most European countries, the share of the elderly population already exceeds 14%. Serbia is among them. In the period between the two censuses, there was no significant change in the share of people under 15 years of age in the total population – 14.3% (2011 Census), compared to 14.4% (2022 Census), but there was a significant decrease in the share of people aged 15–64 – from 68.3% (2011) to 63.5% (2022), i.e. by about 5 percentage points, while the share of people aged 65 and over increased from 17.4% (2011) to 22.1% (2022). The most unfavorable demographic situation is in the Region of South and East Serbia, where almost every fourth person (23.7%) is older than 64 years. The Republic Statistical Office predicts that the share of people over 65 will increase by another 8% in the next thirty years, so that at the end of the projection period every fourth resident of Serbia (25.2%) would be over 65 years old (21).

Life expectancy

Life expectancy is "the average number of years that a person of a given age is expected to live if current mortality rates continue to hold." Life expectancy is increasing in all regions of the world, but particularly in developed countries, those most affected by the problem of aging. Over the past half century, life expectancy at birth has increased by almost 20 years globally, reaching 68.6 years in 2015, largely due to improved health and reduced mortality among the elderly (1).

However, there are large regional differences in this basic indicator. North America currently has the longest life expectancy at 79.9 years and is projected to continue to lead the region with an average life expectancy of 84.1 years in 2050. Current life expectancy in Africa is only 59.2 years. However, Africa is expected to make major health gains and improve AIDS mortality rates over the next few decades, with life expectancy projected to reach 71.0 years by 2050, narrowing the gap between North America and Africa (11). Looking at individual countries, uneven trends in population health are evident, reflected in differences in life expectancy across countries. In 2019, 24 countries had a life expectancy at birth of 80 years or more. Japan, Singapore, and Macau lead this group, with life expectancies over 84 years. At the other end of the spectrum, 28 countries have life expectancies below 60 years. With the exception of Afghanistan, the remaining 27 countries are in Africa. For example, life expectancy in South Africa is 49.7 years and in Japan it is 84.7 years, a difference of 35 years in life expectancy at birth between the two countries (11). Life expectancy has also increased in older ages. In the United States, for example, life expectancy for people aged 65 increased from 11.9 years in 1900–1902 to 19.1 years in 2009. Life expectancy for people aged 80 almost doubled during the same period, from 5.3 years to 9.1 years (22). According to the results of the last Population Census in 2022 the life expectancy of live-born men and women in the Republic of Serbia is 72.30 and 77.49 years, respectively. In relation to the period 2010-2012. year, an increase of 0.3 years was recorded for men and 0.4 years for women, while the difference in average life expectancy of 5.2 years in favor of women was maintained. The expected increase in average life expectancy of about two years, which was achieved in the inter-census period from 2002 to 2011,

was not repeated due to the significant impact of the coronavirus pandemic, especially in 2021, which belongs to the three-year interval around the 2022 census. In terms of this indicator, Serbia lags behind developed European countries (21,23).

Fertility

Today, there is a consensus scientific opinion that the process of population aging is primarily caused by low fertility (11). European demographers have been warning for decades about possible declines in the total population that will accompany the inevitable aging of the population in some European countries, due to persistent "low-low" fertility levels. In some European countries, such as Belarus, Bulgaria, Romania, Serbia and Ukraine, population decline began two decades ago (24). The total fertility rate was halved from 5 to 2.5 in the period 1950–2009. In 2015, the fertility rate was close to or below the replacement level in all regions of the world, with the exception of Africa. In developed countries, this indicator is significantly lower than the world average and is 1.6. Record low fertility rates are observed in Russia (1.6), China and Switzerland (1.5), Germany and Japan (1.4), South Korea (1.2) and Taiwan (1.1). In Asia and Latin America, on the other hand, fertility rates have declined more recently but more rapidly than in Europe. The overall total fertility rate in Asia and Latin America fell by 50% (from 6 to 3 children per woman) during the period 1965–1995. In 2015, the average total fertility rate was at replacement level (2.1) and is projected to continue to decline over the next 35 years to 2050, albeit at a reduced rate (11). In Africa, fertility is still very high (4.4), with the record-breaking countries being Niger (6.8), Mali and Burundi (6.1), Somalia (6), and Uganda (5.9).

Demographers have noted that Africa has undergone a different trajectory of fertility transition than the rest of the world (25). They argue that the slow decline in fertility in Africa is due to the still high ideal family size, which stems from the distinctive pro-natalist cultural norms of African societies, the pervasiveness of fertility control regimes that focus on delaying rather than preventing, and the unmet need for family planning (26-28). The current total fertility rate in Serbia is 1.35, which is one-third lower than the fertility level required for simple population replacement (2.1 live births). For more than half a century, Serbia has been experiencing a downward trend in fertility, which is an excellent indicator of changes in reproductive norms and behavior of the Serbian population, as well as of the difficult socio-economic situation (1).

Accelerated modernization, migration from rural to urban areas, high participation of women in the full-time labor force, unemployment, unsatisfactory economic standard, inadequate support for balancing work and family responsibilities, childcare problems, birth control, etc., are just some of the factors behind the low birth rate in Serbia (29).

Economic consequences of population aging

As one of the consequences of changing economic relations in society compared to the pre-industrial period, there is a decrease in the working-age population above a certain age, which in conditions of permanent population aging can have serious socio-economic consequences (1). As a result of the extension of life expectancy and the secular decline in fertility, which leads to inevitable population aging, the number of pensioners as part of the elderly population is constantly increasing, so pensioners are clearly distinguished as the economically dominant category that appears among the elderly population (30). The pension system is an indispensable factor in the economic and social stability of a state, and its implications are particularly pronounced in countries in transition and economically less developed countries such as Serbia (31).

Recently, many governments have expressed concerns about the adequacy and sustainability of pension systems by modifying the parameters of these schemes. These measures include: raising the statutory retirement age; increasing the contribution rate for defined benefit schemes, the tax or social security rates on pension contributions, and minimum service requirements; removing incentives for early retirement; and introducing automatic adjustment mechanisms such as linking the age at which pension benefits can start to life expectancy. Governments have also introduced reforms that strengthen private pension funds and improve their complementary role in providing adequate retirement income. While measures to raise the statutory retirement age and pension system reforms can improve the sustainability of pension schemes, it is also important to bear in mind the potential consequences of these broad reforms on poverty and inequality among older persons (32).

Population ageing is often framed in terms of sustainable health systems, ensuring well-being and economic growth, while ignoring the significant social, economic and cultural contributions that older people make (33). Ageing is a concern from the perspective of the cost of health care systems, as well as the cost of health care for older people, particularly in settings where institutional, human and financial resources to meet the basic needs of older people are limited and where social safety nets are lacking. High-income countries may differ from low- and middle-income countries in terms of their readiness or availability of resources to provide health care for an ageing population. Institutionalised long-term care, combined with informal care, are some of the options for addressing this challenge (34, 35). As part of the post-Millennium Development Goals set by the United Nations (UN), universal health coverage has become a focus for the post-2015 Sustainable Development Goals (36).

Health profile of the elderly

The burden of chronic noncommunicable diseases on society is a major public health challenge worldwide, and the epidemic of these diseases is significantly associated with population aging (37, 38). The prevalence of most chronic

conditions increases with age, and what is particularly worrying in older people is the high prevalence of comorbidity, the assessment of disease complications, injuries and diseases and the disease of the long term. As preventable, noncommunicable diseases are today the leading cause of morbidity, disability, premature death, and one of the main reasons for the use of health care, especially among older people, where they are, in relation to the community, in relation to the general community. (39, 40). All this has the consequence of increasing public spending on health and social protection of the elderly, and population aging is seen as a global threat to economic stability in the 21st century (41).

In its global report on the status of chronic noncommunicable diseases, WHO points out that in 2012, chronic noncommunicable diseases were responsible for 38 of the 56 million deaths, or 68% of deaths worldwide. Approximately three-quarters of deaths from chronic noncommunicable diseases (28 million) and 82% of premature deaths occurred in low- and upper-middle-income countries, and of the total number of people with mental illnesses that are chronic diseases. 42% were under 70 years of age (37).

According to the results of the Global Burden of Disease Study in 2010, 23.1% of the total burden of disease (574 million out of 2490 million DALYs) was caused by diseases in people aged 60 years and older. In the overall ranking of age-adjusted disability (ADA) diseases, the ten leading causes of disease burden in the elderly are: ischemic heart disease (77.7 million ADIDAs), cerebrovascular disease, obstructive disease66, stroke (43.3 million), diabetes (22.6 million), pain in treatment (19.1 million), lung and bronchial cancer (18.6 million), falls (12.4 million), visual impairment (10.4 million), dementia (10 million, 9 million), hypertension (10 million, 9 million, tuberculosis) (41). The order of occurrence of these diseases does not change much depending on the region (42), with infectious and parasitic diseases contributing more to the burden in low-income countries (43), while mental and neurological diseases will make a more pronounced contribution in high-income regions (44).

The per capita burden of disease in older people is higher in low- and middle-income countries (827 DALYs per 1000) than in high-income countries (590 DALYs per 1000) (45). The most common mental disorders in this age group are depression and dementia. It is estimated that 10–15% of the elderly population suffers from depression, while 25–30% of people aged 85 years or older have some degree of cognitive decline (46). Depression in older age results from the loss of functional abilities, self-esteem, significant roles and people in life, devaluation of social contacts, and a decline in socioeconomic status due to retirement or disability (47). Older adults with physical health problems have higher rates of depression than healthy adults, as depression is comorbid with other physical and mental illnesses and is often underdiagnosed. Depression is often considered a normal and natural response to chronic illness and the changes that come with aging (48, 49). As life expectancy increases, the number of people with dementia, such as Alzheimer's disease, is expected to increase. Alzheimer's disease is currently the sixth leading cause of death in the United States, but recent estimates indicate that it will become the third leading cause of death for older adults and the third leading cause of death from dementia by 2030. The risk of dementia increases sharply with age in people over 60 years of age (50, 51).

The goal is to ensure that people of all ages receive the health services they need without unnecessary financial hardship. The combination of a high burden of chronic diseases and low incomes in older populations means that their patterns of health care use differ from those observed in younger populations (52).

The World Health Organization (WHO) has defined healthy aging as the process of maintaining functional capacity to enable well-being in old age. WHO, Member States and partners for the Sustainable Development Goals have developed the Global Strategy and Action Plan on Ageing and Health 2016–2020 and its follow-up to the WHO Decade on Healthy Ageing 2020–2030. WHO has established key priorities such as supporting country planning and action, collecting better global data and promoting research on healthy ageing, aligning health systems with the needs of older people, laying the foundations and ensuring the human resources necessary for long-term integrated care, undertaking a global campaign to combat ageing, and advancing the global network for age-friendly cities and communities (53).

Ageism in older people

Older people face numerous problems, stereotypes and discrimination in society. One of the most important reasons is the existence of ageism in the population and autoageism in older people. The World Health Organization defines ageism as stereotypes, prejudices and discrimination directed towards other people or towards oneself solely on the basis of age. Although ageism is believed to be present only in younger generations, there is autoageism, which refers to stereotypes, prejudices and discrimination that older people display towards other older people. Just like other forms of prejudice, ageism is a bias that devalues individuals based on their perceived membership in a group (10, 11).

Ageism is a significant social issue in all European societies, affecting individual and societal well-being. The emergence of ageism is most often associated with a generalized opinion about the loss of psycho-physical abilities or mental and physical slowness of older people. The right to dignity is guaranteed by the highest international and national regulations. The United Nations stated in Article 1 of the Universal Declaration of Human Rights that “All human beings are born free and equal in dignity and rights”. The fundamental rights in the declaration include, but are not limited to, the right to life, liberty, non-discrimination, due process, property ownership, education, political participation, work and leisure. Also, in 2002, at the Second World Assembly on Ageing in Madrid, the United Nations adopted the Madrid International Plan of Action on Ageing,

which contains a strategy aimed at providing practical assistance to countries facing demographic change. Three domains have been identified that will be given special attention in the formulation and implementation of policies: (1) older persons and development; (2) improving health and well-being in old age; and (3) ensuring an enabling environment. The existence of ageism is one of the most important obstacles to achieving the set goals of protecting older persons (12).

The United Nations has declared the decade from 2021 to 2030 as the "Decade of Healthy Ageing", which aims to mobilize states, the non-governmental sector, international organizations, professionals, academia, the media and the private sector to improve the lives of older persons, their families and the communities in which they live. Its aim is to raise awareness of ageing, draw attention to the need for urgent action and generate changes that turn the ageing process from a challenge into an opportunity (10).

In 2015, when the 2030 Agenda for Sustainable Development was adopted by all member states, the United Nations came out with a universal plan for achieving sustainable development that would ensure peace, prosperity and the realization of the rights of all people. The Agenda calls for leaving no one behind, for the Sustainable Development Goals to be achieved in all segments of society, at all ages, with a special focus on particularly vulnerable groups, including older people. Of the 17 Sustainable Development Goals, 11 relate, among others, to the protection of older people (11).

Research conducted in the EU shows that 44% of European Union citizens consider age discrimination to be very serious, and 35% have reported discrimination based on age (more than on gender or race), while 51% of them expressed concern that employers give preference to 20-year-olds when hiring. Also shocking is the fact that 57% of the population believe that people over 70 do not contribute economically to society, and 53% of all respondents do not have any friends over 70 (54-56).

Ageism has numerous negative consequences for older people. It reduces their chances of employment, diminishes respect for older people, threatens their right to age with dignity, and contributes to discrimination. In addition, ageism has a significant negative impact on the physical and mental health of older people. Negative attitudes about aging and old age shorten life expectancy by an average of 7.5 years (57). Research has shown that the cost of treating older people for diseases caused by ageism is as much as US$63 billion per year worldwide (58). Interventions to reduce negative prejudices against older people should include educating young people about aging and maintaining contact between generations. It is also important to discuss the negative effects of aging on older people and to implement innovative strategies to reduce the detrimental effects of aging on older adults. These effects may result in the need for effective interventions for older adults, such as positive aging education, emotional management, body confidence building, and flexible goal setting, which can serve as factors to mitigate or perhaps reverse the negative effects of aging on their psychological well-being. Unlike other stressors, ageism cannot be addressed at the individual level alone. All age groups should be involved in addressing ageism, as it is one of the most socially prevalent and institutionalized forms of prejudice that is reflected in many areas of society (59).

There are numerous scales that are used in propose of assessing the ageism. The main issue with scales for ageism is that psychometric properties of these scales are not available which can give wrong estimates of ageism prevalence. Some of the most used scales are: Aging perceptions questionnaire, Aging semantic differential, Anxiety about ageing questionnaire, Attitudes to aging questionnaire, Expectations Regarding Aging, Facts on aging quiz, Fraboni scale of ageism, Image of aging scale, Kogan's attitudes towards older people scale, Reactions to aging questionnaire, Tuckman and Lorge questionnaire and Willingness to Deliver Pharmaceutical Care to Elderly (60). The development and validation of a new ageism scale that covers all dimensions of ageism could be very useful for future studies.

Conclusion

In light of the rapid growth of the aging population, research on aging needs to be given greater attention. The role of demographics, social factors, health needs and barriers to economic resources and disparities in health care utilization among older people needs to be systematically addressed and monitored. The goal is to ensure that people of all ages receive the health services they need without unnecessary financial hardship. The combination of a high burden of chronic diseases and low incomes in the older population means that their patterns of health care utilization differ from those observed in younger populations. A well-organized health and social care system is important for improving the health of older people. Public health policies need to address the diversity of health and functional conditions experienced by older people and maximize the number of people who achieve positive aging trajectories. Integration initiatives require actions at macro-levels (legislation, financing), meso-levels (age-friendly environment) and micro-clinical levels. Creating and implementing innovative strategies to improve the health and quality of life of older people would enable well-being in old age.

CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

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THE ANTITUMOR AND TOXICITY EFFECTS OF RUTHENIUM(II) COMPLEXES ON HETEROTOPIC MURINE COLON CARCINOMA MODEL

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ABSTRACT

The aim of the present study was to examine the antitumor and toxicity effects of ruthenium(II) complexes, [Ru(Cl-tpy)(en)Cl][Cl] (Ru-1) and [Ru(Cl-tpy)(dach)Cl][Cl] (Ru-2) on heterotopic murine colon carcinoma model.
For tumor induction, 1×10^6 CT26 cells suspended in 100 μl of DMEM were injected subcutaneously into flank of male BALB/c mice. Treatment groups were as follows: Ru-1, Ru-2, oxaliplatin and control (saline). The intraperitoneal administration of the tested complexes began on 6th day after CT26 cells inoculation. Each complex was administered at dose of 5 mg/kg, twice weekly, four doses in total. To assess toxicity, serum values of urea, creatinine, AST and ALT were determined and histopathological analysis of organs and tumor were performed. In order to assess the effects of Ru(II) complexes on markers of oxidative stress and antioxidant defense system, we determined the TBARS, GSH, SOD and CAT in the homogenate of tumor, heart, liver, lungs and kidney tissues.
The findings indicate that Ru-1 and Ru-2 exerts equal or better antitumor activity in comparison with oxaliplatin, but with pronounced toxic effects such as reduced survival rate, cardiotoxicity, nephrotoxicity and hepatotoxicity. The increased index of lipid peroxidation in the tissues of the kidneys and heart, but decreased in tumor tissue, after Ru(II) complexes administration, indicates the importance of the induction of oxidative stress as a possible mechanism of nephrotoxicity and cardiotoxicity, but not the mechanism by which they realize antitumor activity.
Additional studies are needed to elucidate the mechanism of antitumor activity and toxicity of the Ru(II) complexes.

Keywords: *Ruthenium(II) complexes, antitumor activity, colon carcinoma, oxaliplatin, oxidative stress, toxicity, BALB/c mice.*

INTRODUCTION

Colorectal cancer is the second leading cause of cancer-related death, with the mortality rates of patients rank from 13% to 89%, indicating the limitations of current treatments and urgent needs for more effective therapies (1-2).

Platinum-based drugs, cisplatin and it's analogues, carboplatin, and oxaliplatin are the most effective chemotherapeutic agents in treatments of solid cancer. Beside their clinical success, there has been some limitatios observed: systemic toxicity (nephrotoxicity, liver toxicity, peripheral neuropathy, myelotoxicity and gastrointestinal toxicity) and the incidence of drug resistance (3-5). These limitations have initiated new investigations for other transition metal complexes that will show similar antitumor activity but being less toxic.

So far four ruthenium complexes, NAMI-A, KP1019, NKP1339 and TLD1443, have entered clinical trials. NAMI-A is selective anti-metastasis agent, whereas KP1019 acts only on primary colorectal tumors. These ruthenium complexes showed similar efficacy to platinum-based anticancer agents, but they are not completely free of toxicity. NAMI-A can damage the kidneys and have led to elevated levels of serum creatinine in animal, while the main target organs for toxic effects of KP1019 are the kidneys and bone marrow (6-8). Within this context, it is necessary to evaluate antitumor effects, toxicity and side effects in parallel when screening new metal-based drugs. For this reason, researchers choose organic ligands to increase selectivity for specific tissues and minimize side effects, because both metal and ligands play an important role in recognizing targets. Ruthenium(II/III) complexes with different ligands have attracted attention in recent years because of their antitumor activity, which they realize through interaction with proteins and DNA, induction of apoptosis, inhibition of metastasis, and at the same time exhibit low toxicity in healthy tissues in *in vitro* and *in vivo* assays (9).

Drug-induced oxidative stress is implicated as a mechanism of toxicity in numerous tissues and organ systems, including liver, kidney and cardiovascular system. Cisplatin and oxaliplatin are the most commonly used metallopharmaceuticals that exhibits multiorgan toxicity associated with induced redox imbalance as a possible mechanism (10, 11). Drug-induced oxidative stress also can interferes with many cellular functions, such as cell cycle progression and apoptotic pathways, that can reduce the ability of antineoplastic agents to kill cancer cells (12). Hence the importance in examining the pro-oxidative action of potentially cytostatics.

We have recently published a study demonstrating the antitumor activity of the ruthenium(II) terpyridine complexes (Ru-1 and Ru-2 complexes, Scheme 1), in *in vitro* and *in vivo* colon carcinoma models, with moderate toxicity (13). These findings encouraged us to examine the antitumor activity, oxidative status and toxicity of higher doses of Ru(II) complexes in syngeneic model of CT26 colon carcinoma in BALB/c mice. The potentially toxic effects of Ru-1 and Ru-2 complexes were evaluated concurrently and compared to oxaliplatin that is effective platinum drug for colorectal cancer.

MATERIALS AND METHODS

Chemicals

The compounds [Ru(Cl-tpy)(en)Cl][Cl] (Ru-1) and [Ru(Cl-tpy)(dach)Cl][Cl] (Ru-2) were synthesised as reported previously (14). All other chemicals were used as purchased without further purification.

Animals

Male BALB/c mice of 6–8 weeks of age, with weights of 20–25 g were used in all experiments. Mice were housed in a temperature controlled environment (22-24˚C) with a 12-h light–dark cycle and were given standard laboratory food and water *ad libitum.* All animals received humane care, and all experiments were approved (01-8461/2) by and conducted in accord with, the Guidelines of the Animal Ethics Committee of the Faculty of medical sciences of the University of Kragujevac (Kragujevac, Serbia).

Animals were randomized into 8 groups with 6 mice that were ear-tagged and followed-up individually throughout the study. Each complex was administered intraperitonealy at dose of 5 mg/kg dissolved in 200 µl saline, twice weekly for four times in total. Treatment groups were as follows: healthy (tumor free) mice that recived: Ru-1, Ru-2, oxaliplatin and sterile saline (control); and tumor-bearing mice who received the same. The dosage of oxaliplatin was selected as 5 mg/kg according to the literature (15,16).

Cell line

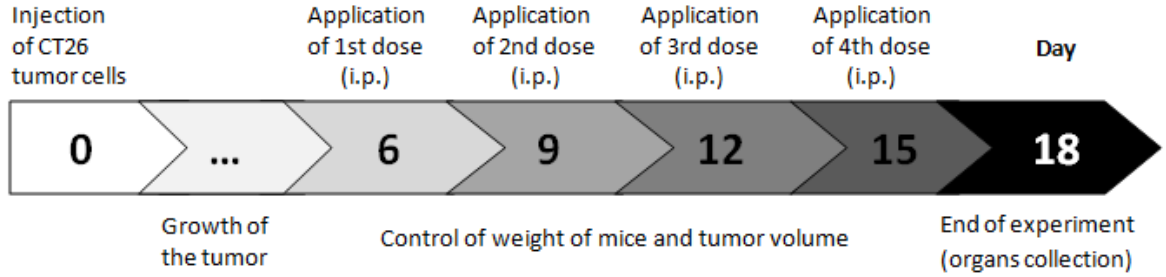
Mouse colon carcinoma cell line (CT26), was obtained from the American Type Culture Collection (ATCC). The cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, 200 mM l-glutamine 10,000 units/ml penicillin and 10 mg/ml streptomycin (all from Sigma, Germany). The cells were cultivated at 37 °C in absolute humidity in an atmosphere containing 5% CO_2.

Tumor implantation and drug injection

Subcutaneous tumors are for many years the mainstay of murine tumor models, with direct measurement of tumor size utilized to study effects of drugs and targeted therapies (17). A BALB/c mice bearing syngeneic CT26 mouse colon carcinoma has been selected as the test system. For tumor induction, 1×10^6 CT26 cells suspended in 100 µl of DMEM were injected subcutaneously into the left flank. The administration of complexes or saline began on 6th day after injection of CT26 cells. Each drug was administered intraperitoneally at doses of 5 mg/kg dissolved in 200 µl saline, twice weekly for four times in total (Scheme 1). Groups were as follows:

Ru-1, Ru-2, oxaliplatin and control (sterile saline). Tumor-bearing mice were examined every 3 days for tumor development and progression and monitoring body weight. Tumor size was measured with a caliper and tumor volume was calculated as follows: TV (mm^3) = (L × W^2)/2, where L is the longest and W the shortest radius of the tumor in millimeters. Results were expressed as means of tumor volumes ± SD.

Scheme 1. Schematic representation of the protocol of the *in vivo* experiments performed on CT26 tumor-bearing BALB/c mice treated with ruthenium(II) complexes and oxaliplatin.



Relative Organ Weight

On the 18th day from tumor induction, all the animals were sacrificed in an atmosphere saturated with diethyl ether. Different organs such as heart, liver, lungs and kidneys were carefully dissected out and weighed in miligrams (absolute organ weight). The relative organ weight of each animal was then calculated as follows:

$$\text{Relative Organ Weigh} = \frac{\text{Absolute organ weight (mg)}}{\text{Body weight of rat on sacrifice day (mg)}} \times 100$$

Assessment of toxicity

In order to assess the tolerability of the application of Ru(II) complexes, the survival and weight of mice were monitored during the experiment. The 72 h after the last treatment, all mice were sacrificed in an atmosphere saturated with diethyl ether. Blood samples were collected in tubes without anticoagulant, processed and analyzed by chemistry analyzer Roche Cobas Mira Plus for blood urea nitrogen, creatinine, as well as liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT). After sacrificed, organs (heart, liver, lungs and kidneys) as well as tumor were isolated for histopathological analysis. The tissues fixed in 4% paraformaldehyde were embedded in paraffin, cut into thin sections and mounted on glass slides. The tissue sections were stained with hematoxylin and eosin for microscopic examination.

Oxidative stress assays

At the end of experiment isolated tissue of tumor, heart, liver, lungs, and kidney from all experimental animals were frozen at -80 °C. The each tissue was homogenized in phosphate buffer pH 7.4 (1:10 ratio) using an electrical homogenizer, on ice. Then, tissue homogenates were centrifuged at 1200 *g* for 20 min at 4 °C. The resulting supernatants were isolated and stored at -80 °C until determination of biochemical parameters (18). Index of lipid peroxidation, measured as TBARS (thiobarbituric acid reactive substances) was determined as marker of oxidative stress. The antioxidative defense system was estimated by determination of levels of reduced glutathione (GSH) and activity of antioxidative enzymes in tissue homogenats: superoxide dismutase (SOD) and catalase (CAT). All parameters were determined spectrophotometrically (UV-1800 Shimadzu spectrophotometer).

Index of lipid peroxidation (measured as thiobarbituric acid reactive substances-TBARS)

The degree of lipid peroxidation in homogenate of tissue was estimated by measuring of thiobarbituric acid reactive substances (TBARS) using 1% thiobarbituric acid (TBA) in 0.05 sodium hydroxide (NaOH) incubated supernatant of homogenized tissue at 100 °C for 15 min and measured at 530 nm (18, 19).

Determination of reduced glutathione (GSH)

The level of reduced glutathione (GSH) was determined based on GSH oxidation with 5.5-dithio-bis-6.2-nitrobenzoic acid using the method reported by Beutler. Detection was performed at 420 nm. The amount of GSH was expressed as nmol/g tissue (20).

Determination of catalase (CAT)

CAT activity was determined according to Aebi. Diluted homogenate of tissue (1:7 v/v) was treated with chloroform-ethanol (0.6:1 v/v). CAT buffer, prepared sample, and 10 mM H_2O_2 was used for determination. Detection was performed at 360 nm. The amount of CAT was expressed as U/g tissue (21).

Determination of SOD

SOD activity was determined by the epinephrine method of Beutler. Homogenate of tissue was mixed with carbonate buffer, and then epinephrine was added. Detection was performed at 470 nm. The amount of SOD was expressed as U/g tissue (22).

Statistical analysis

Statistical analysis of experimental data included the following basic descriptive statistics: the mean value and standard deviation (SD). For testing the normality of the distribution parameters, the Shapiro–Wilk and Kolmogorov–Smirnov test were used. To test the statistical significance of the results and to confirm the hypothesis, the Student's t test was used. A database analysis of the results was performed using software package SPSS 20 (SPSS Inc., Chicago, IL, USA). A p value < 0.05 was considered statistically significant.

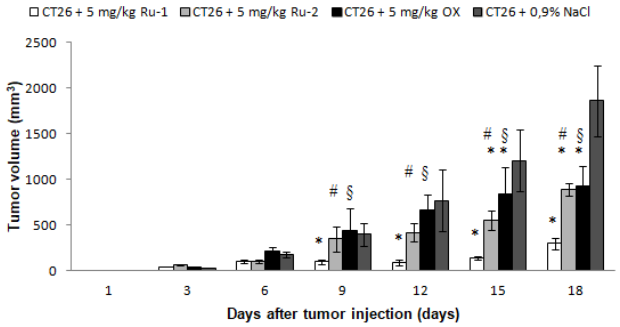
RESULTS

The Ru-1 and Ru-2 inhibits tumor growth in CT26 tumor bearing mice

To detect the antitumor activity of Ru-1 and Ru-2 *in vivo*, we used a CT26 xenograft tumor model. CT26 cells ($1x10^6$) were subcutaneously injected into the left flank of BALB/c mice. Six days later, the tumors were palpable in all animals. The complexes Ru-1, Ru-2 and oxaliplatin, were injected intraperitoneally in a dose of 5 mg/kg every three days, starting from the 6th day after the inoculation of CT26 cells, and a control group was received saline. After being treated for 12 days, the mice were sacrificed and the tumors were stripped off.

The difference in tumor volume was observed from the 9th day after the inoculation of CT26 cells, respectively after the first dose of the examined Ru-1 complex. The difference in tumor volume between the mice that was treated with 5 mg/kg Ru-1 and all other groups is retained until the end of the experiment, so that after the second administered dose, a statistically significant difference was established in relation to the control, but also in relation to the other ruthenium complex - Ru-2, as well as in relation to oxaliplatin. On day 15 of the experiment, the difference between the group treated with 5 mg/kg of Ru-1 and all other groups was maintained, the Ru-2 complex also statistically significantly inhibiting tumor growth compared to the control. On the last day of the experiment, all experimental groups had a statistically significantly smaller tumor volume compared to the control group treated with saline alone. The tumor growth is shown in Figure 1, which suggested the tumors grew the slowest in the group treated by Ru-1 and Ru-2.

Figure 1. The tumor volume per group through days of experiment.



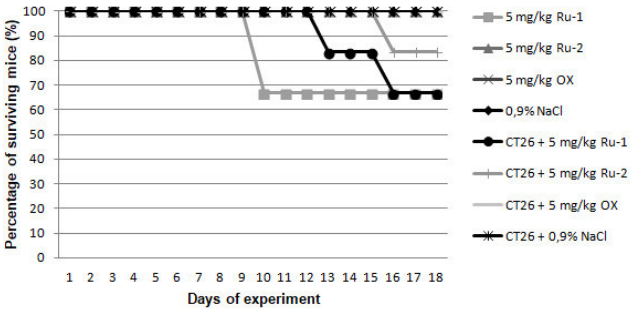
The data are expressed as mean ± SD and were analysed by *Student`s t* тест, $p<0.05$, * saline treated cells *vs*. cells treated with Ru-1, Ru-2 or oxaliplatin; # Ru-1 *vs.* Ru-2; § Ru-1 *vs*. OX; ¶ Ru-2 *vs*. OX.

Evaluation of general toxicity

The survival rate, body weight and relative organ weights of heart, liver, lungs and kidneys were determined for each animal for investigating the general toxicity during the *in vivo* antitumor evaluation. The organ index was calculated as % body weight.

All tumor-free and tumor-bearing mice from control and oxaliplatin group survived until the end of study, while there was a mortality after the second dose of Ru-1 in group of healthy mice, and after third and fourth dose in groups of tumor-bearing mice who received Ru-1 or Ru-2 (Figure 2).

Figure 2. Survival rate of healthy mice and tumor-bearing mice after the administration of Ru(II) complexes and oxaliplatin.



From first day to 18th day of experiment, there were changes in the body weight of mice in all the groups. The control group of mice as well as the treated group of mice, who received oxaliplatin and Ru-2 gained weight throughout the duration of treatment, whereas weight loss was observed in mice who received Ru-1, in healthy and in tumor-bearing mice. However, these changes in the body weights of treated mice were not significantly different at the beginning and at the end of the experiment (Figure 3).

There were no significant changes in the relative weights of isolated organs (heart, liver, lungs and kidney) between the groups. It is interesting that the relative weight of the liver in healthy mice who received Ru-1 was the highest, while in tumor-bearing mice who received the same complex was the lowest (Figure 4).

Figure 3. Body mass of healthy (**A**) and tumor-bearing mice (**B**) before and after treatment with Ru(II) complexes and oxaliplatin. The data are expressed as mean ± SD and were analysed by Student's t test, * $p < 0.05$.

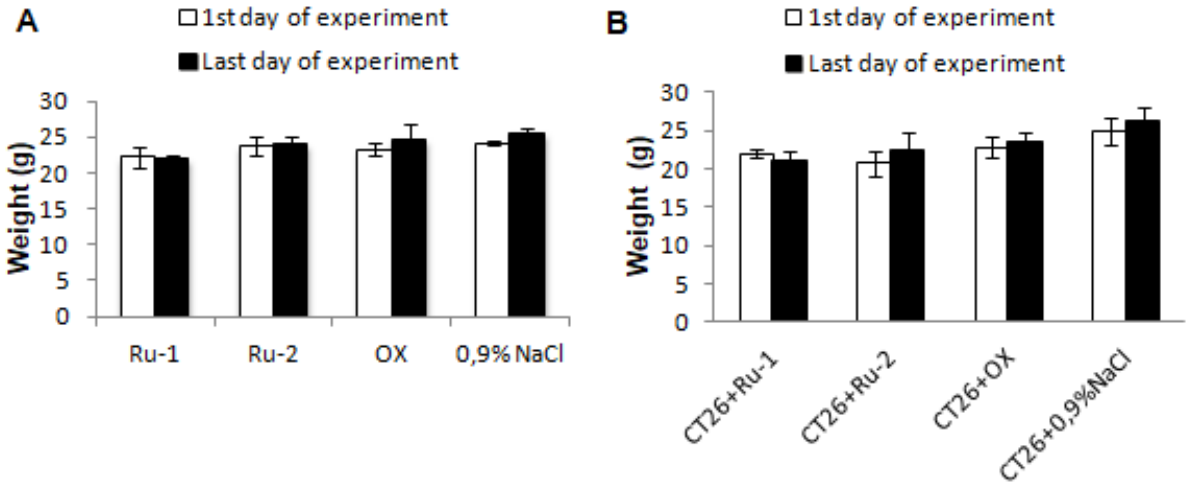
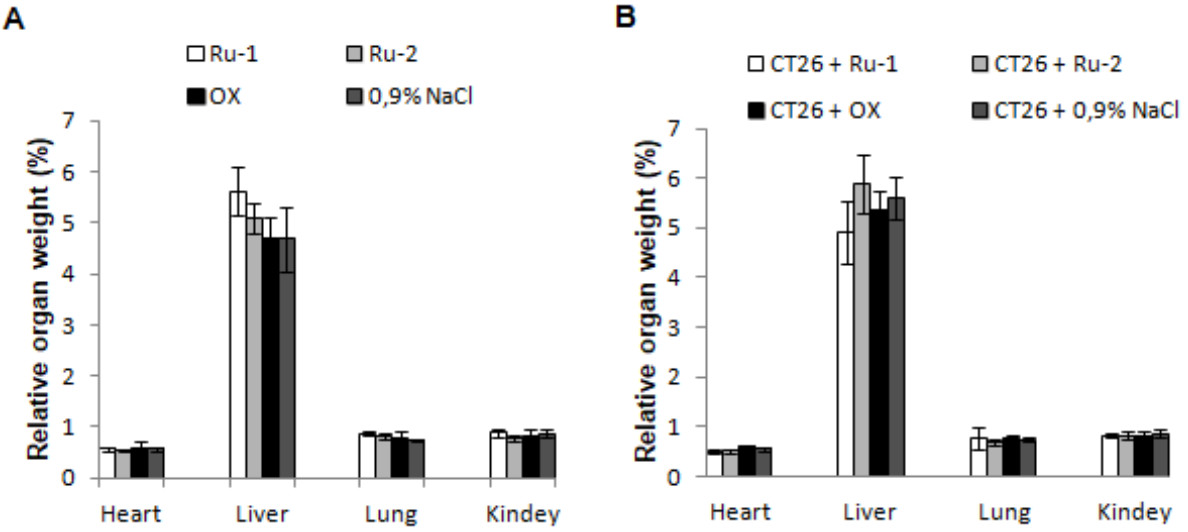


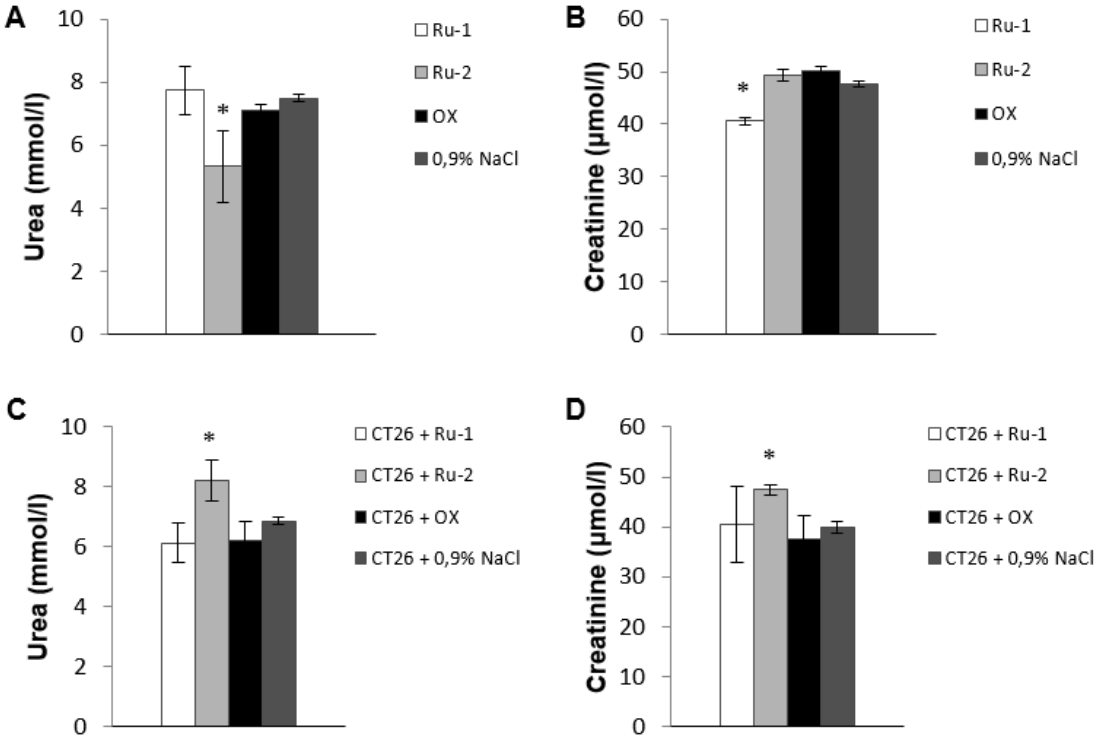
Figure 4. The relative mass of isolated organs of healthy mice (**A**) and tumor-bearing mice (**B**) after treatment with Ru(II) complexes and oxaliplatin. The data are expressed as mean ± SD and were analysed by *Student's t* test, * $p < 0.05$.



The serum concentrations of the biochemical markers ALT, AST, urea and creatinine were obtained to evaluate liver and renal functions. The obtained serum urea and creatinine values indicate that the use of Ru(II) complexes and oxaliplatin did not affect the uree or creatinine concentration in relation to the group of healthy mice who received saline. Even statistically significantly lower urea concentrations were observed in the group that received Ru-2 in relation to the group of healthy mice who received Ru-1 (p=0.038; Figure 5A). Also statistically significantly lower creatinine concentrations were observed in the group that received Ru-1 versus all other groups of healthy mice (p=0.046; Figure 5B).

The concentration of urea and creatinine in the serum of tumor-bearing mice after treatment with the tested complexes in a dose of 5 mg/kg were slightly different. A group of mice that received intraperitoneal Ru-2 had statistically significantly higher urea concentrations compared to all other groups (significant difference from: Ru-1 group (p=0.005); oxaliplatin group (p=0.019); control group (p=0.027); Figure 5C). Also, there were statistically significantly higher creatinine concentrations in Ru-2 group comparing to groups of mice receiving intraperitoneal oxaliplatin (p=0.021); or saline (p=0.001) (Figure 5D).

Figure 5. Concentration of urea and creatinine in the serum of healthy (**A, B**) and tumor-bearing mice (**C, D**) after treatment with Ru(II) complexes and oxaliplatin. The data are expressed as mean ± SD and were analysed by *Student's t* test, * $p < 0.05$.



For the evaluation of liver function, the activity of transaminases (ALT and AST) in serum of healthy mice as in tumor-bearing mice was determined after intraperitoneal administration of the Ru(II) complexes, oxaliplatin or saline. The obtained ALT and AST values clearly indicate that the intraperitoneal application of the Ru(II) complexes leads to a statistically significant elevation in the value of these two parameters. The group of healthy mice as in tumor-bearing mice who intraperitoneally received 5 mg/kg Ru-2 had statistically significantly higher ALT concentrations compared to all other groups (significant difference from: Ru-1 group (p=0.000); oxaliplatin group (p=0.000); control group (p=0.000); Figure 6 A, C), and statistically significantly higher AST concentrations than mice intraperitoneally receiving oxaliplatin (p=0.007) or saline (p=0.000), (Figure 6 C, D). The healthy mice as in tumor-bearing mice who received intraperitoneally 5 mg/kg Ru-1 had statistically significantly higher ALT and AST concentrations than mice intraperitoneally receivied oxaliplatin (p=0.000; p=0.000) or saline (p=0.000; p=0.000), (Figure 6 C, D). There was no difference in the values of ALT and AST between the group that received intraperitoneally oxaliplatin comparing to the control group (Figure 6 A, C).

The fields of necrosis were observed in sections of the primary tumor in all experimental groups (Figure 7A). A cross section of the lungs showed the presence of passive hyperemia, rupture of alveolar septum (emphysema), desquamation of the alveolar epithelium, necrosis and desquamation of the respiratory epithelium. Only in the group treated with Ru-1 complex all mentioned changes were present, while in others groups there were mostly present passive hyperemia and rupture of alveolar septum (Figure 7B). Dilated interstitium was observed in tissue sections of the heart in all treated groups. Passive hyperemia, degenerative changes of cardiomyocytes and hypertrophy of cardiomyocyte were present in the group treated with Ru-2 complex, while in group treated with Ru-1 complex there were present focal coagulation necrosis of the myocardium and degenerative changes of cardiomyocytes too (Figure 7C). Passive hyperemia of kidney tissue sections was found in control group and oxaliplatin treated group. While more severe changes in the kidney tissue were observed in the groups treated with ruthenium (II) complexes, such as glomerular hypercellularity, parenchymal degeneration of the tubular epithelium, intraluminal cylinders and bleeding in the interstitium (Figure 7D). Passive hyperemia of liver tissue sections was found in all groups. Anisocytosis and anisocoria of hepatocytes, hydrops and ballooning degeneration of hepatocytes, were present in liver tissue sections from oxaliplatin trated group, while in Ru-1 group in addition to the mentioned changes, there were additionally present focal and confluent necrosis, portal space infiltration, portal and periportal hepatitis (Figure 7E).

In order to assess the effects of Ru(II) complexes on markers of oxidative stress and antioxidant defense system, as possible mechanisms of action or toxicity, we determined the TBARS, GSH, SOD and CAT in the homogenate of tumor, heart, liver, lungs and kidney tissues.

Figure 6. Concentration of transaminases (ALT and AST) in the serum of healthy (**A, B**) and tumor-bearing mice (**C, D**) after treatment with Ru(II) complexes and oxaliplatin. The data are expressed as mean ± SD and were analysed by *Student's t* test, * $p < 0.05$.

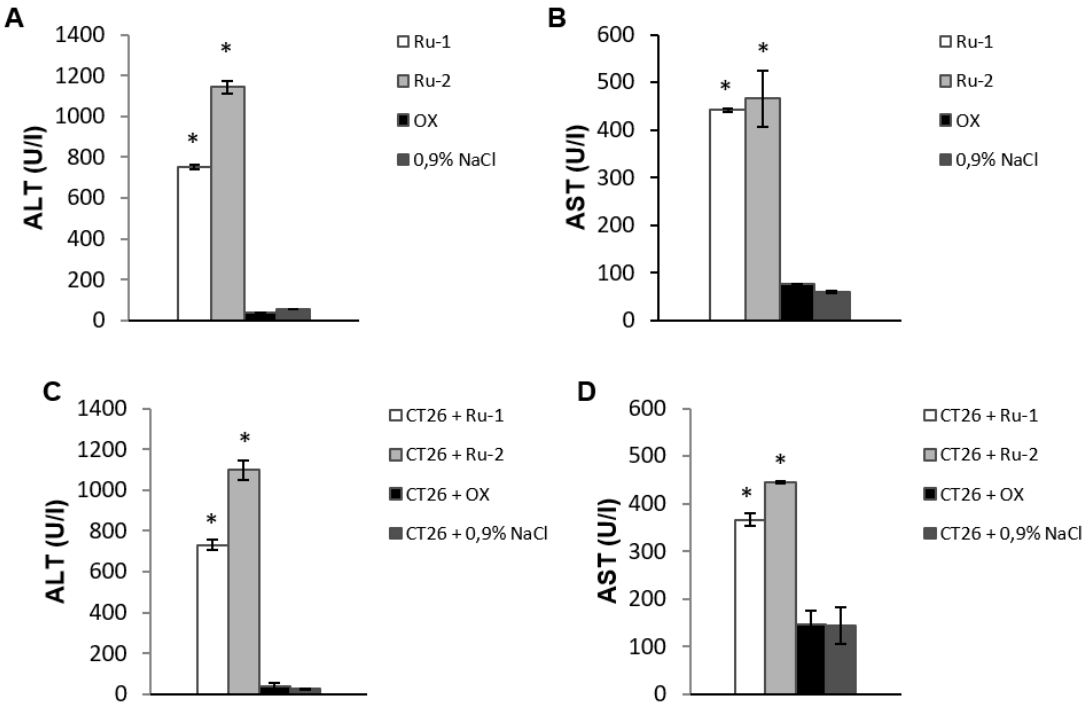
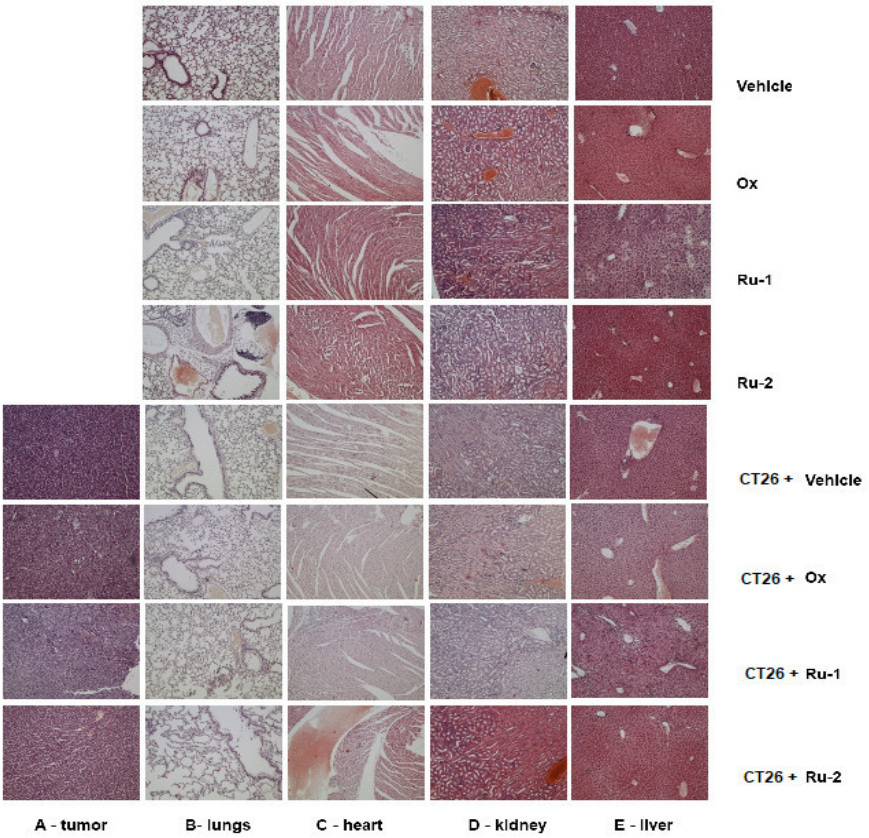


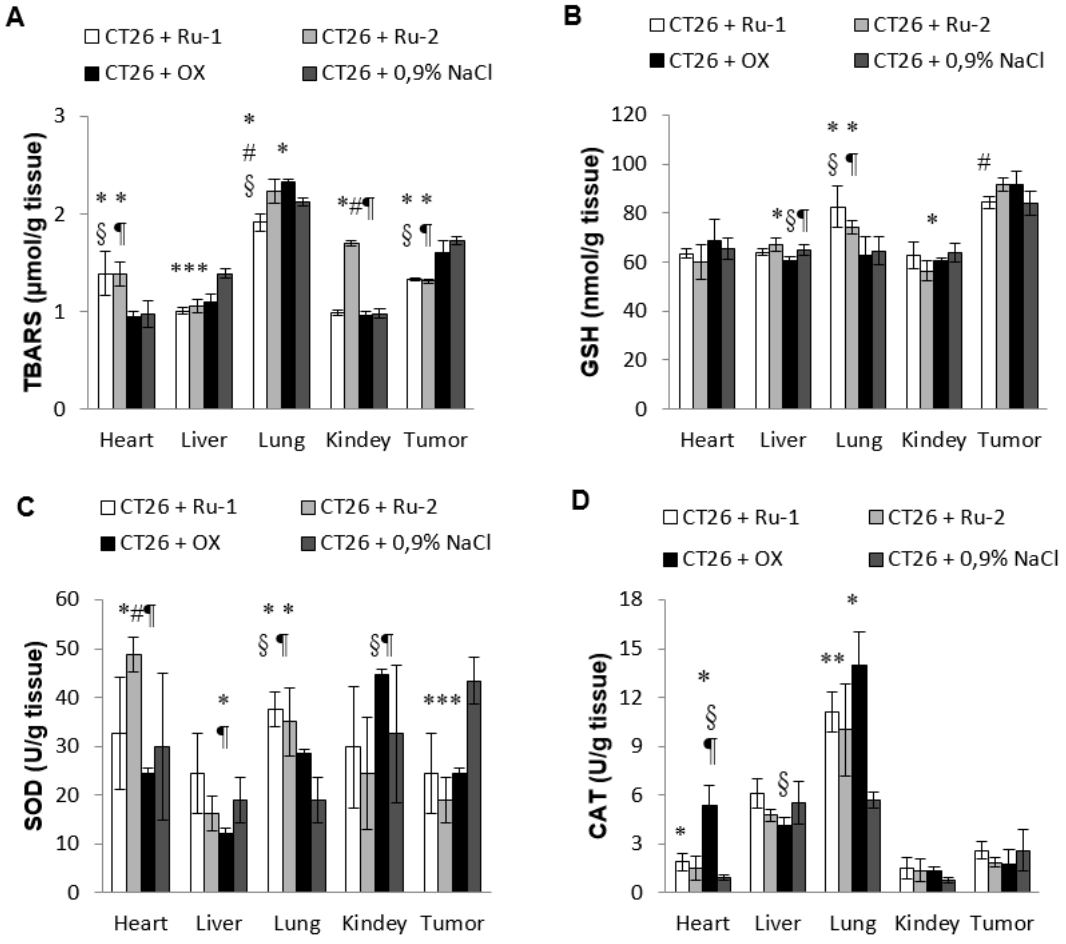
Figure 7. Histological examination. Representative hematoxylin and eosin staining of **(A)** tumor sections, **(B)** lungs, **(C)** heart, **(D)** kidney and **(E)** liver tissue of healty and CT26-tumor bearing mice treated with 5 mg/kg of Ru-1, Ru-2, oxaliplatin or saline (magnification at x100).



Index of lipid peroxidation values were statistically significantly increased in the heart of mice treated with Ru(II) complexes compared to those receiving oxaliplatin or saline. A significant reduction in TBARS in the liver of mice from all experimental groups was observed in comparison to control. The use of oxaliplatin resulted in a statistically significant increase in index of lipid peroxidation in the lungs compared to control and in comparison to the group of mice treated with Ru-1. In a kidney tissue of mice treated with Ru-2 was recorded a significant leap of TBARS, compared to all other groups. The application of the Ru(II) complexes led to a significant reduction in the value of TBARS in the tumor tissue compared to the group receiving oxaliplatin or saline (Figure 8A).

Levels of non enzymatic antioxidant – GSH was significantly increased in lung of mice who received Ru-1 and Ru-2 compared to group who received oxaliplatin and control group, but on the other hand Ru-2 significantly decreased activity of GSH in kidneys in comparison to control group. While, application of oxaliplatin has led to significantly decreased activity of GSH in liver in comparison to groups that were treated with Ru(II) complexes. There were no statistically significant differences in the values of GSH between groups in the heart and tumor (Figure 8B).

Figure 8. Values of **(A)** TBARS, **(B)** GSH, **(C)** SOD and **(D)** CAT in the homogenate of tumor, heart, liver, lungs and kidney tissues. The data are expressed as mean ± SD and were analysed by *Student's t* тест, $p<0.05$, * saline treated cells *vs*. cells treated with Ru-1, Ru-2 or oxaliplatin; # Ru-1 *vs*. Ru-2; § Ru-1 *vs*. OX; ¶ Ru-2 *vs*. OX.



Values of SOD was significantly increased in lungs of mice who received Ru-1 and Ru-2 compared to control group and in group of mice who received Ru-1 compared to oxaliplatin group. In heart of mice who received Ru-2, it was measured significantly increased activity of SOD in comparison to other groups. Activity of SOD was significantly decreased in liver tissue of mice who oxaliplatin compared to Ru-1 and control groups. The values of SOD were significantly decreased in tumor tissue of mice who received Ru(II) complexes and oxaliplatin compared to control group. While the highest SOD values in renal tissue were observed after oxaliplatin administration in comparison to groups treated

with Ru(II) complexes (Figure 8C). Activity of antioxidant enzyme CAT was significantly increased in heart of mice who received oxaliplatin in comparison to all others groups, and in heart of mice who received Ru-1 compared to control group. Also activity of CAT was significantly increased in lungs in all three experimental groups compared to control group, but it was significantly decreased in liver of mice who received oxaliplatin in comparison to mice who received Ru-1. There were no statistically significant differences in the values of CAT between groups in the kidneys and tumor (Figure 8D).

DISCUSION

The efficacy of cisplatin and its analogues as anticancer agents has prompted the search for cytotoxic compounds that contain transition metals other than platinum and have lower systemic toxicity and higher efficacy. Ruthenium(II) complexes attracted significant attention as anticancer candidates, however, only few of them have been reported comprehensively (23-25). Since oxaliplatin and some ruthenium complexes have been reported to cause liver and kidney damage (3-8, 26, 27), in addition to the antitumor potential, the toxicity associated with their use was examined.

To study the toxicity of Ru(II) complexes we performed tests of liver and kidney function by evaluating serum levels of biochemical markers (Figure 5, 6) following the treatment of normal healthy mice and CT26 tumor-bearing mice. Hepatocytes produce liver enzymes such as AST and ALT in minute quantities to begin the transamination reactions in amino-acid metabolism. When liver inflammation occurs because of hepatocyte damage, injury or cancer, these enzymes are released in large quantities into the blood stream leading to increased levels in the plasma. AST and ALT levels correlate with the severity of liver damage and after hepatocyte healing or repair, these enzyme levels usually decrease again (28). We also performed histopathological evaluation, as it is considered the primary test for assessing of potentially toxicity of *in vivo* applied substances. And as a possible mechanism of toxicity, the oxidative status in the tissue was examined.

Both examined complexes, Ru-1 and Ru-2, in a dose of 5 mg/kg exerted significant antitumor effects against CT26 colorectal carcinoma *in vivo*. Ru-1 inhibited tumor growth better than oxaliplatin, while Ru-2 showed equally good effect as oxaliplatin (Figure 1), but both were more hepatotoxic and caused a higher incidence of mortality (Figures 2, 6, 7). In our previous study, we used these same Ru(II) complexes (Ru-1 and Ru-2) at lower dose (2mg/kg), wherein the Ru-1 complex exhibiting moderate antitumor activity and mild toxicity (13). Therefore, comparing the current and previous results, we can conclude that by increasing the dose of Ru(II) complexes, their antitumor potential increases significantly, but unfortunately also systemic toxicity.

Although it is known that elevated levels of ROS permit cancer cells to promote pro-tumorigenic signaling, excessive production of ROS is usually associated with anti-tumorigenic pathways that can cause oxidative stress-induced death of cancer cells (29). The modulation of oxidative stress in tumors may be related to the antitumor effect of Ru(II) complexes in mice. ROS play different roles in tumors than in healthy tissue because of variations in pH, hypoxia, and an increase in transferrin carriers in the tumor microenvironment (30). Tumors rapidly utilize oxygen and other nutrients, and the development of new blood vessels, often fails to keep the pace with tumor growth, therefore, there is usually lower O_2 content in tumor cells (31, 32). Superoxide dismutase is the primary antioxidant enzyme, which provides protection of cells from oxidative damage by catalyzing dismutation of O_2^- to H_2O_2 and O_2 and CAT scavenges intracellular H_2O_2 to further reduce oxidative cell damage (28, 30).

Ru-2 complex exhibited pro-oxidant properties, which was manifested by depletion of GSH in renal tissue and consequently increased values of TBARS (Figure 8A, C). Renal damage caused by the use of Ru-2 complex was also confirmed by biochemical and histopathological analysis (Figure 5, 7D). Both, Ru-1 and Ru-2 have led to a marked leap of serum levels of ALT and AST in healthy as well as tumor-bearing mice (Figure 6). These results suggest the hepatotoxic potential of ruthenium(II) complexes, which is confirmed by histopathological analysis (Figure 7E) and is in accordance with existing results indicating the toxicogenic potential of ruthenium(II) complexes against hepatocytes (9). Although our results unequivocally indicate the toxicity of ruthenium complexes, there is many studies of similar design, which speak of their low toxicity (24, 33-35).

TBARS is considered an indicator of lipid peroxidation produced by peroxidation of fatty acids by reactive oxygen species and leads to irreversible demage of the cells (36). Increased TBARS levels in heart tissue of mice treated with Ru(II) complexes, indicate to cell damage, which is in agreement with ours results of histopathological analysis and with the previous research (37). Mihajlović et al., also examined the redox potential of ruthenium(II) terpyridine complexes, but in the blood and heart of rats, whereby they showed that this complexes have low potential to cause redox imbalance (38). Although at first glance it seems contradictory that the application of Ru(II) complexes led to significantly increase activity of antioxidant enzymes SOD and CAT and non enzymatic antioxidant – GSH in lungs, which would indicate their possible antioxidant properties, enhanced antioxidant protection can also be interpreted as defense mechanisms (28). This hypothesis is further supported by the fact that diethyl ether damages lung tissue, and it was used as an anesthetic in sacrificing mice (39). This is additionally indicated by the present changes in the lungs of mice that did not receive the examined complexes. Nevertheless it is not impossible that examined Ru(II) complexes possess certain antioxidant potential, given that they significantly decreased the levels of pro-oxidative marker – TBARS in liver, and so far such properties have already been described (28, 40).

Oxaliplatin did not show toxic effects if we observe the survival rate, body weight and biochemical parameters, but if we analyze the histopathological results and parameters of antioxidant protection, we see that oxaliplatin exhibited a hepatotoxic effect (Figure 2-7). These data are in acordance with existing data about confirmed hepatotoxicity of oxaliplatin. Results of several studies indicate that systemic chemotherapy with oxaliplatin can cause sinusoidal dilation at even 78% of patients. Basic mechanisms of oxaliplatin induced sinusoidal obstruction syndrome remains poorly understood. It is believed that increased generation of ROS and GSH depletion from sinusoidal endothelial cells causes the increased apoptosis in these cells allowing the damage to occur (11).

CONCLUSION

The present results suggest that examined Ru(II) complexes, in dose of 5 mg/kg exerts equal or better antitumor activity in comparison with oxaliplatin, but with pronounced toxic effects such as reduced survival rate, cardiotoxicity, nephrotoxicity and hepatotoxicity. Further research is needed to elucidate the mechanism of antitumor activity and toxicity of the Ru(II) complexes.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

ACKNOWLEDGEMENT

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FACTORS INFLUENCING PHARMACOKINETIC/PHARMACODYNAMIC INDEX OF MEROPENEM IN CRITICALLY ILL PATIENTS

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ABSTRACT

There is a growing consensus that, in critically ill patients, meropenem dosing should aim to achieve 100% fT > MIC. The primary objective of our study was to evaluate the frequency of meropenem underdosing—considering its widespread use in this population—and to identify factors associated with such underdosing. This prospective, cross-sectional study included two groups of critically ill patients: a control group, comprising patients who received optimal meropenem dosing (100% fT > MIC), and a case group, consisting of patients who were underdosed (fT > MIC < 100%). A validated high-performance liquid chromatography (HPLC) method was employed to measure meropenem concentrations in plasma. The effects of various independent and confounding variables on the dichotomous dependent variable were assessed using univariate and multivariate logistic regression analyses. We recruited a total of 63 critically ill patients. The results of our study demonstrated that the majority of critically ill patients (n = 52; 82.5%) received an adequate meropenem dose, achieving fT > MIC of 100%, whereas 11 patients (17.5%) were underdosed, with fT > MIC below 100%. Finally, Acinetobacter spp. and Pseudomonas aeruginosa, as causative pathogens of bacterial infections, were identified as significant risk factors for meropenem underdosing in critically ill patients. Clinicians should exercise caution when selecting the meropenem dosage for critically ill patients with infections caused by Acinetobacter spp. and Pseudomonas aeruginosa.

Keywords: *Meropenem, critically ill patients, PK/PD index, fT > MIC.*

INTRODUCTION

Meropenem is a broad-spectrum carbapenem antibiotic widely used in the treatment of severe and hospital-acquired infections in hospitalized patients, particularly those caused by most Gram-negative and certain Gram-positive bacteria (1-3). Its advantages stem primarily from its broad-spectrum antibacterial activity, notable efficacy against resistant Gram-negative strains, and excellent tissue penetration (4). Notably, meropenem remains effective against extended-spectrum β-lactamase (ESBL)-producing and AmpC β-lactamase-producing *Enterobacteries*, as well as against *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (5,6). It is administered via intravenous infusion and is generally associated with a favorable safety profile.

Meropenem is a time-dependent antibiotic, and the pharmacokinetic/pharmacodynamic (PK/PD) index that most accurately reflects its bactericidal efficacy is the proportion of the dosing interval during which free plasma concentrations exceed the minimum inhibitory concentration (MIC) of the target pathogen (fT > MIC) (1,7). To achieve optimal antibacterial activity, fT > MIC values should be maintained at ≥ 40% of the dosing interval (8). However, dosing meropenem is particularly challenging in critically ill patients, for whom some clinicians advocate dose adjustments aimed at achieving 100% fT > MIC (9-12).

Antibiotic dosing recommendations for critically ill patients often differ substantially from those for the general population (13). This is primarily due to profound pathophysiological alterations in critically ill individuals, which can significantly affect drug pharmacokinetics (13,14). Moreover, inadequate antimicrobial therapy is a well-established risk factor for in-hospital mortality in this patient population (15). Accordingly, the aim of our study was to assess the frequency of meropenem underdosing—given that it is among the most frequently used antibiotics in critically ill patients—and to identify the factors contributing to such underdosing.

MATERIAL AND METHOD

Study design

This prospective, cross-sectional study included two groups of critically ill patients: a control group, comprising patients who received optimal meropenem dosing (100% fT > MIC), and a case group, consisting of patients who were underdosed (fT > MIC < 100%) (9-12).

Study population

The study population comprised critically ill patients with severe, life-threatening infections treated in the intensive care units of the University Clinical Center Kragujevac (UCCKG) between June 2021 and October 2022. Critically ill patients in our study had the diagnoses of meningitis, pneumonia, sepsis, septic shock, and febrile neutropenia caused by multidrug-resistant gram-negative bacteria, including *Enterobacteriaceae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and strains of *Escherichia coli* that produce extended-spectrum beta-lactamases. We recruited only critically ill patients in whom steady-state meropenem levels were achieved, that is, those in whom meropenem had been administered for at least 3 days before recruitment. On the other hand, we excluded all patients who were not critically ill, those in whom steady-state meropenem was not achieved, pregnant women, lactating women, and patients who refused to participate in the study. We recruited patients by the principles of consecutive convenience sampling.

Before initiating the study, we secured approval from the Ethics Committee at the UCCKG (No. 01-21-63). Written informed consent was obtained from all participants prior to their inclusion in the study.

Study variables

Main study outcome was achieved value of fT > MIC PK/PD index of meropenem that was dichotomized to < 100% and >100%. To calculate fT > MIC of meropenem, we measured at least two plasma concentrations of meropenem in dose interval, and based on the authors-made calculator in Excel (available on demand from the corresponding author) the plasma concentration / time curve was reconstructed and used for determining value of fT>MIC. The concentrations of meropenem in plasma were measured by a validated high-performance liquid chromatography (HPLC) method. Details of the equipment, reagents, and the HPLC method used to measure the concentration are presented in the publication Rančić et al (16).

We obtained data on MIC values in patients in two ways: by reviewing the medical documentation of those patients in whom these values were previously measured in the microbiology laboratory of the UCCKG or by reviewing data from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (17).

The following independent and confounding variables were taken into account for adjustment of their effects on the study outcome: gender, age, body weight, body height, body mass index, dose of meropenem, meropenem dosage interval, values of laboratory parameters (creatinine, aspartate aminotransferase, alanine transaminase, red blood cell count, white blood cell count, platelet count, hemoglobin), causative agent of infection (*Klebsiella spp*., *Staphylococcus aureus*, *Proteus mirabilis*, *Acinetobacter spp.*, *Pseudomonas aeruginosa*, *Enterobacter* and *Escherichia coli*), type of bacterial infection (pneumonia, urinary tract infection, intra-abdominal infection), comorbidities (hypertension, chronic renal insufficiency, cerebral infarction, neoplasma), concomitant administration of other antibiotics (vancomycin, colistin).

Statistical analysis

Data were initially analyzed using descriptive statistical methods. Categorical variables were presented as frequencies (percentages), while continuous variables were summarized

using means, standard deviations, and ranges. The effects of various independent and confounding variables on the dichotomous dependent variable were assessed using univariate and multivariate logistic regression analyses. Potential risk factors were evaluated based on their regression coefficients (B), along with corresponding 95% confidence intervals (CIs). A p-value of <0.05 was considered statistically significant. Results were reported as crude and adjusted odds ratios (ORs) with their respective 95% CIs.

RESULTS

We recruited a total of 63 critically ill patients whose socio-demographic and clinical characteristics are shown in Table 1.

Table 1. Basic socio-demographic and clinical characteristics of recruited patients

Varaible	Mean ± standard deviation (range) or number (%)
Gender (M/F)	37 (58.7%) / 26 (41.3%)
Age (years)	63.92±1.62
Body weight (kg)	78.46±12.73
Charlson comorbidity index	4.25±2.66
Hypertension	20 (31.7%)
Chronic renal insufficiency	8 (12.7%)
Cerebral infarction	11 (17.5%)

M-male; F-female

The most common reasons for the use of meropenem in our patients were complicated urinary tract infections (n=23; 36.5%) and pneumonia (n=20; 31.7%), while in the remaining 20 patients (31.7%), meropenem was used to treat other severe bacterial infections. Table 2 shows a list of the most common bacteria causing infections in our patients. The vast majority of patients received meropenem in a total daily dose of 3 grams (n=53; 84.1%); 8 patients (12.7%) received meropenem in a total daily dose of 2 grams, while in one patient (3.25) meropenem was administered in a total dose of 6 grams per day.

Meropenem was most commonly administered three times daily (n=55; 87.3%), while in 12 patients (12.7%) the dosing regimen involved administering meropenem twice daily.

Table 2. The most common causes of infections in our patients

Bacteria	Number (%)
Klebsiella spp.	17 (27%)
Staphylococcus aureus	12 (19%)
Proteus mirabilis	12 (19%)
Acinetobacter spp.	9 (14.3%)
Pseudomonas aeruginosa	6 (9.5%)
Enterobacter	4 (6.3%)
Escherichia coli	3 (4.8%)

The results of our study showed that in most of our critically ill patients (n=52; 82.5%), the administered dose of meropenem was adequate (fT>MIC =100%), while 11 patients (17.5%) were underdosed (fT>MIC <100%). In the group of underdosed critically ill patients, there were significantly more female patients ($\chi2$=3,982; p=0,046) and those whose infection was caused by *Acinetobacter spp.* ($\chi2$=21.849; p=0,000) compared to the group of optimally dosed patients. The study patient groups did not differ significantly from each other in terms of other characteristics.

Table 3 shows the results of univariate and multivariate logistic regression, which we used to identify factors that predispose to meropenem underdosing in critically ill patients. In the final logistic regression model, we entered the following variables: age, gender, pneumonia, urinary tract infection, *Acinetobacter spp.*, and *Pseudomonas aeruginosa*. Our multivariate logistic regression model (enter method) showed satisfactory goodness of fit (Cox & Snell R Square 0.383, Nagelkerke R Square 0.634). Finally, *Acinetobacter spp.* and *Pseudomonas aeruginosa*, as causative agents of bacterial infections, have been identified as risk factors for the occurrence of meropenem underdosing in critically ill patients.

Table 3. Crude and adjusted odds ratios (OR) of the risk factors for meropenem underdosing in critically ill patients

Risk factors	Univariate model Crude OR with 95% CI p	Multivariate model Adjusted OR with 95% CI p
Gender	5.037 (1.188-21.359) p=0.028*	4.505 (0.562-36.087) p=0.156
Age	1.004 (0.954-1.057) p=0.878	0.988 (0.891-1.095) p=0.818
Acinetobacter spp.	3.778 (1.942-6.725) p=0.000*	4.637 (1.365-14.961) p=0.000*
Pseudomonas aeruginosa	1.812 (1.147-5.125) p=0.044*	2.938 (1.392-7.116) p=0.035*

Risk factors	Univariate model Crude OR with 95% CI p	Multivariate model Adjusted OR with 95% CI p
Pneumonia	1.286 (0.329-5.021) p=0.718	4.945 (0.393-62.148) p=0.216
Urinary tract infection	0.600 (0.142-2.532) p=0.487	0.676 (0.075-6.088) p=0.727

*-statistically significant; CI- confidence interval; p- statistical significance

DISCUSSION

Hydrophilic drugs such as meropenem exhibit considerable pharmacokinetic variability in critically ill patients, primarily due to pathophysiological alterations including capillary leak and augmented renal clearance (18). These changes often lead to reduced systemic drug concentrations, thereby diminishing therapeutic efficacy (19). Numerous studies have demonstrated that beta-lactam antibiotic levels frequently fall below therapeutic thresholds in a substantial proportion of critically ill patients (19). Underdosing of antibiotics has been linked to increased mortality in patients with sepsis or septic shock (19) and plays a critical role in the emergence of antibiotic resistance among various bacterial species (20). Consequently, therapeutic drug monitoring (TDM) is increasingly advocated to optimize dosing of meropenem and other beta-lactam antibiotics in this population (21-24). Several analytical techniques are currently available for the detection and quantification of meropenem in biological fluids (25). The majority of validated methods employ chromatographic approaches coupled with ultraviolet or mass spectrometry detection (25), which was the methodology utilized in our study.

The results of our study demonstrated that meropenem was underdosed in approximately 20% of critically ill patients. Similarly, Angelini et al. reported a 26% underdosing rate in this patient population (26). The lower incidence of underdosing observed in our study may be attributed to the standard practice at UCCKG, where clinical pharmacologists are routinely involved in dosing calculations for critically ill patients (27). The involvement of clinical pharmacologists in treatment planning has been shown to improve clinical outcomes in this vulnerable population (27).

Our findings indicate that the treatment of infections caused by *Acinetobacter* species is associated with an increased risk of meropenem underdosing in critically ill patients, likely due to the elevated minimum inhibitory concentrations (MICs) observed in these infections. The role of carbapenems in managing infections caused by *Acinetobacter baumannii* and related species has undergone substantial changes (28). *Acinetobacter baumannii* is a Gram-negative, aerobic, non-fermenting bacterium responsible for severe healthcare-associated infections, including pneumonia, bacteremia, and urinary tract infections (29,30). For many years, carbapenems were considered the treatment of choice for multidrug-resistant *A. baumannii* infections (30). However, this organism has developed multiple mechanisms of resistance to carbapenems (30,31). In some regions, up to 75% of *A. baumannii* strains exhibit resistance to meropenem (28). The predominant resistance mechanisms involve the production of OXA-type β-lactamases and metallo-β-lactamases (31). Carbapenem-resistant *Acinetobacter baumannii* (CRAB) represents a significant nosocomial threat due to the high mortality rates associated with infections caused by these strains (30,31). Two randomized clinical trials have demonstrated that meropenem, even when combined with colistin, is no longer effective against CRAB infections (32,33). Furthermore, the addition of vaborbactam—a β-lactamase inhibitor—to meropenem does not improve clinical outcomes in *A. baumannii* infections, as the β-lactamases produced either do not hydrolyze the parent carbapenem or are inadequately inhibited by vaborbactam (17).

In contrast to infections caused by *Acinetobacter baumannii*, meropenem remains highly effective against *Pseudomonas aeruginosa* infections (34). Nevertheless, meropenem-resistant strains of *P. aeruginosa* are increasingly reported (34). Consequently, combination therapy with meropenem and either colistin or amikacin is recommended for infections caused by these resistant strains (35). A key determinant of meropenem's efficacy in treating *P. aeruginosa* infections is the minimum inhibitory concentration (MIC) (35). According to EUCAST breakpoints, resistant *P. aeruginosa* strains exhibit elevated meropenem MICs (>8 mg/L) (17). This necessitates the administration of high meropenem doses to achieve optimal pharmacokinetic/pharmacodynamic (PK/PD) targets. Accordingly, doses as high as 12 grams per day have been suggested for the treatment of septic shock caused by P. aeruginosa (36).

This study has several limitations. First, it was conducted at a single center and involved a relatively small sample of critically ill patients, which may limit the generalizability of the findings. Additionally, we were unable to identify cases of meropenem overdose, as the minimum (trough) concentrations of the drug were not measured.

In conclusion, clinicians need to pay special attention when administering meropenem to treat infections caused by *Acinetobacter spp.* and *Pseudomonas aeruginosa* in critically ill patients. Given the elevated MIC values, particularly among resistant bacterial strains, there is a substantial risk of meropenem underdosing, which may lead to unfavorable clinical outcomes in patients.

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CONFLICT OF INTEREST

None.

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RADIOFREQUENCY CATHETER ABLATION OF LEFT ACCESSORY PATHWAYS USING CONVENTIONAL METHODS

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ABSTRACT

Radiofrequency catheter ablation of accessory pathways is considered the treatment of choice for patients with atrioventricular reentry tachycardia due to its proven safety and effectiveness. The aim of this study was to compare the outcomes of this treatment for left-sided accessory pathways in 58 consecutive patients with manifest preexcitation who were treated at the Institute for Cardiovascular Diseases Dedinje. The procedure duration varied significantly ($p<0.05$) depending on the localization of accessory pathways along the mitral annulus (anterior 60.0±26.0 min; anterolateral 45.2±28.4 min; lateral 73.9±27.9 min; posterolateral 59.1±25.5 min; posterior 83.1±29.6 min), as did the number of applied radiofrequency pulses (anterior 9.0±4.5; anterolateral 4.3±4.7; lateral 8.1±5.8; posterolateral 4.0±2.9; posterior 8.1±4.2; $p<0.05$). Fluoroscopy exposure time did not differ significantly ($p=0.078$). Atrial fibrillation was recorded in 15.5% of patients prior to the procedure. For the ablation of left-sided accessory pathways, the transaortic approach was used significantly more often (74.1%) than the transseptal approach. There were no significant differences among the groups in terms of primary procedural success (100% for anterior and anterolateral, 82.1% for lateral, 100% for posterolateral, and 88.9% for posterior; $p=0.672$), recurrence rate (10.7% for lateral and 5.5% for posterior; $p=0.023$), or final success rate, defined as definitive cure (100% for anterior, anterolateral, and posterolateral, 94.8% for lateral, and 89.3% for posterior; $p=0.421$). Despite the increased time and more radiofrequency energy pulses necessary for ablating laterally and posteriorly positioned accessory pathways, the procedure's success rate remained comparable regardless of accessory pathways location along the mitral annulus.

Keywords: *Accessory pathway, radiofrequency ablation.*

INTRODUCTION

The atria are electrically isolated from the ventricular myocardium by the tricuspid and mitral annuli. The only connection between these two electrical units is the specialized conduction system, consisting of the atrioventricular (AV) node and the His-Purkinje system1.

In cases of incomplete embryonic separation, an additional connection, referred to as an accessory pathway (AP), persists between the atria and ventricles in a form of muscular bundle[2]. This congenital anomaly results in atrial electrical impulse activating part or the entire ventricular myocardium earlier than expected if activation were to occur exclusively via the AV node[3].

Preexcitation is diagnosed based on a standard 12-lead electrocardiogram (ECG) that reveals: a delta wave—a characteristic initial portion of the QRS complex, PQ interval <120 ms, and QRS duration >120 ms. The prevalence of manifest preexcitation in the general population is 0.3%[5].

Electrophysiological Characteristics of Accessory Pathways (APs)

The mechanism of atrioventricular reentry tachycardia (AVRT) in Wolff-Parkinson-White (WPW) syndrome, involves both the AV node and the APs, which connect the atria and ventricles forming a circular impulse movement-macroreentry (Figure 1). AVRT can be classified as:

Orthodromic - characterized with anterograde conduction through the AV node and retrograde conduction via the AP, referred to as a *concealed* AP since it is not detectable in sinus rhythm (20-30% of cases).

Antidromic - characterized with anterograde conduction through the AP and retrograde conduction via the AV node, which is associated with manifest preexcitation.

Bidirectional - characterized with conduction occurring in both directions.

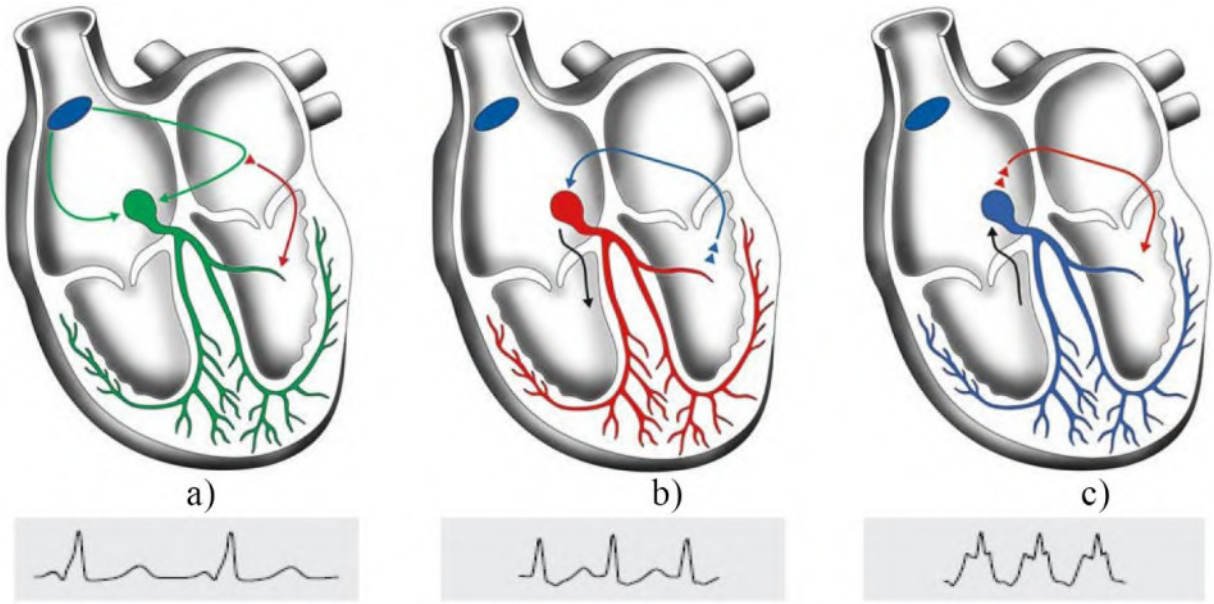

Figure 1. a) **Sinus rhythm with preexcitation** - Anterograde conduction occurs through the AV node (*green*) and the AP (*red*). Due to the faster conduction through the AP, part of the ventricular myocardium depolarizes earlier than the rest, which manifests as a delta wave on the surface ECG. b) **Orthodromic AVRT** - During tachycardia, anterograde conduction occurs exclusively through the AV node (*red*), as the AP has a longer refractory period. However, the impulse returns from the ventricles to the atria via the AP (*blue*). c) **Antidromic AVRT** - In this case, the AV node reaches its refractory period earlier than the AP, so anterograde conduction occurs exclusively via the AP (*red*), while retrograde conduction occurs through the AV node (*black*).

Orthodromic tachycardias are most prevalent (90-95%). They present with regular, narrow QRS complexes, at a heart rate of 120-250 bpm and a retrogradely conducted P wave, that appears after the QRS complex with a prolonged RP interval (*long RP tachycardia*)[8].

Anterograde AVRT represents a minority of cases (5-10%). However, affected patients face an elevated risk of sudden cardiac death (0.1-0.6% annually), which rises to 40% in patients who develop concurrent atrial fibrillation (AF) or rapid atrial flutter (*Figure 2*).

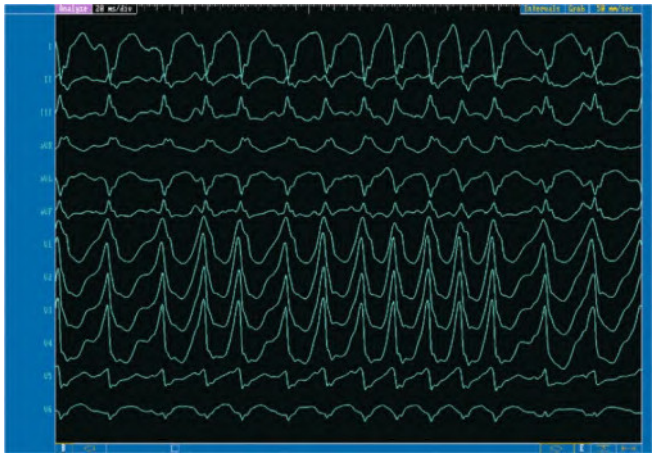

Figure 2. 12-lead surface ECG recording showing atrial fibrillation and antidromic AVRT (minimum RR interval 225 ms).

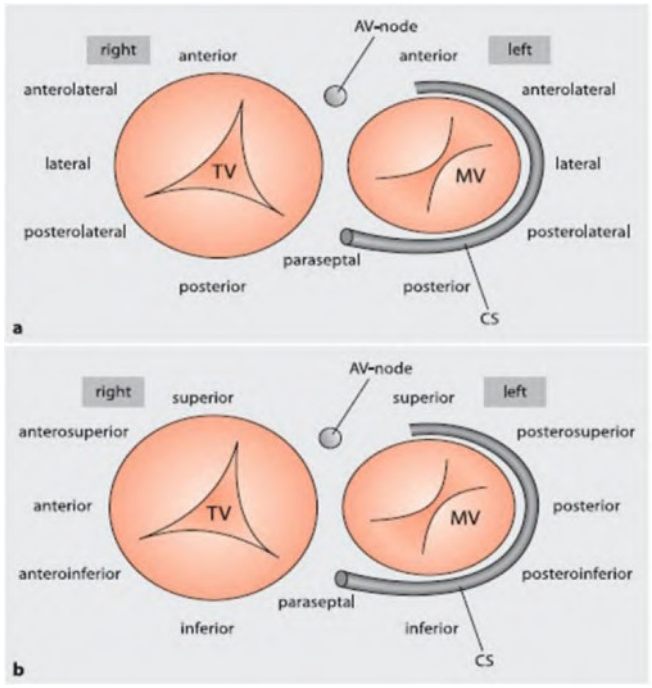

Figure 3. Old (a) and new (b) nomenclature for the localization of APs around the AV junction.

The atrial insertion of an AP on the MA is typically discrete and closely aligned with the annulus. In contrast, ventricular connections are often more extensive, exhibiting branching patterns with multiple connection points that may be located farther from the annulus. This can result in activation of different ventricular regions, thereby affecting the ECG morphology and delta wave polarity. Although several algorithms have been proposed (e.g., Avila A, 1995; Milstein S, 1987; Arruda et al.[12,13]), these tools provide general guidance rather than a precise anatomical localization tool of the AP.

Direct mapping of the MA is performed by systematically advancing the mapping catheter along the annular circumference to identify sites with optimal signals (Figure 4). This procedure can be carried out using either of the following approaches:

- Transaortic (retrograde) approach - via femoral artery puncture
- Transseptal (anterograde) approach - via femoral vein puncture, under fluoroscopic and intracardiac echocardiography (ICE) guidance.

Both techniques are considered complementary, and it is recommended that the operator should be proficient in both approaches to optimize procedural success. Previous studies have demonstrated no significant differences between the two in terms of procedure duration, fluoroscopy time, complication rates, or success rates, which range from 90% to 100%, with a recurrence rate of 5%[15,16].

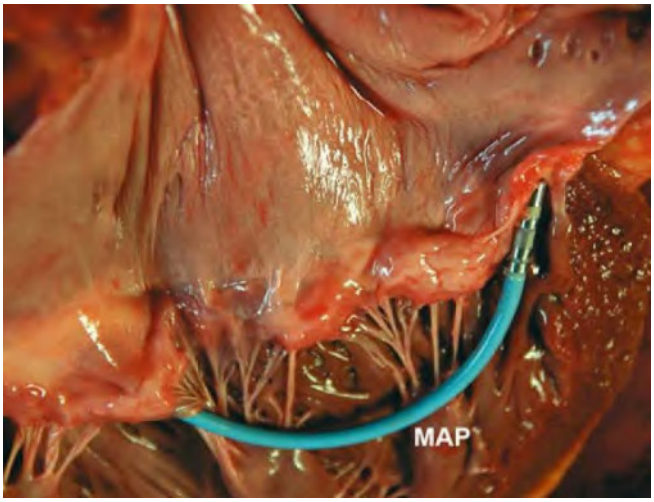

Figure 4. Positioning of the ablation catheter (MAP) through the AV junction at the lateral position of the mitral annulus.

Study Objective

The objective of this study was to evaluate and compare the clinical outcomes of RFA in the treatment of manifest WPW syndrome in patients with left-sided APs (anterior, anterolateral, lateral, posterolateral, and posterior). The study aimed to assess and compare the mean procedure duration, fluoroscopy exposure time, the number of applied RF pulses, and the incidence of intervention complications. Additionally, it sought to determine the primary procedural success rate and the three-month recurrence rate, stratified by the anatomical location of the APs along the MA.

Methodology

This study enrolled 58 consecutive patients diagnosed with WPW syndrome who underwent RFA at the Institute for Cardiovascular Diseases Dedinje in Belgrade between February 1st, 2018, and June 30th, 2019. Indications for RFA were determined in accordance with American College of Cardiology/American Heart Association (ACC/AHA) guidelines.

All patients exhibited manifest preexcitation on surface ECG, and experienced frequent episodes of paroxysmal supraventricular tachycardia (PSVT) that were resistant to pharmacological prophylaxsis with at least one antiarrhythmic drug. In all cases the AP was localized along the MA, consistent with left-sided AP.

All antiarrhythmic medications were discontinued 10 days prior to the procedure.

Written informed consent was obtained from all patients after being thoroughly informed regarding the nature of the procedure, its expected efficacy, and potential risks and complications.

The ablation procedures were conducted in a dedicated electrophysiology laboratory, utilizing either a retrograde transaortic or an anterograde transseptal approach, depending on anatomical and procedural considerations. Procedural success was defined as the complete elimination of the AP, confirmed by the absence of preexcitation on post-ablation ECG.

Follow-up evaluations were conducted three months following the ablation procedure. Primary ablation success, or clinical cure, was defined as the absence of tachycarrythmia recurrence and no evidence of preexcitation on a both 12-lead ECG and 24-hour Holter monitoring. In cases of recurrence, patients underwent a repeated ablation procedure.

Patients were stratified into five groups based on the anatomical localization of the AP: anterior, anterolateral, lateral, posterolateral, and posterior localization.

Statistical analysis was performed to compare the following variables across these five groups: type of AP access (retrograde vs. anterograde), presence of AF, total procedure duration, fluoroscopy exposure time, number of applied RF pulses, primary procedural success, complication rates, recurrence rates, and final procedural success.

Differences in continuous variables (procedure duration, fluoroscopy exposure, number of applied RF pulses) across all five AP localization groups were analyzed using analysis of variance (ANOVA). Differences between specific features were assessed using the Student's t-test.

For categorical variables (type of AP access, presence of AF, acute procedural success, recurrence, need for reablation, final success, and complication rates), statistical significance was determined using the Chi-square (χ^2) test.

RESULTS

Between February 2018. and June 2019., a total of 58 patients with manifest WPW syndrome and left-sided APs localized along the MA were treated at the Institute for Cardiovascular Diseases Dedinje. Of these, 26 patients were female. The mean age of the study population was 35.2 ± 11.4 years, with ages ranging from 16 to 80 years. There was no significant difference in age distribution between sexes (mean age for women: 35.9 ± 12.4 years; men: 35.8 ± 12.2 years; $p = 0.784$).

All patients demonstrated manifest preexcitation on surface ECG, accompanied by PSVT that was refractory to pharmacological therapy. During the electrophysiological study (EPS), left sided APs localized along the MA were confirmed, with anterograde or bidirectional conduction of impulses.

The distribution of patients according to AP localization was as follows: anterior (n=2), anterolateral (n=6), lateral (n=28), posterolateral (n=5), and posterior (n=18).

There were no significant differences among these groups with respect to mean age (anterior: 35.7 ± 13.2 years; anterolateral: 36.1 ± 12.9; lateral: 36.4 ± 13.1; posterolateral: 35.4 ± 12.7; posterior: 35.9 ± 13.1; $p = 0.973$) or sex distribution ($p = 0.237$).

AF was documented before the procedure in 9 patients (15.5%).

The transaortic (retrograde) approach was utilized significantly more frequently (43 patients, 74.1%) than the transseptal (anterograde) approach (15 patients, 25.9%; $p < 0.05$) (*Figure 5*).

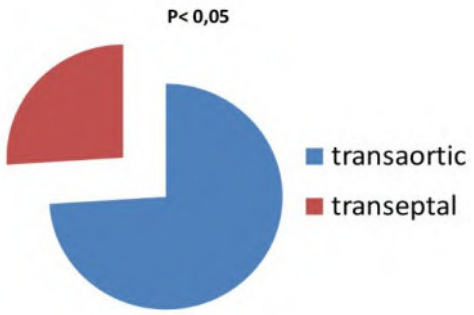

Figure 5. Access routes to the AP along the mitral annulus for radiofrequency ablation (RFA).

The mean procedure duration varied by AP localization as follows: anterior - 60.0 ± 26.0 min, anterolateral - 45.2 ± 28.4 min, lateral - 73.9 ± 27.9 min, posterolateral - 59.1 ± 25.5 min, and posterior - 83.1 ± 29.6 min ($p < 0.05$; *Figure 6*). The procedure duration involving posterior APs was significantly longer compared to those involving posterolateral, anterolateral, and anterior APs ($p < 0.05$). In addition, lateral APs were associated with a significantly longer procedure time compared to anterolateral APs ($p < 0.01$).

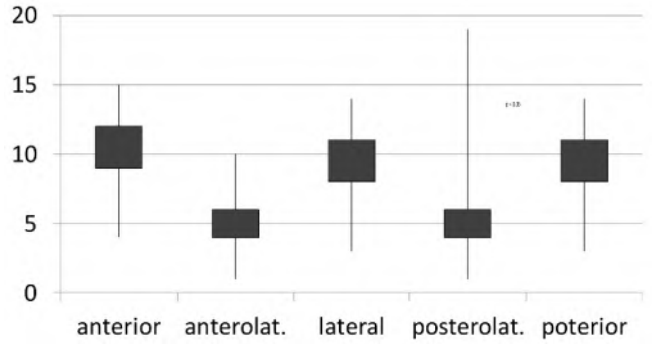

Figure 6. Procedure duration (in minutes) for different localizations of left-sided APs; p< 0,05.

Fluoroscopy exposure times by AP localization, with the following averages: anterior - 12.1 ± 9.3 min, anterolateral - 8.5 ± 7.4 min, lateral - 20.2 ± 10.9 min, posterolateral - 14.6 ± 8.7 min, and posterior - 16.0 ± 9.8 min for APs (p = 0.078;

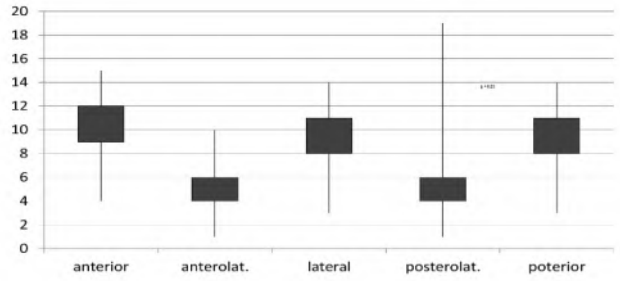

Figure 7. The difference in fluoroscopy exposure duration during ablation, among the five groups, was not significant (p = 0.078).

Ablation required an average of 9.0 ± 4.5 pulses for anterior, 4.3 ± 4.7 for anterolateral, 8.1 ± 5.8 for lateral, 4.0 ± 2.9 for posterolateral, and 8.1 ± 4.2 for posterior APs (p < 0.05; Figure 8).

Significantly fewer RF pulses were delivered for anterolateral and posterolateral APs ablation than for other left-sided localizations (p < 0.05).

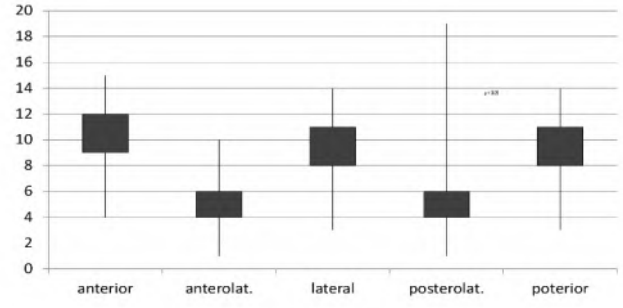

Figure 8. Number of applied RF pulses depending on AP localization; (p < 0.05).

The primary success rate of the procedure was achieved in 51 patients (87.9%). The first ablation was successful in all patients with posterolateral, anterolateral, and anterior APs (100%), compared to 88.9% for posterior and 82.1% for lateral APs. However, intergroup differences were not significant (p = 0.348; *Figure 9*).

Recurrence after three-month follow-up occurred in 4 patients (6.8%) of initially successful ablations. A higher recurrence rate was noted in lateral (10.7%) and posterior APs (5.5%) versus other localizations along the MA, although this difference did not reach statistical significance (p = 0.672).

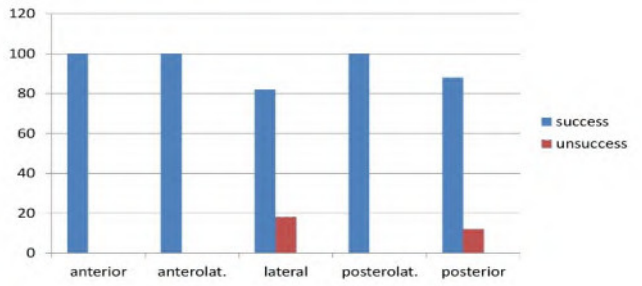

Figure 9. Primary success rate of the procedure by group, depending on AP localization along the mitral annulus; p=0,672

Reablation, a repeat ablation procedure, was required in 12 patients (20.7%), including 8 patients with initial procedural failure and 4 patients with WPW syndrome recurrence. The transseptal approach was employed in reablation procedures.

Stratified by anatomical location, reablation rate was 15% (7 patients) with lateral APs 17.7% for posterior APs (5 patients), demonstrating a statistically significant difference between these groups (p < 0.05).

A complete elimination of the APs, indicating final treatment success, was achieved in 55 patients (94.8%). Ablation was unsuccessful in three patients, all of whom were with lateral AP localization, resulting in a success rate of 89.3% within this subgroup. However, this difference in ablation success across various AP localizations was not statistically significant (p = 0.421).

Single complication was observed in a patient with lateral AP localization, who underwent a successful ablation via the transseptal approach. The complication was manifested as a small pericardial effusion, which resolved spontaneously.

DISCUSSION

This study involved 58 consecutive patients with WPW syndrome who underwent RFA at the Institute for Cardiovascular Diseases Dedinje. All patients exibited manifest preexcitation on surface ECG, experienced PSVT unresponsive to medical therapy, and had an AP pathway localized to the left of the AV node.

The most common AP localization along the MA was on the free wall of the left ventricle (LV) and atrium (28 lateral

APs identified). This was followed by 18 posteroseptal APs in the posterior region, while only 2 APs were found in the anteroseptal region. These findings are fully consistent with previously published studies.[33,34]

One female patient was diagnosed with two APs: a posterior AP exhibiting anterograde conduction and a lateral AP with bidirectional conduction. The presence of multiple APs is clinically significant due to their association with an increased risk of sudden cardiac death, particularly when the effective refractory period (ERP) of the AP is short (less than 270 ms), indicating high conductivity[35]. When such high conductivity APs are present alongside AF, there is a risk of bypassing the normal AV conduction, potentially leading to the degeneration of AF into ventricular fibrillation. The annual risk of sudden cardiac death in patients with WPW syndrome is estimated to range from 0.1% to 0.6%[35].

There was no significant age difference between male (35.8 ± 12.2 years) and female patients (35.9 ± 12.4 years). In addition, no differences in age or sex were observed among the five study groups.

Although the transaortic (TA) and transseptal (TS) approaches are generally considered complementary - with previous studies[24,25] reporting no significant differences in procedure duration, fluoroscopy exposure time, number of RF energy applications, or complication rates (Montenero: 100% TA and TS; Katritis: 87% TA, 90% TS; Lesh: 85% TA and TS)[21,24] - in our study, the TA approach was used significantly more often accounting for 74.1% of procedures.

The TS approach was avoided due to the risk of potential complications, such as atrial wall perforation with consequent tamponade or aortic bulb perforation (major complications rates reported at 1.3%)[20,21]. It was used only in cases in which catheter positioning via the TA approach was not feasible or in reablation procedures following a primary ablation failure with the TA approach.

One complication (1.7%) occurred during a TS puncture for ablation of lateral AP, presenting as a minor effusion along the posterior wall of the LV. It resolved spontaneously within 7 days, as confirmed by echocardiographic follow-up. Minor effusions without consequent tamponade or the need for invasive intervention have been reported in 0.7% of cases in the literature[19].

Minor vascular complications, such as hematomas, were observed in 2.4% of cases. The incidence of complications during ablation of left-sided APs is reported to range range from 0 to 8%, with most common reported rate of around 4%[23]. The majority of these are vascular in nature including hematomas, aneurysms, and AV fistulas. Thromboembolic events, such as catheter tip thrombosis, occur in approximately 2% of cases, despite the use of anticoagulation therapy[22]. Other complications like tamponade, cardiac perforation, and transient ischemic attacks are reported in 1.5% of cases.

Additional complications include thermal injury to the circumflex artery and, in rare cases, the left main (LM) artery[21], as well as vein strictures and mural thrombi formation during ablation within the coronary sinus (CS)[22].

Due to the exceptional expertise and experience of the operators at the Institute for Cardiovascular Diseases Dedinje, none of the aforementioned complications were observed during this study.

A comparison of procedure duration and fluoroscopy exposure time showed a significant difference among the groups ($p < 0.05$). Lateral and posterior APs required longer times for localization, catheter stabilization, and ablation, as well as greater number of RF energy pulses compared to other AP localizations.

The extended procedure duration for ablations targeting AP on the free wall is also documented in the literature[22,24]. This is attributed to complex anatomy of mitral valve (MV), including the presence of mitral chordae and papillary muscles.

Unlike the TA approach, the TS approach offers improved catheter maneuverability, particularly along the lateral atrial wall. However, it is associated with reduced catheter stability, which can lead to transient AP disruption. In our study, the TS approach was employed in 80% of lateral AP ablations, which is in line with recommendations from the literature[23].

The posteroseptal region presents a greater level of complexity, as it includes the CS[29], which is surrounded by muscle fibers from both atria. This anatomical arrangement allows muscular bundles to pass through and establish electrical connections between the atria and ventricles. These connections often involve the vein[30] and ligament of Marshall (*Figure 10*), as well as the circumflex artery, frequently requiring epicardial mapping.

Although rare, epicardial connections (more commonly found on the right side) pose a challenge since they account for 10% of unsuccessful ablations. On the other hand, proximal CS cisterns and diverticula contribute to failure in about 8% of cases[30,31].

In our study, one patient exhibited atrial insertion of a left posteroseptal AP within a CS diverticulum.

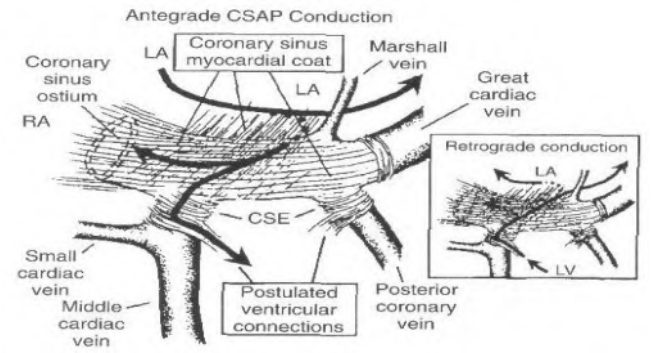

Figure 10. Schematic representation of possible anatomical connections between the musculature of the left atrium (LA) and right atrium (RA) via the coronary sinus (CS) and the myocardium of the left ventricle (LV).

Challenging access to the ventricular insertion is the primary factor contributing to prolonged procedure durations and a higher number of RF energy pulses in cases where the APs are localized on the free wall of the LV. Comparable procedure durations have been documented in previous studies[23]. Notably, even longer durations have been reported when using the transseptal approach, with Fisher and Swartz reporting an average duration of 2.8 ± 0.9 hours and Manolis et al. 5.4 ± 1.9 hours[32,33].

Consistent with these findings, the lowest primary success rate of RFA was observed in lateral (82.1%) and posterior APs (88.9%), in contrast to 100% success rate in all other AP localizations. Similar variations in acute RFA success rates based on AP localizations have also been previously reported in the literature[34,35,36].

At the three-month follow-up, the overall recurrence rate among patients with initially successful ablation was 6.8%. When analyzed by AP localization, the highest recurrence rate was observed in the group with laterally positioned APs (10.7%), followed by posterior APs (5.5%).

Primary RFA success and recurrence rates are influenced by multiple factors. Reported recurrence rates for left-sided APs vary across studies. For instance, Vora et al. reported a reccurence rate of 16% with the TA approach, while Manolis et al. observed 11% with the same approach. In contrast, Katritis reported rates of 4% with the TA and 5% with the TS approach, Yip et al. reported 4% with the TS approach, and De Ponti documented a significantly lower rate of 1.2% using the TS approach[25,32,33].

The most commonly reported causes of ablation failure include inaccessibility of the optimal ablation site in 25% of cases, catheter instability in 23%, the presence of epicardial APs in 8%, mapping inaccuracies in 11%, and AF recurrence in 3%. Furthermore, both the operator's experience and the technical capabilities of the medical represent critical determinants of procedural success.

According to published data, the highest RFA successs rate are observed in left lateral APs, with reported rates reaching 90%[34,35]. In our study, the success rate for this group was 89.3%, while for posterior APs the success rate equaled to 94.5%. Although the highest final success rate (100%) in the treatment of WPW syndrome was observed in patients with anterior, anterolateral, and posterolateral APs, the difference in RFA success among various AP localizations along the MA were not statistically significant.

Limitations of the study

The study demonstrated high overall procedural success and a low complication rate, supporting the effectiveness and safety of catheter ablation in examined population. However, some limitations should be considered when interpreting the findings of this study. The study was conducted at a single tertiary care center (single-center study), which may limit the generalizability of the results to other institutions with differing patient populations, procedural protocols, and operator expertise. Although the study included 58 consecutive patients with manifest preexcitation and left-sided APs, providing a well-defined and clinically relevant sample, patients were divided across five anatomical subgroups of APs. Since some groups included relatively few patients, the statistical power to detect subtle differences, particularly in procedural success or recurrence rates, may be limited.

CONCLUSIONS

There were no significant differences in patient age or sex distribution across the various AP localizations along the MA. However, both the procedure duration and the number of RF pulses were significantly higher for ablations targeting lateral and posterior APs compared to other AP localizations. Although the fluoroscopy exposure time tended to be longer in these groups, the difference was not statistically significant.

While some variation in primary ablation success and recurrence rates was observed among patients with WPW syndrome and different AP localizations along the MA, these differences did not reach statistical significance.

Overall, RFA remains a safe and effective treatment modality for the elimination of left-sided APs, demonstrating a high success rate and low incidence of complications.

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A COMPARATIVE ANALYSIS OF THE INFRAPATELLAR FAT PAD AND SUBCUTANEOUS ADIPOSE TISSUE AS PROVIDERS OF MESENCHYMAL STEM CELLS WITH CHONDROGENIC POTENTIAL: QUANTITATIVE ASSESSMENT THROUGH IMMUNOHISTOCHEMICAL METHODS

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ABSTRACT

The limited regenerative capacity of articular cartilage necessitates effective treatment methods for its repair. Infrapatellar fat pad (IPFP)-derived adipose stem cells (ASCs) demonstrate superior chondrogenic potential compared to subcutaneous adipose tissue (SCAT)-derived ASCs, making IPFP a promising source for cartilage regeneration. This study aimed to compare the quantitative expression of chondrogenic markers in mesenchymal stem cells (MSCs) derived from the IPFP and SCAT. Biopsy samples were collected from 25 patients undergoing knee osteoarthritis treatment. Histological and immunohistochemical analyses were performed on IPFP and SCAT samples, focusing on CD44, CD166, and SOX9 markers. The IPFP samples exhibited significantly higher relative numbers of CD44+ (13.28%), CD166+ (10.34%), and SOX9+ (7.30%) cells compared to SCAT samples, where values were 1.40%, 1.10%, and 0.90%, respectively. The differences were statistically significant ($p < 0.05$). IPFP-ASCs showed enhanced stability in marker expression, suggesting their specialization for chondrogenic differentiation. IPFP is a superior source of MSCs for cartilage repair, with a significantly higher presence of CD44+, CD166+, and SOX9+ cells compared to SCAT. These findings highlight the potential of IPFP-derived ASCs in regenerative cartilage therapy and underscore the importance of their anatomical proximity to cartilage tissue.

Keywords: *Articular cartilage, infrapatellar fat pad, mesenchymal stem cells, chondroprogenitors, cartilage regeneration.*

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INTRODUCTION

The physiological structure of articular cartilage limits its intrinsic regenerative capacity [1]. This constraint underscores a clinical need for effective and safe treatment methods. Consequently, it drives the ongoing development of therapies aimed at cartilage regeneration and repair. However, two-stage surgical procedures pose significant challenges. One notable issue is the potential dedifferentiation of chondrocytes cultured *ex vivo*. These drawbacks have heightened the demand for alternative strategies, with a primary emphasis on novel, predominantly one-stage procedures [2].

For many years, researchers have aimed to identify a substantial population of suitable cells for repairing damaged or diseased hyaline cartilage. Several promising cell-based strategies for cartilage therapy have been investigated. These include the use of autologous mature chondrocytes, bone marrow-derived stem cells, and adipose tissue-derived cells. Despite advances, these sources face limitations, such as variable chondrogenic potential in bone marrow (mesenchymal stem cells) MSCs, scalability issues with chondrocytes, and lower regenerative capacity in subcutaneous adipose-derived cells compared to intra-articular sources. Nevertheless, many current methods – along with those not yet fully implemented in clinical practice – encounter evident or anticipated limitations. Such constraints may hinder their effectiveness in cellular cartilage repair. The application of chondroprogenitors in cell therapy offers a potentially innovative and more effective solution. This approach addresses at least some of the existing challenges [3].

Over the past decade, numerous studies have evaluated the immunophenotype and differentiation potential of adipose-derived stromal cells from the infrapatellar fat pad (IPFP) [4–6]. A study by SONG Sai-sai et al. (2020) found no significant differences in the expression of stem cell surface protein markers between human subcutaneous adipose tissue stem cells (SC-ASC) and infrapatellar fat pad stem cells (IPFP-ASC). However, the proliferation and chondrogenic potential of human IPFP-ASC were superior to those of SC-ASC in vitro. Their therapeutic effect on rat osteoarthritis in vivo also outperformed SC-ASC [7]. IPFP-ASC demonstrate a clear advantage in chondrogenic differentiation, whereas SC-ASC exhibit greater osteogenic differentiation. This finding is supported by the detection of SOX-9, a chondrogenic transcription factor, during differentiation assays [8]. Stem cells expressing this marker are present in the IPFP. Due to its anatomical location, the IPFP may positively contribute to cartilage regenerative processes. However, several questions remain unresolved. These include the quantitative characteristics of these cells, their comparison with other common adipose tissue sources, and their potential for clinical application.

MATERIALS AND METHODS

The study was conducted in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of Zaporizhzhia State Medical and Pharmaceutical University (Protocol No. 8, December 26, 2024). All patients participating in the study were informed about the surgical intervention plan and the use of their biopsy samples for scientific research. They provided signed informed consent.

For this study, data analysis was performed on 25 patients. This group included both male and female participants who underwent surgical or combined treatment for knee osteoarthritis. Patients were selected based on the following inclusion criteria: confirmed diagnosis of knee osteoarthritis (Kellgren-Lawrence grade 2–3), age 40–70 years, and absence of systemic inflammatory diseases or malignancies. Exclusion criteria included prior knee joint replacement, severe comorbidities, or use of immunosuppressive therapy within six months prior to surgery. Histological examination was conducted on biopsy samples from Hoffa's fat pad and abdominal subcutaneous tissue. Macroscopic evaluation and standard tissue processing were performed, including hematoxylin-eosin staining. Microscopy was carried out using a Carl Zeiss Scope.A1 microscope (Germany). The microscope was equipped with a Progres Gryphax Jenoptik 60N-C1" 1.0x426114 camera (Germany), connected to a computer. The Progres Gryphax 1.1.4.2 digital analysis software (Jenoptik Optical System, Germany) was used to capture microphotographs and perform subsequent image analysis.

Morphological study

Immunohistochemical analysis was performed on serial paraffin sections of biopsy samples from Hoffa's fat pad (n = 15) and abdominal subcutaneous tissue (n = 10). Monoclonal antibodies used included CD44 (clone SP37, Vitro, Spain), CD166 (clone F48292, BIOZOL, Germany), and SOX9 (clone EP317, Vitro, Spain). For the analysis, paraffin blocks containing tissue fragments were sectioned into 4 μm slices. These slices were deparaffinized and rehydrated. High-temperature antigen retrieval was conducted by heating the sections in Tris-EDTA buffer (pH 9.0) in a water bath to unmask the antigens. Endogenous peroxidase activity was blocked with a 3% hydrogen peroxide solution. This was followed by the application of a blocking serum. Incubation with primary antibodies was carried out according to the manufacturers' instructions. The DAKO EnVision+ System with diaminobenzidine (DAB) (DAKO, USA) was used to visualize the immunohistochemical reaction. The sections were then counterstained with Mayer's hematoxylin, dehydrated, and mounted with Canadian balsam.

Mesenchymal stem cell populations were identified by the parallel detection of multiple positive and negative markers. This identification was based on the minimal criteria established by the International Society for Cellular Therapy. Evaluation was conducted in five standardized fields of view. A Carl Zeiss Scope.A1 microscope (Germany) with a Progres Gryphax Jenoptik 60N-C1" 1.0x426114 camera (Germany) was used at ×200 magnification for each case. Digital images of microscopic specimens were obtained. The relative quantity (%) of cells expressing CD44, CD166, and SOX9

was calculated for each standardized field of view at ×200 magnification.

Statistical processing of research results

Statistical processing of the obtained results was performed using a personal computer. The statistical package Statistica® for Windows 13.0 (StatSoft Inc., license No. JPZ804I382130ARCN10-J) was employed. Non-parametric statistical methods were used for the analysis.

To assess the hypothesis of normality for the distribution of the studied variables, the Shapiro–Wilk test was applied. The results showed that the data in the comparison groups were not normally distributed. Therefore, the median and interquartile range (Me [Q1; Q3]) were reported. The non-parametric Mann–Whitney U-test was used to compare independent samples. In all analyses, differences were considered statistically significant at $p < 0.05$.

Results. According to the results of immunohistochemical and statistical analyses (Table 1, Fig. 1), biopsy samples from the infrapatellar fat pad (IPFP) and subcutaneous adipose tissue (SCAT) showed notable differences. The relative number of cells expressing CD44, CD166, and SOX9 markers in a standard field of view (×200) was 30.92% in IPFP and 3.4% in SCAT. This suggests that the relative abundance of these cells in IPFP was 9.09 times higher than in SCAT. The observed difference was statistically significant ($p < 0.05$) across the entire marker profile. Statistically significant differences were also identified for individual markers (Fig. 1).

The study results revealed a significant difference in the relative number of CD44-positive cells between the IPFP and subcutaneous adipose tissue SCAT. In the IPFP biopsy, the relative number of CD44-positive cells was 13.28% (8.57; 12.78). In the SCAT biopsy, it was 1.40% (1.00; 2.00) (Table 1, Figs. 1, 2). This represents a 9.48-fold increase. The difference was statistically significant ($p < 0.05$).

Table 1. The relative number of cells expressing CD44+, CD166+, SOX9+ in the biopsy material of infrapatellar fat pad (IPFP) and abdominal subcutaneous adipose tissue (SCAT)

GROUP	MARKER	ME (%)	Q1 (%)	Q3 (%)	IQR (%)	CV (%)
SCAT	CD166+	1.10	0.80	1.50	0.70	63.64
	CD44+	1.40	1.00	2.00	1.00	71.43
	Sox9+	0.90	0.60	1.20	0.60	66.67
IPFP	CD166+	10.34	8.22	12.78	4.56	44.10
	CD44+	13.28	8.57	15.11	6.54	49.25
	Sox9+	7.30	6.71	8.02	1.31	17.95

Similarly, the relative number of CD166-positive cells was 10.34% (8.22; 12.78) in the IPFP biopsy. In the SCAT biopsy, it was 1.10% (0.80; 1.50) (Table 1, Figs. 1, 2). This indicates a 9.4-fold increase. This difference was also statistically significant ($p < 0.05$).

For SOX9-positive cells, the relative number in the IPFP biopsy was 7.30% (6.71; 8.02). In the SCAT biopsy, it was 0.90% (0.60; 1.20) (Table 1, Fig. 2). This reflects an 8.1-fold increase. The difference was statistically significant ($p < 0.05$).

The obtained data highlight several findings. In subcutaneous adipose tissue, the coefficients of variation (CV) range from 63.64% to 71.43%. This reflects a significant spread of values within this group. The greatest variation occurs for CD44-positive cells. In the infrapatellar fat pad, the CV is lower than in SCAT. This is particularly evident for SOX9-positive cells (17.95%), suggesting greater data stability in IPFP. The greatest spread in IPFP is observed for CD44-positive cells (49.25%).

The obtained data confirm the presence of adipose-derived mesenchymal stem cells (MSCs) with an immunohistochemical profile of CD44, CD166, and SOX9. These cells were identified in biopsies from both Hoffa's fat pad and subcutaneous adipose tissue. The difference in the number of these cells between the two tissues was statistically significant.

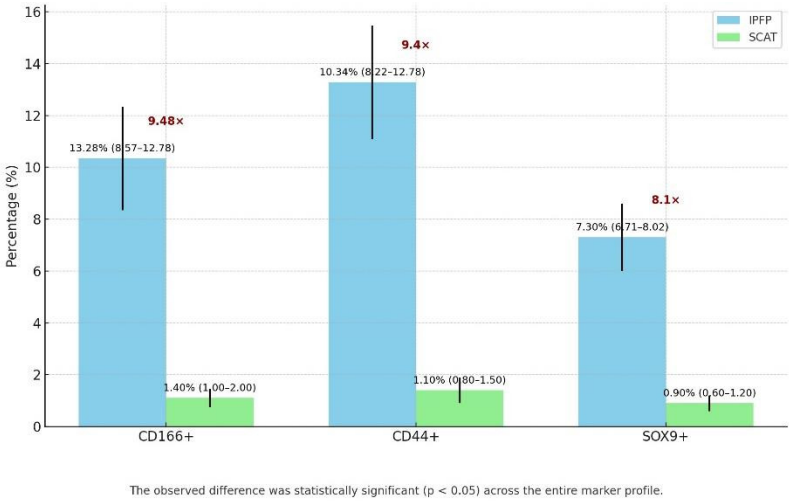

Figure 1. Comprasion of Marker-Positive Cells in the infrapatellar fat pad (IPFP) and abdominal subcutaneous adipose tissue (SCAT)

Analysis of the results based on individual marker expression revealed distinct patterns. The relative number of SOX9-positive cells was significantly higher in the infrapatellar fat pad (IPFP) than in subcutaneous adipose tissue. Similarly, the expression of CD44 and CD166 was statistically significantly greater in the IPFP. This difference may be due to the greater diversity of cellular composition in the IPFP. It could also reflect a higher number of inflammatory cells, possibly resulting from traumatic or non-traumatic changes in the knee joints of the donors.

The anatomical location of the tissue likely plays a crucial role. MSCs from subcutaneous adipose tissue show moderate SOX9 expression, consistent with basal levels. In contrast, the infrapatellar fat pad is a specialized tissue located intracapsularly in the knee joint. It serves unique functions, including load cushioning, maintenance of cellular homeostasis in the joint, and participation in reparative processes. A key difference between the two adipose tissues lies in functional adaptation. MSCs in Hoffa's fat pad are specialized for maintaining and restoring articular tissues, such as cartilage, which requires elevated SOX9 activity. Conversely, subcutaneous adipose tissue primarily functions in energy storage and insulation, reducing the need for SOX9-mediated processes.

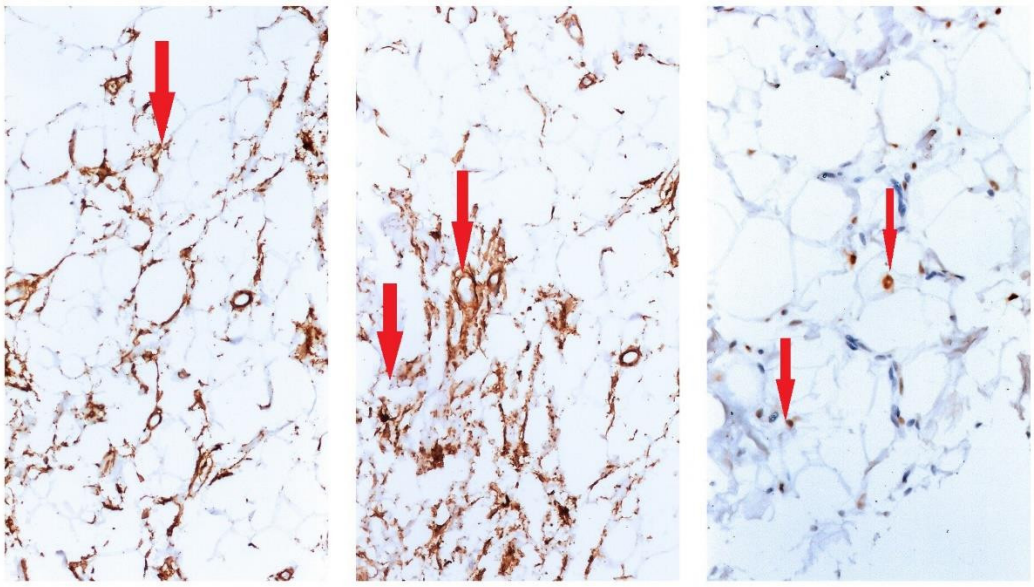

Figure 2. Immunohistochemical staining of biopsy samples from infrapatellar fat pad showing expression of CD44, CD166, and SOX9 markers (brown staining). Arrows

The microenvironment also contributes significantly. The proximity of cartilage tissue and other joint structures influences the receptor profile and growth factors in the IPFP. These factors stimulate chondrogenic differentiation pathways. Mechanical signals during joint loading further enhance this effect.

However, the study has limitations that must be acknowledged. The sample size was limited, with 15 tissue samples from the infrapatellar fat pad and 10 from subcutaneous adipose tissue. Additionally, the use of the non-parametric Mann–Whitney test has drawbacks. While it does not require normality of distribution or equality of variance, it is less rigorous than the parametric Student's t-test for independent samples.

Discussion. The infrapatellar fat pad was, until recently, viewed primarily as a large vascular structure. It is innervated by intracapsular and extrasynovial tissue and plays a biomechanical role in the anterior compartment, protecting the knee joint. Over time, researchers recognized that the IPFP and the synovial membrane function as a single unit. Their close molecular interactions have become evident. This discovery has highlighted new roles for the IPFP in the pathophysiology of joint diseases, including osteoarthritis. It has also revealed characteristics of resident cells associated with the immune system, which exhibit diverse phenotypes.

This structural complex is increasingly recognized as a potential therapeutic target for patients with various knee joint pathologies. The presence of mesenchymal stem cells (MSCs) as perivascular cells in the IPFP is notable. These cells demonstrate immunomodulatory, antifibrotic, and neutralizing activities. Such properties align with those of key mediators in the mesentery. As a result, the IPFP emerges as an alternative source of MSCs for clinical cell-therapy protocols [9]. The molecular basis for these therapeutic effects involves the secretion of bioactive molecules, such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β), which modulate inflammation and promote tissue repair. Studies have shown that IPFP-derived MSCs suppress proinflammatory cytokines like IL-1β and TNF-α, contributing to an anti-inflammatory microenvironment conducive to cartilage regeneration [10].

The intraarticular location of the IPFP and its anatomical proximity to cartilage are significant. It is not surprising that IPFP-derived adipose stem cells show a strong affinity for chondrogenic differentiation in vitro. Specifically, IPFP-ASC exhibit a greater capacity for chondrogenic differentiation compared to adipose-derived stem cells from other anatomical locations. This capacity also surpasses that of MSCs from bone marrow and umbilical cord. However, some studies suggest that the chondrogenic potential of IPFP-ASC is at least comparable to that of bone marrow-derived MSCs. Still, it remains lower than that of native chondrocytes and perivascular IPFP-ASC. The enhanced chondrogenic potential of IPFP-ASCs is likely driven by the upregulation of key transcription factors, such as SOX9, and the production of extracellular matrix (ECM) components, including type II collagen and aggrecan, under the influence of TGF-β signaling pathways. TGF-β activates the SMAD2/3 signaling cascade, which directly regulates SOX9 expression, a master regulator of chondrogenesis [11].

Research based on these findings indicates that selective isolation of specific IPFP-ASC subpopulations can enhance their chondrogenic differentiation capacity. Additionally, perivascular IPFP-ASC produce significantly more extracellular matrix than heterogeneous, untreated IPFP-ASC cultures. This difference may be attributed to the enriched expression of perivascular markers, such as CD146, which correlate with higher proliferative and differentiation potential. Recent studies further confirm that CD146+ IPFP-ASC subpopulations demonstrate superior chondrogenic potential due to their enhanced expression of cartilage-specific genes, such as Col-2A1 and ACAN, highlighting their promise for targeted regenerative therapies [12].

In vivo studies show promising results. Freshly isolated, uncultured CD44+ IPFP-ASC were seeded in a TGF-β3-derived extracellular matrix (ECM). When subcutaneously implanted into nude mice, these cells generated cartilage-like tissue. This tissue was rich in sulfated glycosaminoglycans (sGAG) and type II collagen. Chondroprogenitor cells, a subpopulation of multipotent progenitor cells, are primed for chondrogenesis. These cells differ from articular chondrocytes. They exhibit a high affinity for fibronectin, high colony formation efficiency, and expression of the Notch1 gene. Notch1 signaling has been implicated in maintaining stemness and promoting early chondrogenic commitment, further supporting the therapeutic promise of these cells [13].

Several markers are commonly linked to the chondrogenic potential of mesenchymal stem cells and chondroprogenitor cells in current scientific literature. These include CD44, CD49c, CD166, SOX9, and Col-2 [14-16]. Due to their strong proliferative and chondrogenic differentiation capacities, these cells can play a significant role. They contribute to the regeneration and repair of cartilage in osteoarthritis. CD44, a hyaluronic acid receptor, facilitates cell-matrix interactions critical for chondrogenesis, while SOX9 orchestrates the expression of cartilage-specific genes. The molecular interplay between these markers and signaling pathways, such as Wnt/β-catenin and Hedgehog, further modulates chondrogenic differentiation and ECM production [14].

CD44 is a common marker for both chondrocytes and chondroprogenitor cells in normal cartilage, while CD90, CD105, and CD166 are common markers for chondroprogenitor cells in both normal and damaged cartilage [17].

In the current literature, there are very few reports on the quantitative characterization of chondroprogenitor cells in adipose tissue, which could potentially be used in one-step procedures for treating musculoskeletal diseases. This gap underscores the need for standardized protocols to quantify chondroprogenitor subpopulations in IPFP, which could

streamline their clinical translation for single-stage cartilage repair procedures.

In healthy cartilage tissue, CD166+ cells are present at a level of 15.3 ± 2.3%, according to FACS analysis [18]. This value closely aligns with the results of our study on IPFP biopsy material. It supports the theory of their similarity to surrounding cells in regenerative restoration.

In our study, SOX9+ cells were identified in freshly isolated tissue samples from Hoffa's fat pad. We used immunohistochemical analysis with digital image processing. The relative number of SOX9+ cells was 7.30% (6.71; 8.02). Literature data, based on PCR analysis of SOX9 gene expression in intra-articular structures like Hoffa's fat pad, indicate higher levels of chondrogenic gene expression compared to extra-articular structures, such as subcutaneous tissue. These findings are consistent with our results. They emphasize the need for further studies on the therapeutic potential of these cells [19]. The elevated SOX9 expression in IPFP may be influenced by the local microenvironment, including synovial fluid-derived growth factors like TGF-β and BMP-2, which enhance chondrogenic gene expression [20]. Recent quantitative proteomic analyses have identified BMP-2 as a critical regulator of SOX9 expression in IPFP-ASCs, suggesting that targeted modulation of BMP signaling could enhance their chondrogenic potential in clinical applications [21].

Stem cell therapy using cartilage progenitor stem cells shows promise as an important approach for treating osteoarthritis. These cells are a subset of stem cells with enhanced proliferation, chemotaxis, and significant potential to differentiate into chondrocytes. Stem cells isolated from the infrapatellar fat pad benefit from their anatomical location. This location influences the characteristics of adipose stem cells and enhances the chondrogenic differentiation potential of IPFP-derived ASCs. The close proximity of IPFPs to the synovial membrane and fluid likely contributes to this higher potential by exposing cells to a milieu rich in chondrogenic inducers. For instance, synovial fluid contains high levels of hyaluronic acid and TGF-β, which synergistically promote MSC differentiation into chondrocytes. As a result, IPFPs can be considered a valuable resource for regenerative cartilage therapy. The therapeutic potential of mesenchymal stem cells from Hoffa's fat pad makes it an important source of adipose-derived stem cells. These cells can be utilized for regenerative engineering treatments, especially for cartilage tissue.

To maximize the clinical impact of these findings, key takeaways include the superior chondrogenic potential of IPFP-ASCs compared to other MSC sources, driven by their unique molecular profile and anatomical advantages. The identification of specific subpopulations, such as CD146+ and SOX9+ cells, offers a pathway for developing targeted cell-based therapies for osteoarthritis. However, challenges remain, including the need for standardized isolation techniques and larger-scale clinical trials to validate efficacy and safety.

Future research should focus on several critical directions. First, optimizing the isolation and expansion of chondroprogenitor subpopulations from IPFP could enhance their therapeutic efficacy. Second, longitudinal studies are needed to assess the durability of IPFP-ASC-derived cartilage repair *in vivo*, particularly in human osteoarthritis models. Third, integrating advanced biomaterials with IPFP-ASCs could improve cell delivery and retention at the defect site, addressing current limitations in cartilage regeneration. Finally, exploring the role of novel signaling pathways, such as Hedgehog and BMP, in regulating IPFP-ASC chondrogenesis could uncover new therapeutic targets to enhance cartilage repair outcomes.

In conclusion, the molecular mechanisms underlying the therapeutic effects of IPFP-derived MSCs and their chondrogenic potential involve a complex interplay of signaling pathways (e.g., TGF-β/SMAD, Notch, Wnt) and the expression of cartilage-specific genes (e.g., SOX9, Col-2). These mechanisms are supported by the immunomodulatory properties of MSCs and their ability to produce a cartilage-like ECM. Further exploration of these pathways and their regulation could optimize the use of IPFP-ASCs in clinical settings, offering a promising avenue for OA treatment.

CONFLICT OF INTERESTS

The authors report there are no competing interests to declare.

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SERUM LEVELS OF 25-HYDROXYVITAMIN D AND BONE TURNOVER MARKERS IN FEMALE BASKETBALL PLAYERS: AGE-RELATED DIFFERENCES

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ABSTRACT

The aims of this study were to 1) quantify 25-hydroxyvitamin D [25(OH)D] status during the midpoint of the in-season phase when the sun exposure is lowest (in late Fall); 2) provide age-specific reference data for serum markers of bone turnover in middle adolescent (15-18 years) and late adolescent to early adulthood (19-30 years) female basketball players. Fasting morning blood samples (5 mL) were drawn from an antecubital vein to determine circulating levels of 25(OH)D, osteocalcin, and carboxy-terminal telopeptides of crosslinks of type I collagen (CTx-I). Independent T-test [25(OH)D and osteocalcin] or Mann-Whitney U test (CTx-I) were used to identify differences between groups. Considering 25(OH)D concentration, 21 participants (80.7%) displayed vitamin D insufficiency (50-75 nmol/L), three participants (11.5%) displayed vitamin D deficiency (<50 nmol/L), and two participants (7.7%) were vitamin D sufficient (>75 nmol/L). Significant differences were observed in bone turnover markers (osteocalcin: p = 0.003 and CTx-I: p = 0.03) between groups, whereby middle adolescent female basketball players displayed significantly higher serum levels of osteocalcin (46.1 ± 15.8 ng/ml) and CTx-I (1018.8 ± 271.4 pg/ml) compared to late adolescent to early adulthood female basketball players (osteocalcin: 30.4 ± 7.6 ng/ml and CTx-I: 776.7 ± 240.8 pg/ml). A high prevalence (92.2%) of vitamin D inadequacy (insufficiency and deficiency) was observed in the players examined in our study. Middle adolescent female basketball players possess a higher bone turnover rate than late adolescent to early adulthood female basketball players.

Keywords: *Vitamin D insufficiency, bone health, osteocalcin, bone metabolizam.*

INTRODUCTION

Bone is a metabolically active tissue with constant remodeling throughout the lifespan. Biochemical markers of bone turnover provide insight into dynamic process of bone formation and bone resorption (1). Previous studies (2, 3) observed almost constant levels of bone turnover markers between the age of 19 and 30 in the general population, suggesting that bone turnover is relatively constant in this period of life. On the other hand, rising levels of biochemical bone turnover markers have been observed in children during growth and puberty. While bone turnover markers mainly represent bone remodeling in adults, in children they are associated with the processes of bone modeling, remodeling and growth in length (4).

Although bone turnover markers have been identified as important determinants for the prediction of fracture risk and repair of microinjuries, as well as in monitoring the rate of linear growth in children (3), limited attention has been given to bone remodeling in female basketball players. Most of the available data give reference ranges for serum markers of bone formation and resorption in the general population, but only few studies were undertaken in basketball players [early pubertal male basketball players (5) and late adolescent to early adulthood female basketball players (18-26 years) (6)], with no reference data available in middle adolescent female basketball players (15-18 years). Data collected in the general population cannot be transferred to athletes due to the adaptations (BMD accretion) that occur in response to their high-impact loading during exercise (7). In this way, bone turnover screening may be of clinical utility in monitoring growth in children.

The rapid process of bone modeling and remodeling with progression through puberty could potentially increase the need for vitamin D in this accretive phase of life (8). Vitamin D receptors identified in human cells of the bone and skeletal muscles have been positively associated to bone mineralization and the synthesis of muscle proteins (9). Although vitamin D may originate from the diet, the principal source is obtained through sunlight ultraviolet-B (UVB) exposure of the skin. In this regard, seasonal variations are observed in vitamin D status, with low serum 25-hydroxyvitamin D [25(OH)D] levels in winter and high levels in summer (10). Therefore, it may be presumed that indoor athletes are more susceptible to vitamin D insufficiency in winter, and that this would adversely affect bone health.

The aims of this study were to 1) quantify 25(OH)D status during the midpoint of the in-season phase when the sun exposure is lowest (in late Fall); 2) provide age-specific reference data for serum markers of bone turnover in middle adolescent (15-18 years) and late adolescent to early adulthood (19-30 years) female basketball players. We hypothesized that a high prevalence of vitamin D inadequacy (deficiency and insufficiency) would be observed. Also, it was hypothesized that younger female basketball players posses higher bone turnover rate.

METHODS

Participants

Twenty six female basketball players from two teams competing in the first and second division of the Serbian National League were recruited for this study. Participants from the first division [n = 14, age: 22.8 ± 4.6 (range: 19-30 years)] were training 8 x 90 min and playing a maximum 2 games per week. Participants from the sesond division [n = 12, age: 16.8 ± 1.3 (range: 15-18 years)] were training 5 x 90 min and playing one game per week. Participants provided written informed consent prior to participation, including guardian consent for participants <18 years of age. The procedures were approved by the Human Research Ethics Committee of the Faculty of Medical Sciences at the University of Kragujevac.

Procedures

A cross-sectional, desrciptive research design was adopted with screening conducted at the midpoint of the in-season phase, over a 2-week period in late Fall. Fasting morning blood samples (5 mL) were drawn from an antecubital vein to determine circulating levels of 25(OH)D, osteocalcin (formation marker), and carboxy-terminal telopeptides of crosslinks of type I collagen (CTx-I – resorption marker). To minimize cyclic variations in bone turnover markers (11) and the impact of prior activity (12), all blood samples were collected during the midfollicular phase to earlyluteal phase of the menstrual cycle, 36 h following exercise. Participants were also required to choose the self-perceived stage of pubertal development by comparing their state with illustrations of breast development and pubic pilosity representing five different states (13).

Biochemical analysis

After centrifugation at 3000 rpm for 10 min, serum was aliquoted and analyzed within 3 h. Serum 25(OH)D (Roche, Cobas e 411 analyzer, Roche Diagnostics GmbH, Mannheim, Germany), osteocalcin, and CTx-I (Roche-modular E170 analyzer, Roche Diagnostics GmbH, Mannheim, Germany) were measured using elecrochemiluminescence assay. According to the manufacturer, Elecsys Vitamin D Assay (ref. 07464215190: <7.2% and <10.3%), Elecsys β-CrossLaps Assay (ref. 11972316122: <4.7% and <5.7%) and Elecsys N-MID Osteocalcin (ref. 12149133122: <4.0% and <6.5%) possessed acceptable repeatability and intermediate precision. Vitamin D status was categorized as follows (14): I) optimal 25(OH)D >75 nmol/L; II) insufficiency 25(OH)D 50-75 nmol/L; and III) deficiency 25(OH)D <50 nmol/L.

Statistical analysis

The Shapiro Wilks test confirmed the normality of distribution for 25(OH)D and osteocalcin, while CTx-I was not normally distributed. Independent T-test [with 95% confidence intervals (CI)] was used to identify differences in 25(OH)D and osteocalcin between middle adolescent and

late adolescent to early adulthood female basketball players. Difference in CTx-I between middle adolescent and late adolescent to early adulthood female basketball players was assessed using Mann-Whitney U test. Statistical significance was accepted at $p < 0.05$.

RESULTS

Median, minimum, maximum and corresponding inter-quartile range (25 and 75th centiles) for 25(OH)D, osteocalcin, and CTx-I are presented in Figure 1. Mean, SD, geometric mean, and 95% CI for 25(OH)D, osteocalcin, and CTx-I are presented in Table 1. Three participants had Tanner stage 4, while all remaining participants had Tanner stage 5. Considering 25(OH)D concentration, 21 participants (80.7%; 15-18 years: n = 11; 19-30 years: n = 10) displayed vitamin D insufficiency (50-75 nmol/L), three participants (11.5%; 15-18 years: n = 1; 19-30 years: n = 2) displayed vitamin D deficiency (<50 nmol/L), and two participants (7.7%; 19-30 years: n = 2) were vitamin D sufficient (>75 nmol/L).

Figure 1. Median, mainimum, maximum and corresponding inter-quartile (25 and 75th centile) range for 25(OH)D, osteocalcin, and CTx-I in middle adolescent (n = 12) and late adolescent to early adulthood (n = 14) female basketball players. In the box plots, whiskers indicate the minimum and maximum values, the boundary of the box closest to zero indicates the 25th percentile, a black line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile.

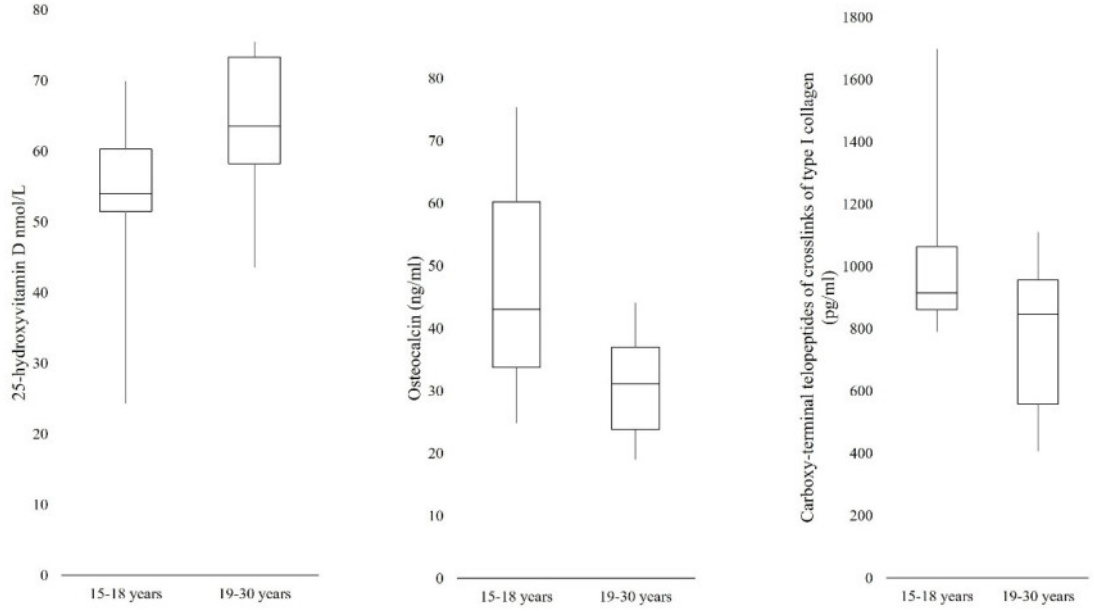

Table 1. Mean, standard deviation (SD), geometric mean, and 95% confidence intervlas (CI) for 25-hydroxyvitamin D, osteocalcin, and carboxy-terminal telopeptides of crosslinks of type I collagen (CTx-I) in middle adolescent (15-18 years) and late adolescent to early adulthood (19-30 years) female basketball players.

Outcome measures	mean ± SD	Geometric mean	95% CI
25-hydroxyvitamin D (nmol/L)			
15-18 years	55.6 ± 12.2	52.7	47.8, 63.3
19-30 years	63.7 ± 10.5	62.8	57.6, 69.8
Osteocalcin (ng/ml)			
15-18 years	46.1 ± 15.8	43.8	32.9, 53.1
19-30 years	30.4 ± 7.6	29.5	26.0, 34.8
CTx-I (pg/ml)			
15-18 years	1018.8 ± 271.4	991.4	846.4, 1191.2
19-30 years	776.7 ± 240.8	737.7	637.7, 915.7

No significant difference in 25(OH)D ($p = 0.09$) was observed between middle adolescent and late adolescent to early adulthood female basketball players. Significant differences were observed in bone turnover markers (osteocalcin: $p = 0.003$ and CTx: $p = 0.03$) between groups, whereby middle adolescent female basketball players displayed significantly higher serum levels of osteocalcin and CTx-I compared to late adolescent to early adulthood female basketball players

DISCUSSION

In support of our hypothesis, a high prevalence of vitamin D inadequacy (insufficiency and deficiency) was observed in female basketball players (92.2%) examined in the present study. In addition, middle adolescent female basketball players possessed a higher bone turnover rate compared to late adolescent to early adulthood female basketball players.

The prevalence rate of vitamin D inadequacy we observed is congruent with results reported in other investigations examining basketball players (9, 15-20). Previously, similar prevalence rate of vitamin D inadequacy, ranging from 77% to 95% has been observed in female and male basketball players (9, 15-20). The limited sunlight exposure in late Fall (November) and within indoor environments from training and competition may negatively affect vitamin D status, underpinning high prevalence of vitamin D inadequacy in female basketball players we observed. In addition to sun exposure, the high percentage of vitamin D inadequacy seen in female basketball players is likely multifactorial and perhaps related to reduced vitamin D intake and lifestyle factors (eg, sunscreen and clothing) yet to be evaluated. Collectively, our results and those made previously (9, 15-20) suggest that female basketball players possess a high risk of vitamin inadequacy in late Fall.

Bone turnover markers complements the static measures of bone tissue (i.e. bone mineral density), while detecting the dynamics of bone remodeling with respect to bone formation and resorption. Our findings showed that middle adolescent female basketball players possess greater bone turnover rate than late adolescent to early adulthood female basketball players. Age-related differences align with findings from previous research (4), showing lower values of bone turnover markers (osteocalcin and CTx-I) in the transition to adulthood. Circulating concentrations of osteocalcin vary by age and pubertal stage, with the highest levels being found throughout puberty (females: 11-12 years; males: 13-14 years) (21). The beginning of pubertal growth spurt is characterized by increases in estrogens and androgens (22). Rising levels of estrogen and other sex hormones are accompanied by the secretion of growth hormone and insulin-like growth factor I, which stimulates greater bone turnover during puberty compared to other phases of life (3).

In addition to variations with chronological age, our geometrical mean concentrations for osteocalcin [15-18 years: 43.8 ng/ml (95% CI 32.9, 53.1) and 19-30 years: 29.5 ng/ml (95% CI 26.0, 34.8)] and CTx-I [15-18 years: 991.4 pg/ml (95% CI 846.4, 1191.2) and 19-30 years 737.7 pg/ml (95% CI 637.7, 915.7)] are higher than those observed in the general population. In females aged 20-24 years and 25-29 years, Hu et al. (23) observed lower geometrical mean concentrations of osteocalcin [21.2 ng/ml (95% CI 18.7-22.5) and 18.5 ng/ml (95% CI 17.2-19.8)] and CTx-I [412 pg/ml (95% CI 354-470) and 335 pg/ml (95% CI 304-367)]. Furthermore, Paldánius et al. (24) also demonstrated lower concentration of osteocalcin (geometrical mean ± SD: 13.0 ± 3.6) in females between the age of 15.5-18.4 years. Collectively, findings from our study support the notion that mechanical loading of bone through long-term basketball training and competition produce an increase in osteocalcin and CTx-I, and there may be a need to use specific reference limits in basketball players to from correct conclusions about exercise-induced bone remodeling.

Some limitations of this study should be acknowledged. First, variations in bone turnover markers are more dependent on pubertal stage than chronological age. Therefore, additional studies are warranted to gather further insight into puberty-related differences in bone remodeling. Second, disparities in training frequency were not accounted across comparison groups. Although the bone turnover response to training frequency suggests an overlap (osteocalcin: $p = 0.07$ and CTx-I: $p = 0.14$) between athletes completing <8 h or >8 h training sessions per week (25), future investigations should include a matched sample size across training frequency to ensure the generalizability of data.

CONCLUSION

A high prevalence (92.2%) of vitamin D inadequacy (insufficiency and deficiency) was observed in the players examined in our study. In addition, middle adolescent (15-18 years) female basketball players possess a higher bone turnover rate than late adolescent to early adulthood (19-30 years) female basketball players.

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PROGNOSTIC SIGNIFICANCE OF *P21* PROTEIN IN BREAST CANCER

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ABSTRACT

Breast cancer is the most common malignancy in women. Bearing in mind these circumstances, a review of new molecular mechanisms underlying breast cancer progression, resistance and various aspects of existing therapeutic methods would lead to new insights for biologists and clinicians. In this regard, we conducted a study covering recent advances in breast cancer biology with a focus on the p21 protein. The study included 147 patients diagnosed with invasive breast cancer. The presence of non-invasive lesions was noted in each invasive breast cancer and surrounding tissue. p21 expression was determined by reading the percentage of nuclear expression in epithelial cells of invasive breast cancer and non-invasive lesions. Results showed that expression of p21 increases with the progression of cytological changes in the epithelium; it is significantly higher in invasive breast cancer compared to non-invasive lesions ($p<0.001$). There is a difference in p21 expression between different molecular subtypes of breast cancer ($p=0.004$). Statistically significantly higher values of p21 expression were observed in those breast cancers that showed overexpression of HER2 compared to HER2-negative tumors ($p=0.001$). Depending on Ki67 expression, the highest p21 expression is in the group with high Ki67 expression values ($p=0.019$). The increase in p21 expression in tumor cells was accompanied by a statistically significantly reduced expression of ER ($p=0.015$, $\rho=-0.225$) and PR ($p=0.027$, $\rho=-0.205$). p21 protein plays an important role in proliferation, malignant transformation, as well as in progression from non-invasive lesions to invasive breast cancer.

Keywords: *Cyclin-Dependent Kinase Inhibitor p21, breast cancer, molecular subtype, immunohistochemistry.*

INTRODUCTION

Breast cancer (*IBC*) is the most common malignancy (30% of all new tumor cases) and the second leading cause of tumor death in women (15% of all tumor deaths) (1) and is an important public health problem worldwide. In 2020, there were 2.3 million cases of illness and 685.000 deaths (2). In the Republic of Serbia, about 4.000 newly discovered cases of this disease are registered every year, which represents more than a quarter of all malignant diseases in women, with most European countries showing a decreasing trend in mortality from breast cancer, while Serbia, Poland and Romania have recorded less in the last period as well favorable trends.Thus, in 2017, the highest mortality rates were recorded in Serbia (20.6/100.000 women) (3).

In the past two decades, new therapies, such as immunotherapy and targeted therapies have emerged as a result of the discovery of new molecules and signaling pathways involved in breast cancer proliferation. However, a significant proportion of patients remain resistant to therapy or relapse, indicating an incomplete understanding of cancer metabolism (4). Bearing in mind these circumstances, a review of new molecular mechanisms underlying breast cancer progression, resistance and various aspects of existing therapeutic methods would lead to new insights for biologists and clinicians. In this regard, we conducted a study covering recent advances in breast cancer biology with a focus on the *p21* protein.

p21, a 21 *KDa* protein encoded by the *CDKN1A* gene, is a member of the *Cip/Kip* family of *CDKIs* in addition to *p27* and *p57*. *p21* is the first discovered transcriptional target of the *p53* protein. It is capable of inactivating all *CDKs,* thereby inhibiting cell cycle progression (5). It inhibits the kinase activity of the cyclin-*CDK* complex by interacting with cyclins via two cyclin binding motifs (*Cy1* and *Cy2*). This leads to inhibition of phosphorylation of the *pRb* family of proteins and subsequent association with *E2F* and formation of the *DREAM* complex, leading to cell cycle arrest (6, 7). *p21* plays a dual role in cell cycle progression depending on its expression level (8). High levels of *p21* inhibit the kinase activity of the cyclin-*CDK* complex leading to inhibition of cell cycle progression, while low levels act as a factor in the formation of the cyclin *D/CDK4,6* complex and promote its activation leading to cell cycle progression (9). Despite its role in arresting cell proliferation and its ability to promote differentiation and cell senescence, recent studies suggest that under certain conditions *p21* can promote cell proliferation and oncogenicity (10). Accordingly, *p21* is often misregulated in human cancers, but its expression, depending on the cellular context and circumstances, suggests that it may act as a tumor suppressor or as an oncogene (10).These conflicting observations have undoubtedly increased the importance of *p2*1 in the field of tumor biology. Moreover, to date, no consensus has been reached on the relationship between *p21* and clinicopathological parameters, and the characteristics of *p21* expression and its clinical/prognostic significance in human breast cancer remain unclear.

Since the data of previous researches were different, and also there is no consensus and clear guidelines regarding the use of *p21* expression in routine clinical diagnosis of breast cancer, we conducted this study to analyze the expression of *p21* in *IBC*, but also in non-invasive breast lesions (*NIL*) with the aim of determining whether *p21* is a useful adjunctive tool for distinguishing between *NIL* and *IBC*.

Furthermore, we examined the possible correlation of this marker with the clinicopathological characteristics of *IBC* as a possible link with its progression.

MATERIAL AND METHODS

Study Design

The study was conducted in accordance with the Declaration of Helsinki, approved by the decision of the Ethics Committee number 01/17/2290, included 147 patients with a diagnosis of breast cancer, who were diagnosed and treated at the University Clinical Center Kragujevac, Serbia in the period from 2012 to 2017.

Pathohistological analysis of tumors on *H&E* stained specimens was performed on the operative material obtained by tumerectomy, quadrantectomy and/or mastectomy with dissection of regional lymph nodes (11).

The presence of non-invasive lesions *(NIL)* [*in situ* lobular and ductal carcinoma *(ISC)*, lobular and ductal atypical hyperplasia (*AH*) and normal ductal and acinar epithelium (*NE*)] was noted in each *IBC* and surrounding tissue. Based on microscopic analysis, *IBCs* were classified into three groups: ductal, lobular, and a group of other histological types. E-cadherin expression differentiated ductal from lobular breast cancer. At the same time, all relevant macroscopic, pathohistological and prognostic parameters (tumor size, histological type and grade, nodal status, presence of necrosis, intra and peritumoral mononuclear infiltrate, perineural, lymphatic and vascular invasion, molecular subtype IBC and disease stage) were defined (12).

IBCs are classified according to the recommendations into four molecular groups: *Luminal A, Luminal B, HER2* + and *TNBC* (13).

Immunohistochemical (*IHC*) procedure

Tissue sections were fixed in 10% Neutral Buffered and stabilized formalin solution, pH 7.0 and embedded into paraffin. *IHC* was performed on a tissue section of a representative paraffin block of each patient. Tissue sections 4 μm thick were applied to adherent slides (*SuperFrost® Plus,* VWR, Leuven, Belgium), then deparaffinized in xylene and rehydrated in decreasing alcohol concentrations. After epitope retrieval, endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 minutes. The preparations were incubated

with primary monoclonal and polyclonal antibodies at room temperature for the recommended duration. The following antibodies were used, ready for use or in appropriate dilution: *p21$^{WAF1/CIP1}$* (SX118, 1:50, M7202, DAKO, Denmark), mAb *ER* (1D5, RTU, IR657, DAKO, Denmark), mAb *PR* (PgR636, RTU, IR068, DAKO, Denmark), pAb *HER2* (1:1200, AO485, DAKO, Denmark), *Ki67* (1:200, MIB-1, IR626, DAKO, Denmark). After washing the primary antibody, tissue sections were incubated with commercial biotinized secondary antibody at room temperature, for the recommended duration (*En Vision* FLEX HRP, RTU, K8000). The *IHC* reaction was visualized using 3,3'-diaminobenzidine tetrahydrochloride (*DAB*). The preparations were finally contrasted with Mayer's hematoxylin (Hematoxylin M, HEMM-OT-1L, Biognost, Croatia), and the coverslips were mounted with Canada balsam. Tissue samples in which incubation with the primary antibody was omitted were used as negative controls of the *IHC* reaction, and *IBCs* with known expression of the analyzed markers were used as positive controls. Slides were analyzed at 100x, 200x, and 400x magnifications, using a light microscope (*AxioScop 40*, Carl Zeiss, Germany). Representative sections were photographed using a digital camera (*AxioCam ICc1*, Carl Zeiss, Germany).

Evaluation of *IHC* Staining

Immunohistochemical stains were rated by two independent pathologists, who were blinded to clinical follow-up data at the time of analysis. In cases of differently assessed expression, agreement was reached by joint analysis of the preparation and consultation with a third pathologist.

Analysis of estrogene (*ER*) and progesterone receptors (*PR*) expression was performed using Allred score (14) as the sum of the percentage of positive nuclei of tumor cells and the intensity of *IHC* staining. The Allred score ranges from 0 to 8.

The expression of human epidermal growth factor receptor 2 (*HER2*) was analyzed based on standard recommendations (15). Depending on the continuity and intensity of membrane staining, all *IBCs* were classified into *HER2* negative (0 and 1+) and *HER2* positive (3+). Equivocal *HER2* (2+) was retested by silver in situ hybridization (*SISH*) technique after which patients were classified as *HER2* positive or negative *IBCs*.

Ki67 IHC expression was defined as the percentage of positive tumor cells per 100 counted in the zone of highest tumor proliferation. According to the previously defined limit value of *Ki67* expression in our laboratory, *IBCs* are classified into 3 groups: low (*Ki67* <15%), medium (*Ki67*: 15-30%) and high proliferative activity (*Ki67*> 30%) (16, 17).

p21 expression was determined as the percentage of nuclear expression in *IBC* and *NIL* epithelial cells. By analyzing the expression, we defined the *cut off* value for *p21*. Based on the obtained result, we divided all *IBCs* into *p21* positive and *p21* negative

Statistical data processing

The commercial software package *SPSS* (version 22.0, *SRSS Inc., Chicago, IL*) was used for statistical processing of the data obtained. In the analysis of the obtained results we used: descriptive statistics methods, *Man-Whitney* test, *Kruskal-Wallis* test, $\chi 2$ test, *Pearson* or *Spearman* correlation coefficient, *ROC* curve (with determination of cut off value, sensitivity and specificity). By determining the sensitivity and specificity of the test, the level of practical reliability of statistical analysis was determined. All reported p values were 2-sided and $p < 0.05$ was considered statistically significant.

RESULTS

The experimental research group included 147 women diagnosed with IBC, average age 58, with the youngest patient being 29 and the oldest 84. In 79 patients, in situ carcinoma (ISC) was present simultaneously with IBC, in 82 atypical hyperplasia (AH), and in 109 cases, fields of glandular parenchyma without signs of epithelial proliferation and atypia were noted (NE - normal epithelium of ducts and acini). The average size of the cancer was 22.5 mm (the smallest 9 mm and the largest 68 mm).The average expression of estrogene receptors is 54.27±35.48%, while the average expression of progesterone receptors is 34.49±34.43. Presented through the Allred score, the average value of the score for estrogens was 5.36±3.17 and for progesterone 3.92±3.21. Other clinicopathological characteristics of IBC are shown in Table 1.

Table 1. Clinicopathological characteristics of breast cancer

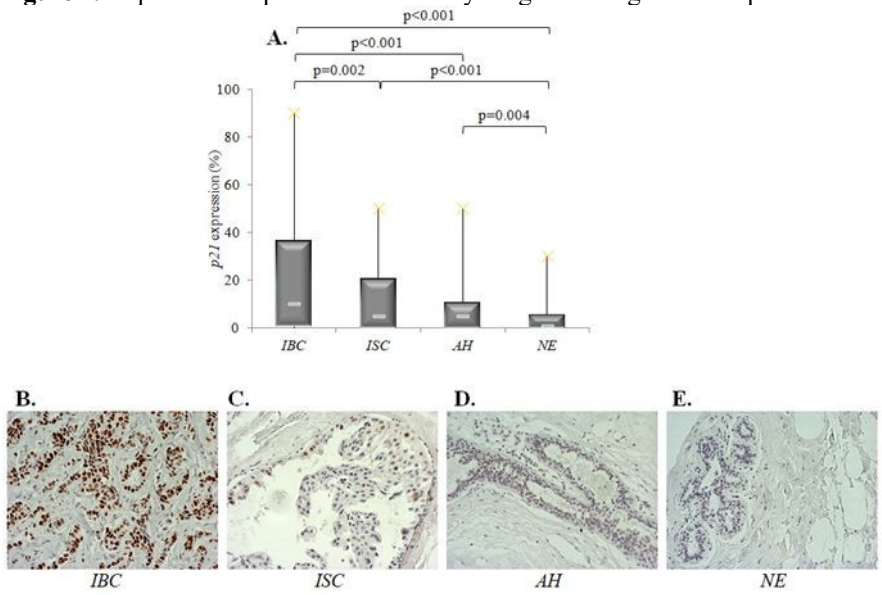
Variables		*N*	*%*
Side	left	66	44.9
	right	81	55.1
Histological type	lobular	18	12.4
	ductal	123	84.8
	other	4	2.8
Histological grade	HG1	17	11.9

Variables		*N*	*%*
	HG2	73	51
	HG3	53	37.1
Nuclear grade	NG1	17	15.2
	NG2	64	57.1
	NG3	31	27.7
Mitotic index	grade 1	19	42.2
	grade 2	20	44.4
	grade 3	6	13.3
Tumor necrosis	absent	26	21.7
	present	94	78.3
Desmoplasia	low	17	16.3
	medium	55	52.9
	high	32	30.8
Periductal elastosis	low	19	20.0
	medium	20	44.4
	high	16	35.6
Perineural invasion	absent	101	68.7
	present	46	31.3
Lymphatic invasion	absent	72	48.9
	present	75	51.1
Vascular invasion	absent	113	76.9
	present	34	23.1
HER2	negative	115	79.3
	positive	30	20.7
Ki67	low	30	20..9
	medium	42	29.4
	high	71	49.7
Molecular subtypes	Lum A	30	20.4
	Lum B	76	51.7
	HER2 +	19	12.9
	TNBC	22	15
T status	*T1*	48	35.8
	T2	64	47.8
	T3	9	6.7
	T4	13	9.7
N status	*N0*	50	37.3
	N1	48	35.8
	N2	19	14.2
	N3	17	12.7

Expression of *p21* in relation to cytological changes in epithelium

The average value of *p21* expression was calculated, and a statistically significant increase was observed with increasing invasiveness (in *NE* 2%, in *AH* 7%, in *ISC* 11% and in *IBC* 23%) (*Kruskal-Wallis, p<0.001*). A statistically significant difference was absent only in the case of the comparison of the *ISC* and *AH* groups (*Mann-Whitney U, p=0.06*), while this difference was shown between all other groups (Figure 1A).Immunohistochemical expression of *p21* in different histo- and cytomorphological changes is shown in Figure 1B-E.

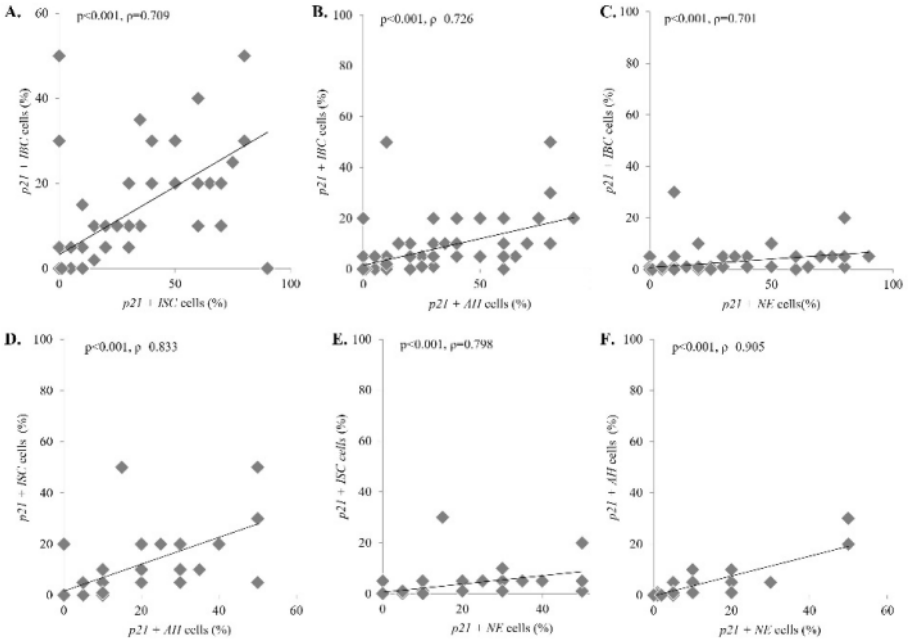
Figure 1. Expression of p21 in relation to cytological changes in the epithelium. A.



A statistically significant difference was observed between each of the mentioned groups except between the *ISC* and *AH* groups (*Mann Whitney U, p=0.06*). The result is shown as the median. Microscopic image of *p21* expression in various histo and cytomorphological changes: **B.** *IBC*. **C**. *ISC*. **D.** *AH*. **E.** *NE* (immunohistochemical analysis, original magnification 200x).

A strong positive correlation was found between *p21* expression in all mentioned changes (*Spearman ρ*). With the increase in *p21* expression in *IBC* cells, the expression in *NIL* cells also increases (Figure 2). Also, the increase in p21 expression in *ISCs* was accompanied by an increase in *AH* and *NE*. With increasing expression in *AH*, expression in *NE* also increases.

Figure 2. Correlation of p21 expression between groups in relation to cytological changes in the epithelium (Spearman ρ).

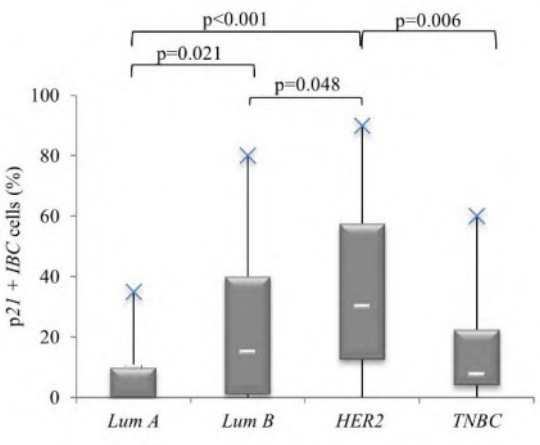


A strong positive correlation was found between all groups: **A**. *IBC* and *ISC* (*p<0.001, ρ=0.709*), **B**. *IBC* and *AH* (*p<0.001, ρ=0.726*), **C**. *IBC* and *NE* (*p<0.001, ρ=0.701*), **D**. *ISC* and *AH* (*p<0.001, ρ=0.833*), **E**. *ISC* and *NE* (*p<0.001, ρ=0.798*) and **F**. *AH* and *NE* (*p<0.001, ρ=0.905*).

Expression of *p21* in tumor cells in relation to the molecular subtype of *IBC*

When looking at the expression of *p21* in relation to the molecular subtypes of *IBC*, a statistically significant difference was observed among the different subtypes (*Kruskal-Wallis, p=0.004*). The highest expression was observed in the *HER2+* subtype of *IBC*. In relation to the *HER2+* subtype of *IBC*, molecular subtypes *Lum B* and *Lum A* had a statistically significantly lower value of *p21* expression, while the lowest value was recorded in *TNBC* (Figure 3).

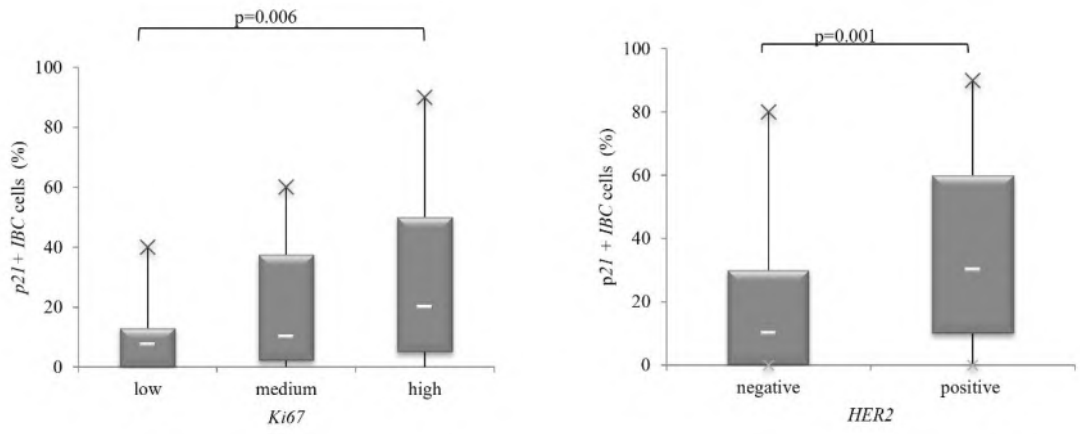
Figure 3. Expression of p21 in different molecular subtypes of IBC.



A statistically significant difference was shown between the following groups: *Lum A* and *Lum B* (*Mann-Whitney U, p=0.021*), *Lum A* and *HER2* (*Mann-Whitney U, p<0.00*), *Lum B* and *HER2* (*Mann-Whitney U, p=0.048*), *HER2* and *TNBC* (*Mann-Whitney U, p=0.006*). The result is presented as median.

Statistically significantly higher values of *p21* expression were observed in those *IBCs* that showed overexpression of *HER2* compared to *HER2*-negative tumors. Depending on *Ki67* expression, the highest *p21* expression is in the group with high *Ki67* expression values (Figure 4).

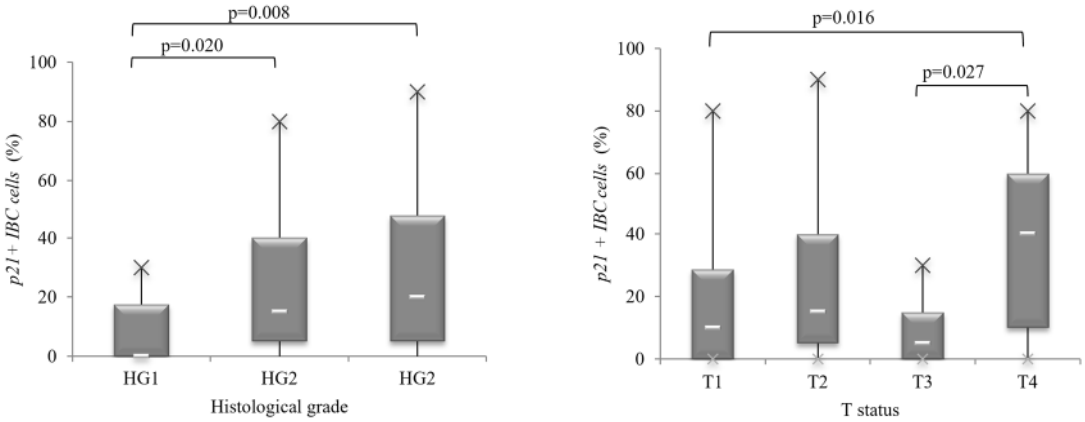
Figure 4. p21 expression depending on Ki67 expression and HER2 expression.



A. *p21* expression in tumor cells depends on *Ki67* expression. The result is shown as the median (*Kruskal-Wallis, p=0.019*). **B**. There is a significant difference in *p21* expression depending on *HER2* expression. The result is shown as the median (*Mann Whitney U, p=0.001*).

In relation to the nuclear grade, a statistically significantly higher expression of *p21* was shown in *IBCs* of nuclear grade 3 compared to nuclear grades 1 and 2. Also, *IBCs* belonging to the *T4* group, in relation to the *T* status, had a significantly higher expression in relation toto groups *T1* and *T2* (Figure 5).

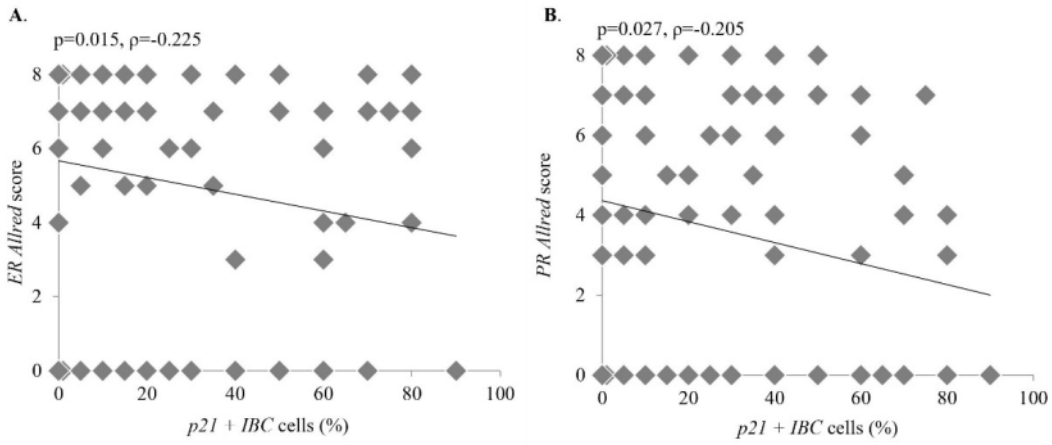
Figure 5. Expression of p21 depending on expression of nuclear grade and T status.



Expression of *p21* in tumor cells depends on **A**. nuclear grade (*Kruskal-Wallis, p=0.026*) and **B**. *T* status (*Kruskal-Wallis, p=0.05*). The result is shown as the median.

When the expression of *p21* was observed in relation to the expression of receptors for estrogens and progesterone, it was observed that with the increase in the expression of *p21* in *IBC*, the expression of receptors for estrogens and progesterone decreases significantly (*Spearman ρ*) (Figure 6).

Figure 6. Expression of *p21* depending on the expression of *ER* and *PR*.

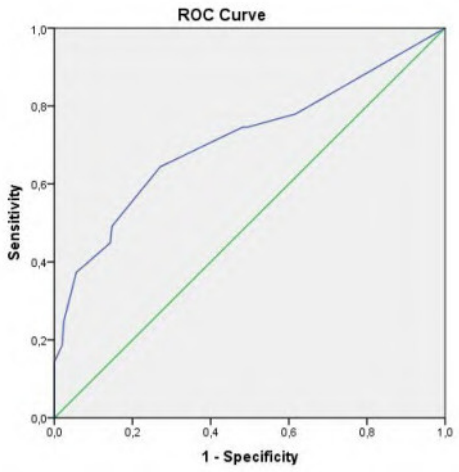


The expression of *ER* and *PR* was analyzed through the *Allred* score. The increase in *p21* expression in tumor cells was accompanied by a statistically significantly reduced expression of **A**. *ER (p=0.015, ρ=-0.225)* and **B**. *PR (p=0.027, ρ=-0.205)* (small negative correlation).

Expression of *p21* as a marker of breast cancer progression

By analyzing the expression of *p21*, it was shown that its increase can be a marker of breast cancer progression, with an expression value of 7.5% as a treshold value, with a sensitivity of 64.4% and a specificity of 72.9% (*AUC=0.712, p<0.001*) (Figure 7).

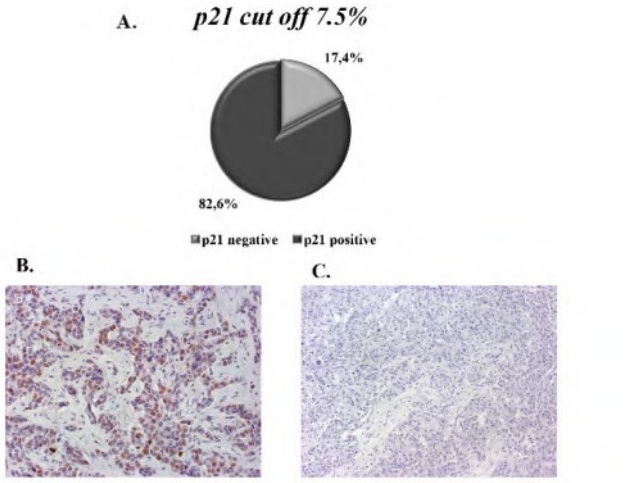
Figure 7. *ROC* curve of *p21* expression in *NIL* and *IBC*.



The calculated value of *AUC=0.712* with a sensitivity of 64.4% and a specificity of 64.4% determined a threshold value of 7.5%.

Using the obtained threshold value, all *IBCs* were further divided into two groups: negative whose *p21* expression value was ≤7.5%, and positive whose *p21* expression was >7.5% (Figure 8A).The immunohistochemical expression of *p21* relative to the threshold value is shown in Figure 8B-C.

Figure 8. A. Frequency of *p21+* and *p21- IBC* in relation to the treshold value of *p21* expression.



Microscopic image of *p21* expression in relation to the threshold value: **B**. *p21+* and **C**. *p21-* (immunohistochemical analysis, original magnification 200x).

A statistically significant association with the molecular subtype of *IBC* as well as with *HER2* expression was demonstrated. A significant difference in the percentage of *IBC* molecular subtypes can be observed in the *HER2+* subtype, where 19.7% are *p21* positive, while no *p21* negative *IBC* belongs to the *HER2+* molecular subtype. Also, when looking at *HER2* status, it was found that all *IBCs* that were negative in relation to *p21* expression were also *HER2* negative, while 33.8% of *p21* positive ones were *HER2* positive. Other clinicopathological characteristics did not show a statistically significant association (Table 2).

Table 2. Association between *p21* expression in *IBC* and examined clinicopathological characteristics.

Variables		*p21 cut off 7.5%*		*Chi-Square*	*p*
		-	+		
Mononuclear infiltrate	absent	1 (9.1%)	3 (5.8%)	0.666	0.881
	low	4 (36.4%)	20 (38.5%)		
	medium	5 (45.5%)	20 (38.5%)		
	high	1 (9.1%)	9 (17.3%)		
Histological type	lobular	1 (6.7%)	5 (6.7%)	0.622	0.733
	ductal	14 (93.3)	67 (89.3)		
	other	0 (0.0%)	3 (4.0%)		
Histological grade	HG1	2 (12.5%)	8 (11.0%)	0.364	0.834
	HG2	7 (43.8%)	38 (52.1%)		
	HG3	7 (43.8%)	27 (37.0)		
Nuclear grade	NG1	0 (0.0%)	6 (9.7%)	2.096	0.351
	NG2	7 (53.8%)	37 (59.7%)		
	NG3	6 (46.2%)	19 (30.6%)		
Tumor necrosis	absent	2 (15.4%)	17 (27.0%)	0.278	0.598
	present	11 (84.6%)	46 (73.0%)		
Perineural invasion	absent	13 (81.3%)	49 (64.5%)	1.015	0.314
	present	3 (18.8)	27 (35.5%)		
Lymphatic invasion	absent	5 (31.3%)	37 (48.7%)	0.993	0.319
	present	11 (68.8%)	39 (51.3%)		
Vascular invasion	absent	12 (75.0%)	57 (75.0%)	0.000	1.000
	present	4 (25.0%)	19 (25.0%)		
Molecular subtypes	*Lum A*	1 (6.3%)	10 (13.2%)	11.490	0.009
	Lum B	8 (50.0%)	42 (55.3%)		
	HER2 +	0 (0.0%)	15 (19.7%)		
	TNBC	7 (43.8%)	9 (11.8%)		
HER2	negative	16 (100.0%)	49 (66.2%)	5.895	0.015
	positive	0 (0.0%)	25 (33.8%)		
Ki67	low	2 (13.3%)	10 (13.5%)	1.075	0.584
	medium	6 (40.0%)	20 (27.0%)		
	high	7 (46.7%)	44 (59.5)		
T status	*T1*	3 (20.0%)	22 (32.4%)	2.924	0.404
	T2	9 (60.0%)	34 (50.0%)		
	T3	2 (13.3%)	3 (4.4%)		
	T4	1 (6.7%)	9 (13.2%)		
N status	*N0*	4 (26.7%)	23 (33.3%)	1.504	0.681
	N1	8 (53.3%)	26 (37.7%)		
	N2	1 (6.7%)	10 (14.5%)		
	N3	2 (13.3%)	10 (14.5%)		

DISCUSSION

Although extensive research related to breast cancer has been conducted in recent years, there is still a need to understand the basic aspect of tumorigenesis. Cell cycle control proteins are known to play an important role in the pathogenesis of breast cancer (18). Each phase of the cell cycle is strictly regulated in normal cells. However, upon exposure to mitogenic stimuli, these regulatory components become deregulated, which predisposes to cellular transformation of breast epithelial cells. Numerous studies have implicated roles for oncogenic and tumor-suppressive components in various types of human cancers, including breast cancer initiation and development (19-21). *p21* was initially considered a tumor suppressor protein because it was recognized as a major mediator of cell cycle arrest. Conversely, it has been proposed that *p21* may also function as an oncogene because it can inhibit apoptosis. Therefore, *p21* is considered a dual-behavior protein, as its expression can cause potential benefits or harmful effects in breast cancer (22).

We calculated the average value of *p21* expression in different breast lesions and found that the highest expression was in *IBC* and gradually decreased from *ISC*, through *AH* to

NE. Identical results were obtained by *Wei et al*., i.e. showed that *p21* protein was highly expressed in cancer samples, compared to normal breast tissue (23). In the same study, they showed that *p21* protein expression is significantly associated with larger tumor diameter, higher grade, and lymph node metastases, which indicates a poor prognosis of the disease. Other authors have also shown that the level of *p21* expression is significantly elevated in patients with breast cancer compared to patients with benign breast lesions (24-28). *Zohny et al* showed that *p21* expression was associated with histological grade 3 tumors and the presence of lymph node metastases, which together indicate that high expression of this marker is associated with advanced breast cancer (25). *Barbareschi* examined the expression of *p21* in changes in the breast and came to the results that show a gradual increase in the expression of this marker from normal epithelium, through well-differentiated and then poorly differentiated *ISC* to *IBC* where the expression was also the highest (26). Furthermore, high expression was accompanied by high histological grade, but there was no correlation with other investigated clinicopathological characteristics of the tumor. If the role of *p21* is only inhibition of the cyclin/CDK complex, which is required for the transition from G1 to S, and inhibition of DNA replication, high expression of p21 could result in reduced cell proliferation. However, in our study, p21 was significantly increased in IBC compared to NIL. We can explain this by the presence of mutated non-functional forms of p21. Balbin et al investigated p21 in 36 primary breast cancers and found a mutation in this molecule due to the replacement of arginine with tryptophan ($p21^{R94W}$) (29). This mutation was attributed to a tumor-specific change, as it was not observed in *DNA* extracted from peripheral blood cells of the same patient. Functional analysis of the $p21^{R94W}$ protein produced in different expression systems revealed that this mutation causes an impairment in the ability of *p21* to inhibit cyclin*A/CDK2*, cyclin*B/CDK1*, cyclin*D/CDK4*, and cyclin*D1/CDK6*. These data suggest that the $p21^{R94W}$ protein may participate in breast carcinogenesis because it cannot inhibit a number of cyclin/*CDK* complexes. Differences in *p21* expression were also observed in changes in other organs. Thus, strong positive nuclear staining with *p21* was observed in full-thickness epithelium in squamous cell carcinoma, while in keratoacanthoma it was limited to the peripheral and suprabasal layers. Both the level and intensity of staining for *p21* were higher in squamous cell carcinoma compared to keratoacanthoma (30).

We showed the association of *p21* expression with high nuclear grade and larger tumor diameter and tumors in *T4* disease status, high *Ki67* proliferative index, positive *HER2* status and negative *ER* and *PR*. Others obtained similar results and showed that overexpression of *p21* is associated with positive nodal status, larger tumor diameter, and worse prognosis in breast cancer patients (31). The association of high *p21* expression with high proliferative activity is particularly interesting in the context of our research. Increased cell proliferation in *p21* positive cases has been previously described (32). High *p21* expression in highly proliferative cells may reflect failed attempts to arrest proliferation. This may result from the presence of other cell cycle regulatory pathways, which bypass the *p21*-mediated cell cycle block, such as c-Myc or B-Myb (33, 34). In addition, overexpression of *CDK2* and cyclin *A* has been reported to reverse the inhibitory effect of high *p21* expression (*35, 36*). Mutation of the retinoblastoma protein (*pRb*) can also lead to increased expression of *p21* via deregulation of the transcription factor *E2F-1 (37, 38)*.

By analyzing the molecular subtypes of breast cancer, we found that *p21* expression was highest in the *HER2* positive subtype of *IBC*. This is further supported by the fact that *p21* expression was highest in *HER2* overexpressing tumors. Others have found that *HER2* overexpression positively correlates with *p21* in breast tumors and that there is a significant correlation of *p21* positivity with worse disease-free survival (39). Studies have shown that in breast cancer cells, *HER2* can contribute to the translocation of *p21* from the nucleus to the cytoplasm, resulting in the loss of its tumor suppressor function (40). There is increasing evidence that the function of *p21* is related to its localization in cells. When localized in the cytoplasm, *p21* functions as an oncogene, thus promoting cell proliferation and progression through the cell cycle, while nuclear localization of *p21* is involved in prodifferentiation and senescence-promoting effects (40, 41).

CONCLUSION

Our results confirmed that increased expression of *p21* may indicate malignant transformation of breast changes and progression of *IBC*. The treshold value of *p21*-positive tumor cells allows the separation of patients with *NIL* from those with *IBC*, so *IHC* analysis of *p21* expression can be used as an additional diagnostic test in separating benign from malignant changes in the breast. Such observations may recommend this marker for use in diagnostic purposes.

ETHICS APPROVAL

The study was conducted in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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None.

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A STUDY OF ABSORPTION AND SELECTED MOLECULAR PHYSICOCHEMICAL PROPERTIES OF SOME ANTIPSYCHOTIC DRUGS

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ABSTRACT

Antipsychotic drugs are commonly prescribed for different mental disorders and can be classified into two main groups: the first which contain originally developed antipsychotics of the first generation or typical antipsychotics and the other group with newly developed antipsychotics or atypical antipsychotics of the second generation. In this study, eleven antipsychotic drugs (chlorpromazine, flupentixol, haloperidol, zuclopenthixol, aripiprazole, clozapine, olanzapine, quetiapine, risperidone, sertindole, ziprasidone) were investigated to evaluate significance of their molecular physicochemical properties (lipophilicity, aqueous solubility, polar surface area, molecular weight, volume value and acidity) for their bioavailability. Relationships between literature available intestinal absorption data of antipsychotic drugs and their lipophilicity descriptor with one additional molecular descriptor, investigated using multiple linear regression analysis provided high correlations for molecular descriptors, Mw, Vol, pKa, as additional independent variables. Values of correlation coefficients (R^2) were ranged from 0.951 (for Vol) above 0.944 (for Mw) to 0.923 (for pKa).

Keywords: *Antipsychotic drugs; absorption; lipophilicity; solubility; polar surface area.*

INTRODUCTION

Psychotic disorders are today relatively frequent among global population. They can be divided into number of mental illnesses as psychoses, neuroses or many other. According to their mechanism of action antipsychotic drugs are generally antagonists of dopamine receptor. However, they can affect cholinergic, α adrenergic, histamine or serotonin receptors, as additional targets, which can influence their side effect.

From the beginning of their implementation until the 90's this drugs had been significantly improved and number of newly synthetized drugs were introduced into medical practice in aim to optimize their efficiency, safety and modes of application (1-5).

Concerning their development antipsychotic drugs can be divided into two main groups. There are originally developed antipsychotics which belong to the first group of drugs, known as typical antipsychotics or antipsychotics of the first generation (chlorpromazine, flupentixol, haloperidol, zuclopenthixol and others), while the other group represents newly developed antipsychotics, known as atypical, namely antipsychotics of the second generation (aripiprazole, clozapine, olanzapine, quetiapine, risperidone, sertindole, ziprasidone and others) (1-5).

It is generally known that medical success and therapeutic efficiency of drugs significantly depend on their absorption, distribution, metabolism and route of elimination namely their ADME data (6-8). On the other hand, drugs ADME properties are critically influenced by physicochemical properties of drugs molecules: lipophilicity, solubility, molecular weight, volume and acidity (8-12).

In our prior researches, influence of several molecular physicochemical properties on absorption (13-15), plasma protein binding (16,17) as well as route of elimination (18-20) were investigated for number of antihypertensive drugs and significant dependence was established. Following in our recently published paper we studied relationship between antipsychotics molecular properties and their plasma protein binding degree (21).

The the aim of our study was to estimate molecular physicochemical descriptors of several antipsychotic drugs and to compare their values with each other and published literature data about their absorption.

MATERIALS AND METHODS

In this investigation eleven antipsychotic drugs were selected, four which belong to the typical or antipsychotics of the first generation and seven atypical or antipsychotics of the second generation. The eleven selected antipsychotics investigated in presented research were: **1.** chlorpromazine, **2.** flupentixol, **3.** haloperidol, **4**. zuclopenthixol, and seven atypical: **5.** aripiprazole **6.** clozapine, **7.** olanzapine, **8.** quetiapine, **9.** risperidone, **10.** sertindole and **11.** ziprasidone.

For calculation of antipsychotics' molecular physicochemical descriptors, several software packages were used: Virtual Computational Chemistry Laboratory (22), Molinspiration Depiction Software (23), as well as DrugBank (24) a database of published data on the pharmacological drugs properties and Chemdraw ultra 12.0.

With application of software package Molinspiration Depiction Software (www.molinspiration.com) three molecular physicochemical descriptors were calculated: electronic descriptor - polar surface area (*PSA*); constitutional parameter - molecular weight (*Mw*) and geometric descriptor - volume value (*Vol*) (23).

Using software package, Virtual Computational Chemistry Laboratory (www.vcclab.org) antipsychotics' lipophilicity descriptors, seven different log*P* values, as well as their aqueous solubility data (log*S*) were calculated (22). The additional lipophilicity parameter was calculated with application of Chemdraw ultra 12.0 software package while experimental lipophilicity parameters (log*Pexp*) of investigated antipsychotics were obtained using DrugBank database (24).

The DrugBank database was also used to obtain antipsychotics' acidity descriptors, pK_a values. Moreover, data about values of intestinal absorption of investigated antipsychotics were obtained using DrugBank database (24).

RESULTS

The calculated lipophilicity descriptors (different log*P* values) and aqueous solubility data (log*S* values) are presented in Table 1, intercorrelations between all colected log*P* values of investigated antipsychotic drugs are presented at Table 2, while antipsychotics data of polar surface area (*PSA*), molecular weight (*Mw*), volume value (*Vol*) and antipsychotics' acidity descriptors (pK_a values) as well as their bioavailability, are presented in Table 3.

All statistical analysis were performed by application the Microsoft Excel 2003 and Origin 7.0 PRO (Origin Lab Corporation, USA).

Table 1. The lipophilicity (logP) and solubility (logS) values of investigated antipsychotic drugs

No.	Compound	logP exp	ClogP	AlogPs	AClogP	milogP	AlogP	MlogP	XlogP2	XlogP3	log*S*
1	Chloropromazine	5.41	5.80	5.18	5.03	5.03	4.74	3.77	4.92	5.19	-4.84
2	Flupentixol	4.51	4.34	4.56	4.45	4.91	4.82	3.89	4.42	4.51	-4.50
3	Haloperidol	4.30	3.85	3.70	4.63	4.30	3.89	4.01	3.98	3.23	-4.81
4	Zuclopenthixol	nd	4.13	4.46	4.30	4.69	4.54	3.58	4.12	4.31	-4.55
5	Aripiprazole	4.50	4.63	5.21	4.58	5.08	5.00	3.61	4.49	4.64	-4.95
6	Clozapine	3.23	4.10	3.67	3.21	4.14	3.95	2.96	3.74	3.08	-3.26
7	Olanzapine	2.00	3.40	3.61	2.98	3.47	3.21	2.31	2.32	2.86	-3.28
8	Quetriapine	2.80	3.37	2.93	2.80	3.49	3.18	2.36	2.83	2.14	-3.22
9	Risperidone	2.50	2.71	2.41	3.37	2.96	3.32	3.61	3.07	2.72	-3.89
10	Sertindole	nd*	5.07	4.29	4.52	3.84	4.68	3.77	4.10	4.07	-5.78
11	Ziprasidone	3.80	3.58	4.64	2.45	4.05	4.26	3.44	3.77	4.02	-4.32

* no data

Table 2. Intercorrelations between different log*P* values of investigated antipsychotic drugs

	logP exp	ClogP	AlogPs	AClogP	milogP	AlogP	MlogP	XlogP2	XlogP3
logP exp	1								
ClogP	0.8548	1							
AlogPs	0.8062	0.8297	1						
AClogP	0.7897	0.7331	0.5279	1					
milogP	0.9099	0.8913	0.9080	0.7504	1				
AlogP	0.8861	0.7914	0.9067	0.6666	0.9418	1			
MlogP	0.7545	0.4027	0.4322	0.7107	0.5626	0.6745	1		
XlogP2	0.9704	0.8353	0.7882	0.7741	0.9065	0.9296	0.7891	1	
XlogP3	0.8693	0.8369	0.9275	0.6699	0.8812	0.9426	0.6495	0.8822	1

Table 3. Intestinal absorption, calculated values of polar surface area, volume, molecular weight, acidity descriptors (pK_a values for p*H* = 7.4) for investigated antipsychotic drugs

No.	Compound	% ABS	PSA	Vol	Mw	pKa
1	Chloropromazine	10-80*	8.17	285	319	9.20
2	Flupentixol	47	26.70	379	435	8.51
3	Haloperidol	65	40.54	337	376	8.05
4	Zuclopenthixol	49	26.70	361	401	8.43
5	Aripiprazole	87	44.81	395	448	7.46
6	Clozapine	65	35.16	292	327	7.50
7	Olanzapine	87	35.16	286	312	7.24
8	Quetriapine	100	48.83	352	384	7.06
9	Risperidone	70	64.17	374	410	8.76
10	Sertindole	75	40.41	390	441	8.59
11	Ziprasidone	60	48.47	352	413	7.09

* absorption values have large individual variation

DISCUSION

Drugs Lipophilicity, Solubility and acidity

In this study, eleven antipsychotic drugs, four which belong to the typical or antipsychotics of the first generation (chlorpromazine, flupentixol, haloperidol, zuclopenthixol) and seven atypical or antipsychotics of the second generation (aripiprazole, clozapine, olanzapine, quetiapine, risperidone, sertindole, ziprasidone) were investigated to evaluate influence of their molecular physicochemical properties on their bioavailability.

The molecular structure and physicochemical properties of drugs molecules, at the first place its lipophilicity, play important role in development of new antipsychotic drugs. The calculation of lipophilicity descriptors, seven different log*P* values (Alog*Ps,* AClog*P,* milog*P*, Alog*P,* Mlog*P,* XLOG*P*2*,* XLOG*P*3) were performed using software package Virtual Computational Chemistry Laboratory. The another software package, Chemdraw ultra 12.0 was applied for calculation of Clog*P* lipophilicity parameter, while DrugBank database was used to obtain experimental lipophilicity parameters, logPexp values.

The relations between all obtained log*P* values were studied (27) and calculated correlation coefficients showed that good agreements were obtained between majorities of collected log*P* values, but the best can be observed for Xlog*P2* data. Also, it can be seen that although all calculated log*P* values correlate well with experimental log*P* data collected for investigated antipsychotic drugs and the best correlation for experimental log*P* was obtained with calculated Xlog*P2* (R^2 = 0.971). All collected log*P* values demonstrate similar trend. The typical, antipsychotic drugs from the first generation are more lipophilic then atypical or antipsychotics from second generation. According calculated log*P* values, the most lipophilic antipsychotic drug is typical antipsychotic chloropromazine, while the less lipophilic ones are atypical antipsychotics olanzapine and quetriapine.

Furthermore, another one molecular property, water solubility has important influence on drug's absorption as well as transport from site of administration to the blood. The low drug's absorption can be result of its insufficient water solubility (25,26). As can be seen, according calculated solubility log*S* values of antipsychotic drugs which are presented in Table 1, the lowest solubility value show atypical antipsychotic sertindole. However, results generally demonstrate that investigated atypical antipsychotics exhibit higher water solubility values than typical ones. Among typical, antipsychotics from the first generation, chloropromazine shows the lowest solubility which correspond to its highest lipophilicity, while the highest water solubility show atypical antipsychotic quetriapine what is in accordance with its low lipophilicity.

The drug's partition are significantly influenced by its acidity and dissociation (28). The calculated p*Ka* values in water at temperature of 25.0°C for all investigated antipsychotics showed general trend. The calculated acidity, p*Ka* values of antipsychotic drugs, show that the lowest value of p*Ka* (7.06) exibit quetriapine and for majority of investigated atypical antipsychotics p*Ka* values are lower than 7.5. The exceptions represent risperidone and sertindole (with p*Ka* values higher than 8.0). However, typical, antipsychotics from the first generation exhibit higher p*Ka* which are ranged from 8.1 for haloperidol to 9.2 for chloropromazine. Among antipsychotics from the first generation (typical) chloropromazine already showed the lowest solubility and highest lipophilicity values.

Polar surface area, molecular weight, volume

Beside lipophilicity, solubility and acidity for investigated antipsychotic drugs, further molecular descriptors, polar surface area (*PSA*), molecular weight (*Mw*) as well as molecular volume (*Vol*) were calculated. All these molecular properties together with lipophilicity, solubility and acidity have significant influence and correlates good with the human intestinal absorption (25,26). According investigation of number of drugs which belong to different groups it was supposed that very high values of *PSA, Mw or Vol* can lead to poor drug absorption (25,26).

Among drugs investigated in present research the lowest value of *PSA* exhibit typical antipsychotics chloropromazine (8.17) while flupentixol and zuclopenthixol show four times higher values of *PSA*. However majority of atypical antipsychotics have showen PSA values in the range 35.16 (for clozapine and olanzapine) to 64.17 (risperidone).

According presented results for all investigated antipsychotic drugs, molecular weight values are in the range 312 (olanzapine) to 448 (aripiprazole) while volume values are ranged from 285 (chloropromazine) to 395 (aripiprazole). For both parameters, molecular weight as well as volume value, the lowest values for typical antipsychotics belong to chloropromazine while highest values have been calculated for atypical antipsychotic aripiprazole.

Oral absorption

The main route of drugs administration generally is oral application. According Lipinski's "the rule of 5" low absorption or permeation can be predicted for drugs with molecular weight larger than 500 and the calculated log*P* higher than 5, but also for those with more than 5 hydrogen-bond donors, 10 hydrogen-bond acceptors (12). Also insufficient water solubility or very high values of *PSA* and *Vol* values can lead to poor drug absorption (25,26). Molecular physicochemical properties of drugs are important factors which can modulate their intestinal absorption.

The intestinal absorption values for all investigated antipsychotic drugs according available literature are in the range 10 to 100%. The majority of investigated antipsychotic drugs exhibit values of intestinal absorption in the range 50-80%. However, typical, antipsychotic drugs from the first generation, show lower values of intestinal absorption than atypical, antipsychotic drugs from the second generation.

Among investigated antipsychotic drugs of second generation, aripiprazole, olanzapine, quetriapine, are those with highest values of intestinal absorption. Their molecular weights are lower than 500 and in accordance with Lipinski's "the rule of 5". Among atypical antipsychotics aripiprazole is molecule with highest lipophilicity while the less lipophilic ones are olanzapine and quetriapine. Their polar surface area values are ranged from 35 to 49 and volume values from 286 to 394. However, the molecular parameter that distinguishes this three antipsychotic drugs of second generation, from other especially typical antipsychotics, is their acidity, namely their p*Ka* values which are in the range 7.46 – 7.06 while physiological pH is 7.4.

On the other hand chloropromazine value of intestinal absorption can differ 10-80% (absorption values have large individual variation). Chloropromazine belongs to typical or antipsychotic drugs of the first generation and already was pointed as a drug with highest p*Ka* value, low solubility and highest lipophilicity. Its value of Xlog*P2* was 4.92, what is very close to value of 5 and it is known that Lipinski's "the rule of 5" predict drugs low absorption for log*P* values higher than 5.

Since values of intestinal absorption for investigated antipsychotic drugs are highest for antipsychotics from second generation, especially for aripiprazole, olanzapine, quetriapine which have shown high solubility, moderate lipophilicity and molecular weight as well as p*Ka* values around 7.4, this can indicate that molecular physicochemical properties, lipophilicity, solubility, molecular weight and acidity, p*Ka*, but also polar surface area and volume are very important factors which are decisive for drug absorption.

In final stage of study, the relationships between all calculated molecular descriptors (lipophilicity, solubility, polar surface area, molecular weight, volume and acidity) of selected drugs and data about their intestinal absorption were examined using simple linear regression. The correlations between intestinal absorption data and antipsychotic drugs calculated molecular descriptors, *PSA, Mw, Vol, logS, pKa* were investigated providing not very good correlations with correlation coefficients (R^2) lower than 0.38.

The slightly better correlations were obtained between antipsychotic drugs intestinal absorption data and several calculated log*P* values. The best correlations were obtained for values of Mlog*P* (R^2 = 0.445) and Xlog*P2* (R^2 = 0.398). Since Xlog*P2* values have already shown the best correlation (R^2 = 0.971) with experimental logP data collected for investigated antipsychotic this lipophilicity descriptor (Xlog*P2*) was chosen for further study. Following, the correlation between antipsychotic drugs intestinal absorption, lipophilicity descriptor Xlog*P2* and one additional molecular descriptor were investigated using multiple linear regression analysis (MLR). As potential additional descriptors *Mw, Vol* and p*Ka* were chosen, since there were found relatively good correlations (R^2 > 0.30) between Xlog*P2* and values of log*S* and *PSA*.

Relationships investigated using multiple linear regression analysis provided considerably higher correlations for all used molecular descriptors, *Mw, Vol*, p*Ka*, as additional independent variables. Values of correlation coefficients (R^2) were ranged from 0.951 (for *Vol*) above 0.944 (for *Mw*) to 0.923 (for p*Ka*). All established correlations with obtained correlation coefficient R^2 higher than 0.80 can be considered as very good, as proposed by Asuero et al. (29), especially due to the limited number of compounds, eleven investigated antipsychotic drugs. The all results obtained using MLR analysis applying two different descriptors (*Mw, Vol*, p*Ka*) as independent variables are presented in Table 4 and at Figure 1.

The selection criteria for drug – like properties of investigated antipsychotic drugs are reported in Table 5 make it obvious that in the aim to obtain a potent antipsychotic drug, appropriate balance between physicochemical and pharmacokinetic properties should be established.

Table 4 . The antipsychotic drugs intestinal absorption data collected from literature (*) and predicted using (A) XlogP and Vol data; (B) XlogP2 and Mw; (C) XlogP and pKa values

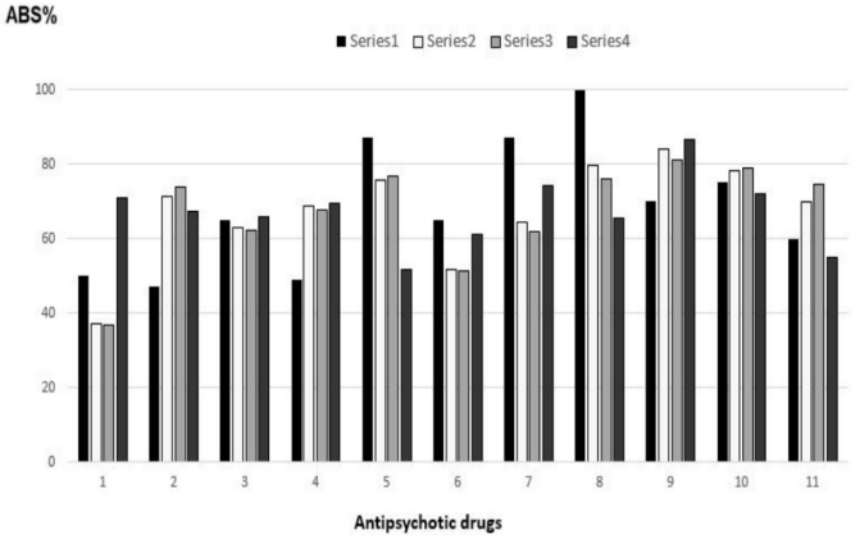
No.	Compound	ABS%(*)	ABS% (A)	ABS% (B)	ABS% (C)
1	Chloropromazine	50*	37	37	71
2	Flupentixol	47	71	74	67
3	Haloperidol	65	63	62	66
4	Zuclopenthixol	49	69	68	70
5	Aripiprazole	87	76	77	52
6	Clozapine	65	52	51	61
7	Olanzapine	87	65	62	74
8	Quetriapine	100	80	76	66
9	Risperidone	70	84	81	87
10	Sertindole	75	78	79	72
11	Ziprasidone	60	70	75	55

*average value used for calculation

Table 5. Drug - like properties of investigated antipsychotic drugs

Lipophilicity descriptor (Xlog*P2*)	2.32 – 4.92
Aqueous solubility (log*S*)	(-5.78) – (-3.22)
Acidity descriptor (pKa)	7.06 – 9.20
Polar surface area (PSA, Å^2)	8.17 – 64.17
Molecular weight (Mw)	312 – 448
Volume value	285 – 395
Percent of oral absorption (%ABS)	10 – 100

Figure 1. The relationship between antipsychotic drugs intestinal absorption data collected from literature (seria 1) and predicted using (seria 2) XlogP2 and Vol; (seria 3) XlogP and Mw data (seria 4) XlogP and pKa values. No denote investigated antipsychotic drugs



CONCLUSION

The investigation of relationship between literature available intestinal absorption data of antipsychotic drugs, and lipophilicity descriptor (Xlog*P2*) with one additional molecular descriptor (*Mw, Vol* or p*Ka*) provided high correlations (with $R^2 > 0.9$) and confirmed that application of in silico achieved molecular descriptors can be valuable technique in drug research and development of new drugs candidates.

CONFLICT OF INTEREST

The author declare no conflict of interest.

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ILIAC VESSELS INJURY DURING DISC HERNIATION SURGERY - CASE REPORT

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ABSTRACT

Iliac vessel injuries during spinal surgery are rare but can be fatal. The incidence of these injuries is approximately 0.04%, with the first description dating back to 1945 by Linton and White. Clinical presentation can range from acute hemorrhagic shock to subtle injuries with asymptomatic progression. We present the case of a 53-year-old previously healthy female undergoing planned surgical treatment for a disc hernia at the L4-L5 level on the left. Postoperatively, she experienced hemorrhagic shock with a decrease in hemoglobin to 35g/l. An emergency MSCT angiography verified a vascular lesion. During urgent surgical intervention, a lesion of the common iliac vein was observed, which was sutured directly, and a lesion of the right common iliac artery, which was managed by interposition of an 8mm Dacron graft. Follow-up MSCT angiography showed normal findings. During spinal surgery, it is essential to be aware of the possibility of vascular lesions. Due to the potential occurrence of delayed massive bleeding, intensive peri- and postoperative monitoring of patients after spinal surgery is necessary.

Keywords: *Iatrogenic iliac artery injury, iatrogenic iliac vein injury, disc herniaton surgery, spinal surgery complications, iatrogenic bleeding.*

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INTRODUCTION

The surgery for lumbar-sacral disc herniation and/or spinal canal stenosis is commonly performed and considered a low-risk procedure. However, peri- and postoperative complications can occur, ranging from superficial infections of the surgical wound to deeper infections, cerebrospinal fluid leakage, persistence or onset of neurological deficits, and even life-threatening iatrogenic injuries to major blood vessels (1-3). Besides serious vascular complications, other non-neurological complications such as ureter necrosis or the occurrence of hemothorax have been described (4-6). Injuries to major blood vessels in the retroperitoneum can occur not only during neurosurgical interventions but also during hip surgery, kidney transplantation, and various interventional radiological procedures (7, 8).

The first vascular injury as a complication of disc herniation surgery was described in 1945 by Linton and White (9). Although these injuries are rare, they can be fatal. The literature reports an incidence of iatrogenic vascular injuries around 0.04%, with a high mortality rate ranging from 15 to 60% (10). Despite their rarity, the frequency of vascular injuries has remained consistent over the last few decades (11).

Injuries to vascular structures represent one of the most dreaded surgical complications due to their consequences. These complications can range from acute hemorrhagic shock requiring urgent intervention to insidious injuries with asymptomatic progression. It is crucial to carefully study these manifestations to determine the most appropriate therapeutic approach (12).

Here, we present a case of injury to the right common iliac artery and right common iliac vein in a patient operated on for disc herniation at the L4-L5 level.

CASE PRESENTATION

A 53-year-old female was admitted for planned operative treatment of neuroradiologically verified disc herniation at the L4/L5 level on the left and spinal canal stenosis. She had been experiencing lumbar-sacral back pain and pain along the left leg for several months. Magnetic resonance imaging (MRI) revealed a disc prolapse at the L4-L5 level with compression of the dural sac and anular rupture at the L5-S1 level with dorzomedial protrusion. The patient had no previous illnesses, allergies, injuries, or surgeries.

The surgery was performed in the typical genitopectoral position, involving interhemilaminectomy. Intraoperatively, the yellow ligament was noted to be extremely hypertrophic. The L5 root and the protrusion of the disc below the root shoulder were visualized. The posterior ligament and anulus were opened, and the nucleus was removed. During the surgical intervention, there was a drop in systolic arterial pressure to 50 mmHg. Administration of therapy by the anesthesia team led to the normalization and stabilization of arterial pressure.

Postoperatively, after 4-5 hours, there was a drop in arterial pressure. Patient was pale, tachycardic, and hypotensive, with gas analysis showing an estimated hematocrit below 15%. After consultation with a vascular surgeon, urgent MSCT angiography was indicated, revealing active bleeding and a rupture of the right common iliac artery (Figure 1). It was also impossible to exclude the presence of iliacocaval fistula or pseudoaneurysm. A large retroperitoneal hematoma on the right side was also observed (Figure 2). Emergency surgical intervention was necessary.

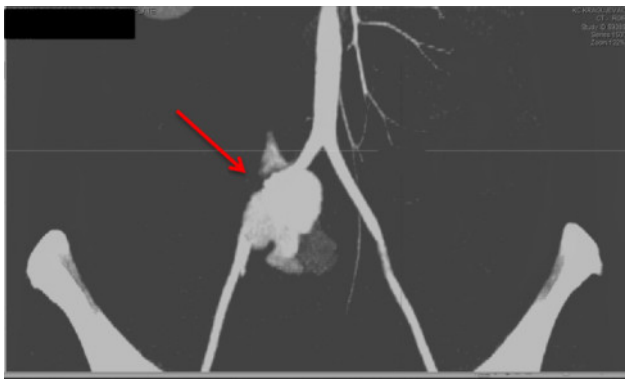

Figure 1. Lesion of the right common iliac artery with signs of bleeding (red arrow shows extravasation of contrast from righit common iliac artery)

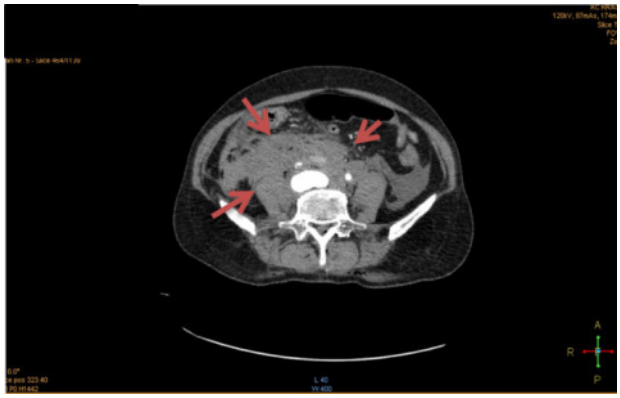

Figure 2. Large retroperitonal hematoma
(red arrows show a big retroperitoneal hemathoma around injured right common iliac artery)

Due to the pronounced hemodynamic instability of the patient, a medial laparotomy was performed, clamping the abdominal aorta initially for temporary hemostasis. During aortic access, a large retroperitoneal hematoma was observed. Subsequently, a defect was noted on the posterior wall of the dissected right common iliac artery, with bleeding, and on the posterior-inner wall of the right common iliac vein was defect too. To stop venous bleeding, we made I decision to cut the artery. The common iliac artery was cut, the defect on the vein was sutured directly, while the right iliac artery, due to the impossibility of end to end anastomosis reconstruction, was reconstructed by interposing an 8mm Dacron graft. Blood derivatives and other resuscitation

therapy were administered to the patient during the surgical intervention and immediately after.

Postoperatively, the patient was hemodynamically stable, with palpable pulses in the main arteries of the right leg. Color Doppler ultrasound examination detected physiological flows in the tibial arteries of the right leg, with no signs of deep vein thrombosis. After 14 days, she was discharged home.

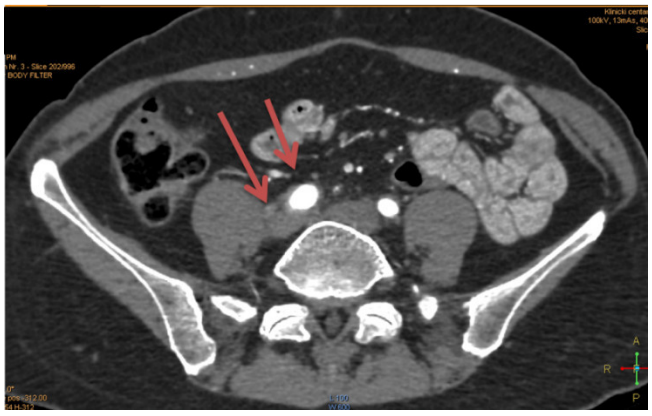

Figure 3: Normal findings on control MSCT angiography (red arrows show right common iliac artery and right common iliac vein on 3 years after intervention

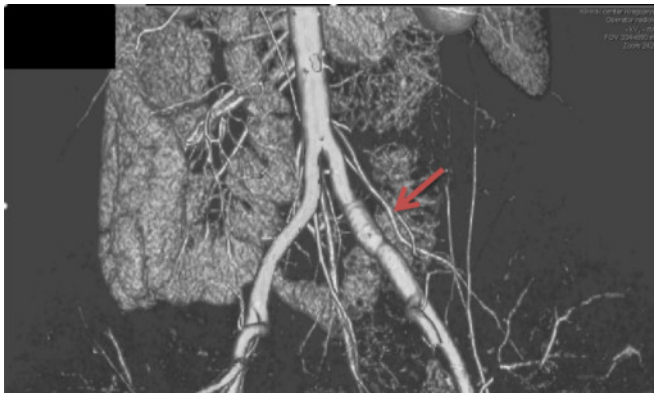

Figure 4. Control MSCT angiography after three years (red arrow shows patent synthetic graft)

On the follow-up MSCT angiography performed 3 years after the intervention, the findings were normal (figure 3) There were no hemodynamically significant stenoses on the right common iliac artery, and the graft was patent (figure 4). The finding on the right common iliac vein was also normal.

DISCUSSION

During spinal surgery, serious vascular injuries can involve laceration of blood vessels, formation of pseudoaneurysms, and arteriovenous fistulas. These injuries are rare, Akhadae et al. in their literature review from 2022 state that from 1969 to 2018, about 100 cases of vascular injuries during posterior approach lumbar disc surgery have been reported (13).

Despite the use of optimal surgical techniques, lumbar disc surgery is often performed in a space with limited maneuvering possibilities. There are numerous risk factors for vascular injuries mentioned by S. Papdoulas et al. in a 2002 study, including previous disc or abdominal surgeries with adhesion formation, disruption or degeneration of the anterior annulus fibrosus and anterior longitudinal ligament, inadequate patient positioning, excessively deep instrument intrusion during surgery, close proximity between the disc and large retroperitoneal blood vessels, further increased by placing a pillow under the abdomen during surgery, as well as vertebral anomalies (14). Although these risk factors are important, none of them individually were pronounced in the case of our patient. Radiological (MRI) and clinical examinations did not reveal the presence of any of the mentioned factors.

The blood vessel that is injured can be arterial, venous, or both simultaneously, as was the case with our patient. Clinically, the injury can manifest immediately intraoperatively, later, or immediately postoperatively. Delayed onset of bleeding in such injuries poses a significant problem, as described in the case report by Moutinho et al. in 2019 (15). Lacerations, pseudoaneurysms, and AV fistulas are the most common, but vasospasm can also occur.

The most commonly injured blood vessels are the common iliac artery, common iliac vein, aorta, inferior vena cava, but also smaller blood vessels such as the lumbar artery or inferior mesenteric artery (16-18) . Clinical manifestations of injuries vary depending on the type of blood vessel and the pathoanatomical substrate. In laceration, hypotension occurs, signs of hemorrhagic shock, a suddenly formed mass in the abdomen, and/or abdominal distension (19).

In contrast to lacerations, arteriovenous fistulas can cause symptoms such as abdominal bruit, leg swelling, pulmonary thromboembolism, abdominal pain, chest pain, hematuria, renal failure, varicose veins in the legs, thrombophlebitis, or deep vein thrombosis, depending on the time when the problem is noticed (20-23). Naouli et al. describe late complications of arteriovenous fistulas due to constant increased inflow into the right heart and increased preload, leading to heart failure of varying degrees, even in younger people (24).

Treatment of such injuries, especially lacerations of large blood vessels in the retroperitoneum, requires urgent surgical intervention. Treatment can be endovascular or open surgery. In endovascular interventions, a stent graft is placed in the area of the injured artery to stop the bleeding. A limiting factor of this type of treatment can be the availability of an angiography suite, availability of suitable stent grafts, as well as difficulties in identifying simultaneous injuries to venous blood vessels if not clearly visible on CT angiography, which may require later reinterventions. One complication described by Sahinoglu et al. in 2019 during endovascular treatment is the occurrence of abdominal compartment syndrome if a large hematoma forms, which may require post-

procedural medial laparotomy and subsequent reconstruction of the anterior abdominal wall (25, 26).

Open surgical treatment for lacerations of large blood vessels can take the form of direct suturing, patch plastic surgery, graft interposition (autovenous or synthetic), anastomosis with the contralateral internal iliac artery, internal arteriotomy suturing, ligation of the blood vessel, or conservative treatment. Papadoulas et al. state that the most common form of open surgical treatment for common iliac artery injuries is direct suturing. According to their review of studies from 2002, covering studies from 1965, almost a third of 30 patients with common iliac artery injuries were treated with direct suturing. However, in the case of our patient, we did not have adequate conditions for this form of surgical treatment (14).

In our case, due to the simultaneous injury to the iliac vein, it was necessary to cut the already injured common iliac artery to stop venous bleeding. Subsequently, considering the patient's very poor general condition and significant bleeding, we opted to use an 8mm synthetic Dacron graft instead of an autologous vein graft, which would have prolonged the duration of the operation. It is important to note that in such injuries, retroperitoneal or intraperitoneal bleeding may be overlooked if there are no clear intraoperative signs of bleeding. In young and healthy individuals, even when bleeding reaches 40% of the circulating volume, a clear clinical picture or signs of hemorrhagic shock may be absent, leading to delays in diagnosis and treatment (27).

Literature indicates that the overall mortality rate from vascular injuries during spinal surgery is around 10%, with a higher percentage if it involves an arterial blood vessel (20-38%). The mortality rate in the presence of arteriovenous fistula is around 5%, while it is very low in chronic pseudoaneurysms. It is important to note that data on mortality outcomes are less frequently published compared to data on successfully completed treatments (15).

In light of the complexities and potential severity of iliac vessel injuries during spinal surgery, we have summarized the critical aspects of this condition in a concise table 1. This table is designed to serve as a quick reference for clinicians, providing essential information on incidence, typical injuries, clinical presentations, diagnostic and surgical interventions, outcomes, risk factors, and management strategies. The aim is to enhance the understanding and readiness of healthcare professionals when faced with such challenging scenarios. Refer to the table below for a comprehensive overview:

Table 1. Summary of Key Clinical Information on Iliac Vessel Injuries During Spinal Surgery

Aspect	Details
Incidence & Severity	Rare but potentially fatal, with a reported incidence of 0.04% and mortality rates between 15-60%.
Common Injuries	Iliac artery and vein injuries are common, with risks of laceration, arteriovenous fistulas, and pseudoaneurysms.
Clinical Presentation	Symptoms range from immediate hemorrhagic shock to asymptomatic progression. In the presented case, postoperative hemorrhagic shock occurred with a hemoglobin drop to 35g/l.
Diagnostic Approach	Utilization of MSCT angiography is critical for identifying active bleeding and vascular lesions.
Surgical Intervention	Emergency surgery often necessary; techniques include direct suturing and graft interposition (e.g., 8mm Dacron graft for iliac artery reconstruction in the case study).
Risk Factors	Previous surgeries, close proximity of the disc to major vessels, and intraoperative challenges such as excessive instrument intrusion and inadequate positioning.
Management Strategies	Vigilant peri- and postoperative monitoring is vital due to the risk of delayed massive bleeding. Both endovascular and open surgical interventions are viable depending on the scenario.

CLINICAL PRACTICE ALGORITHM FOR ILIAC VESSEL INJURIES DURING SPINAL SURGERY

Iliac vessel injuries during spinal surgery represent a significant clinical challenge due to their potential for severe complications, including hemorrhagic shock and even mortality. This clinical practice algorithm has been developed to guide surgeons, anesthesiologists, and perioperative teams through the systematic approach required for the prevention, diagnosis, and management of iliac vessel injuries during spinal procedures. It encompasses a series of meticulously outlined steps that facilitate early detection and effective intervention, thereby minimizing patient morbidity and improving outcomes.

Step 1: Preoperative Assessment

1.1 Evaluate Patient History: Check for prior abdominal or spinal surgeries which might increase risk of adhesions.

1.2 Radiological Examination: Preoperative MRI or CT scan to assess the relationship between vascular structures and the surgical site.

Step 2: Intraoperative Monitoring

2.1 Vital Signs: Continuously monitor blood pressure and heart rate to detect signs of bleeding.
2.2 Surgical Technique: Utilize precise and gentle maneuvers to minimize risk of vascular injury.

Step 3: Identification of Vascular Injury

3.1 Detection: Look for signs of significant blood loss or a drop in blood pressure.
3.2 Initial Response: Stabilize the patient by pausing the surgery and preparing for diagnostic assessment.

Step 4: Diagnostic Confirmation

4.1 Intraoperative Ultrasound: Quick assessment to locate the injury.
4.2 Confirmatory Imaging: Perform MSCT angiography for a detailed view of the vascular injury.

Step 5: Management of Vascular Injury

5.1 Immediate Hemostasis: Apply manual pressure or vascular clamps to control bleeding.
5.2 Surgical Repair:
5.2.1 Minor Lacerations: Direct suturing.
5.2.2 Major Injuries: Use of vascular grafts or stenting.

Step 6: Postoperative Care

6.1 Intensive Monitoring: Monitor hemodynamics and hematocrit levels closely in the immediate postoperative period.

6.2 Follow-up Imaging: Schedule postoperative MSCT angiography within 24-48 hours, then periodically.

Step 7: Long-term Monitoring

7.1 Regular Follow-ups: Perform periodic Doppler ultrasounds to ensure proper blood flow and check for late complications.

7.2 Patient Education: Advise the patient on symptoms of complications such as swelling, pain, or color change in the extremities.

CONCLUSION

Iatrogenic vascular injuries during spinal surgery are rare but can have potentially fatal outcomes. To promptly establish a diagnosis, it is crucial for both the anesthesiology and surgical teams to be aware of the possibility of such injuries during spinal surgery, as clinical manifestations may occur with delayed effects, as was the case with our patient. Due to the potential for life-threatening bleeding resulting from vascular injuries, careful and intensive perioperative monitoring of patients after spinal surgery is extremely important.

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EFFECTS OF SODIUM BICARBONATE SUPPLEMENTATION IN MARTIAL ARTS

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ABSTRACT

The aim of this systematic review was to evaluate the effects of consuming sodium bicarbonate ($NaHCO_3$) and to gain insight into the nature of any changes in performance following $NaHCO_3$ supplementation among combat sport athletes. The analysis of the results provides compelling evidence in favor of acute or chronic $NaHCO_3$ supplementation as an ergogenic substance which could have an impact on several aspects of performance in judo [23, 31, 32], taekwondo [17, 20], karate [17, 33] [28, 29], wrestling [18, 19], jiu-jitsu [32] and boxing [16].
Acute or chronic $NaHCO_3$ supplementation is effective in the improvement of several variables of physical performance in combat sports during testing and simulated matches. Enhanced performance resulted in the increased capacity of the glycolytic system. However, the positive effects of its use are most often visible following the onset of fatigue. In addition, the use of $NaHCO_3$ is associated with an increased concentration of lactate in the blood. This systematic review provides data relevant for sports professionals and athletes alike regarding the use of $NaHCO_3$ as a supplement, prior or during training and matches.

Keywords: *sodium bicarbonate, supplementation, martial arts, physical performance, fatigue, lactate , acidosis. ergogenic effects.*

INTRODUCTION

Combat sports such as judo, taekwondo, karate, Brazilian jiu-jitsu, and boxing require activities of maximum and submaximum intensity, as well as short periods of recovery during the competition itself [1, 2]. In combat sports action comprises a combination of physical fitness and technical-tactical abilities for achieving better performances [3, 4]. However, these sports include various types of effort and rest periods during a competition [4]. Competitions are, in various combat sports, such as judo and taekwondo, divided into weight categories, where we often encounter the problem of deliberate decrease in body mass a few days prior to competition, so as to gain an advantage of over lighter opponents [5].

It is well-known that recovery between bouts is quite brief (lasting from 15 to 30 seconds in judo) [6]. During these brief intervals there is not enough time for the suitable activation of the ATP, which renders the exertion of exercising and recovery dependent on the amount of lactate in the muscles [7]. Sports science strives to apply the scientific principles of food, diet, and supplementation, since they are a necessary requirement in contemporary sport [8]. Combat sport is no exception in this respect, and it is necessary to find ways of maximizing the abilities of athletes [8]. Therefore, most athletes are focused on using supplements and energy drinks such as vitamins, proteins, carbohydrates, sodium bicarbonate, and caffeine to enhance performance [9]. Numerous athletes have indicated a tendency to use caffeine and sodium bicarbonate (better known as baking soda ($NaHCO_3$), which are found on the list of substances banned by the International Olympic Committee [10, 11]. Numerous studies and meta-analyses [12] have shown that an increase in intracellular or extracellular buffering capacity by means of beta-alanine or sodium bicarbonate supplementation can improve the capacity and performance of exercise, especially in activities where acidosis limits performance [13-15]. Numerous studies [16-25] which have focused on the ergogenic activity of alkaline substances in combat sports of high intensity and short duration have confirmed a positive impact on the delay in onset of fatigue and an improvement in exercise [8, 26-29]; still, some studies deny or are unable to confirm the effect of sodium bicarbonate on the performance of combat sports athletes [26, 30]. Therefore, there is a need for further study which might in more detail explain and pinpoint the impact and association between sodium bicarbonate and the activity of combat sports athletes.

To our knowledge, no previous study has included research which focused on the effects of sodium bicarbonate supplementation in combat sports and processed it in the form of a systematic review. Therefore, the aim of this study was to find scientific research which focused on the effects of sodium bicarbonate supplementation in combat sports.

METHODS

Electronic databases such as PubMed, Google Scholar and Medline were used to search for relevant articles. The search was limited to studies published from 2006 to 2021. The key words used for the search included: “sodium bicarbonate”, “judo”, “martial arts”, “supplementation”, “wrestling” and “karate athletes”. In addition, the references of the included studies were analyzed to identify further studies in that field. The database search was limited to articles published in English. During the first phase of the search, the relevance of the titles and abstracts was analyzed. During the second phase of the search, entire articles were accessed and analyzed for inclusion. After all these steps were completed, 19 articles were selected for analysis, all of which were relevant for this study (Diagram 1). The titles and abstracts of all the analyzed articles were reviewed by two researchers, in the form of a double review. All of the studies that met the inclusion criterion were accessed in full-text form.

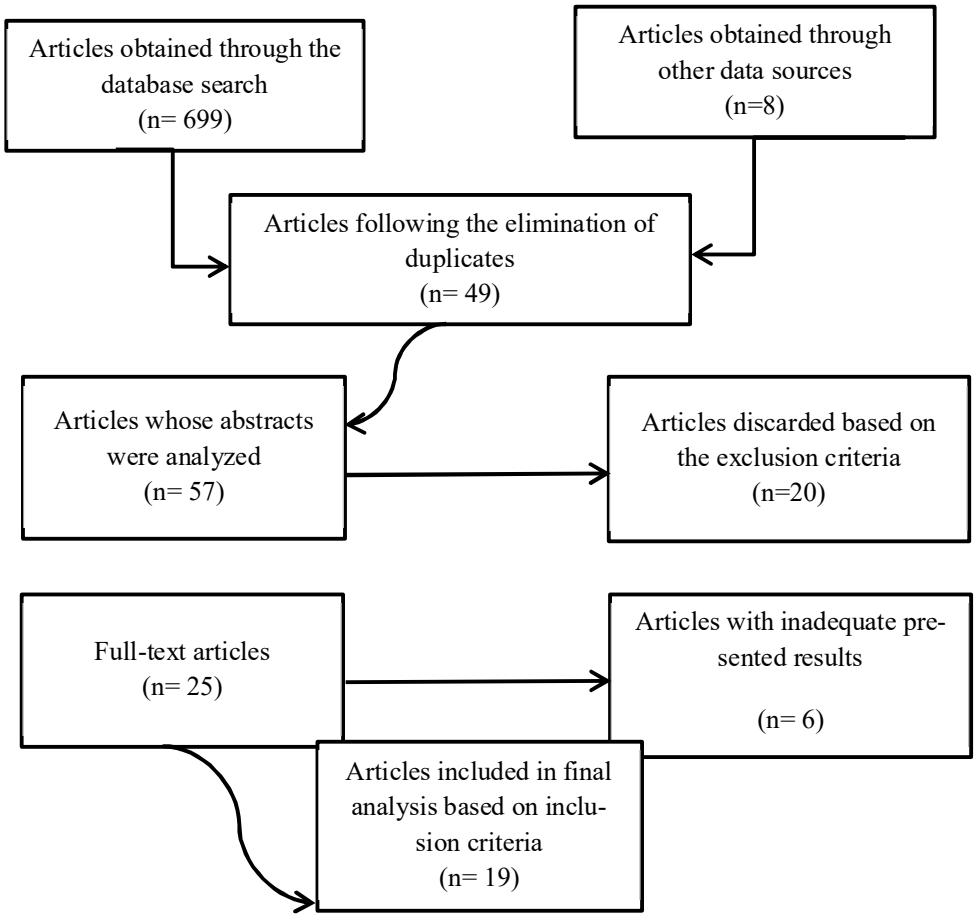
Articles were included in the study based on the following criteria: (1) articles from the field of combat sports; (2) sodium bicarbonate ($NaHCO_3$) supplementation with the aim of evaluating the effects of supplementation on blood lactate concentration, whereby various tests were used (the Wingate test, Boxing specific high-intensity interval running, the Karate-specific aerobic test, Taekwondo Anaerobic Intermittent Kick Test, and the Special Judo Fitness Test).

These criteria were used to form a diagram which presents a schematic overview of the data search (Diagram 1).

Articles were excluded from the study based on the following criteria: (1) review studies; (2) studies in which the results were not adequately presented or parameters for further analysis were lacking; (3) articles in which the tested athletes did not take part in combat sports.

The participants included wrestlers, boxers, judokas, karatekas, taekwondists, and jiujitsukas, both male and female. The data extracted from the full texts are shown in Table 1 in the following order: the author’s last name and year of publication, the participants, the exercise tests, dose of sodium bicarbonate, duration of the treatment, and main findings. Extraction and verification of data were carried out independently by two authors (MM, AS).

Diagram 1. Flow diagram of the data search strategy



RESULTS

Table 1. Summary of study characteristics and findings for studies exploring the effects of sodium bicarbonate supplementation in martial arts

Study	Participants	Implemented physical treatment	Dose of $NaHCO_3$	Timing of intake	Main findings
Artioli et al., 2006	EG= 3, CG= 3, 6 male judo athletes, age: 20 ± 1.9	3 × 5min judo bouts, 15 min. of rest	0.3g/kg	120 min prior to testing	- Standing judo ↔ - Total number of attacks ↔ - BLC (after the fight 1) ↑

Study	Participants	Implemented physical treatment	Dose of $NaHCO_3$	Timing of intake	Main findings
Artioli et al., 2007	23 male judo athletes, SJFT= 9, age: 21.5 ± 3; WT= 14, age: 19.3 ± 2.4	3 × SJFT (5-min rest); 4 × the Wingate test for upper extremities, 3-min recovery period between attempts	0.3g/kg	120 min prior to testing	- Number of throws (attempts 2 and 3, total) ↑ - BLC (after SJFT) ↑ - WT (attempts 3 and 4) ↑ - BLC (after WT) ↔
Siegler & Hirscher, 2010	10 male boxers, age: 22±3	2 two competitive boxing sparring matches one week apart; 4 × 3 min rounds, 1 min seated recovery	0.3g/kg	60 min prior to testing	- Total effectiveness of the punch ↑ - HR ↔ - RPE↔
Tobias, et al., 2013	37 male athletes, judo: n= 16, jiu-jitsu: n= 21 age: 26±4	4 × 30s – the Wingate test for upper extremities with a recovery period of 3 min between attempts	BA+ SB 0.5g/ kg	7 days during week four	- WT (attempts 2, 3 and 4) ↑ - BLC (after WT) ↑ - MP↑ - PP↑ - Total work done↑
Kazemi et al., 2013	16 male taekwondo athletes, age:17.93 ± 0.34	2 × 30 sec of repeated vertical jumps, 60 min rest before the second attempt	0.065g/kg	day of testing	- average value of anaerobic strength (attempts 1 and 2) ↑ - BLC ↔
Felippe et al., 2016	10 male judo athletes, age: 23 ± 5	3 × SJFT (5-min recovery)	0.3g/kg	120 to 60 min prior to testing	- Number of throws in all attempts (CAF+ SB) ↑ - Number of throws (SB) ↔
Yousef et al., 2015	10 elite male taekwondo athletes, age: 26.2 ± 4.26	Interval training, speed training, plyometrics, punching focus mitts for a period of 6 weeks	0.3g/kg	60 min prior to treatment	- SB & BA compared to LD & CK ↔
Šančić et al., 2017	10 judo athletes (6 males and 4 females), age: 19.2 ±1.4	SJFT and JMS for a duration of 4 min	0.3g/kg	120 min prior to testing	- Total number of throws ↔ - BLC ↑

Study	Participants	Implemented physical treatment	Dose of $NaHCO_3$	Timing of intake	Main findings
Oliveira et al., 2017	2 judokas, 5 jiu-jitsu male athletes, age: 26 ± 5	4 × modified Wingate test for the upper extremities, 3 min of recovery	0.5g/kg, 0.125g/kg per day	5 days prior to treatment	- TMW in total ↑, - attempts 1 and 2 p= 0.38 ↔ - attempts 3 and 4 p= 0.001 ↑
Durkalec-Michalski et al. 2018	18 female and 31 male wrestlers age: 19± 4	2 × Wingate test alternating with throwing a testing dummy during recovery	0.025 to 0.1 g/kg day 1-2= 0.025, day 3-5= 0.05, day 6-7= 0.075, day 8-10= 0.1g/kg	10 days prior to treatment	- Time to peak power (first Wingate) ↑ - Time to peak power (second Wingate) ↓ - maximum, average and minimum power: ↔ Number of throws: ↔
Lopes-Silva et al., 2018	9 male taekwondo athletes age: 19.4± 2.2	3x 2 min of a simulated taekwondo match, one minute of recovery	0.3 g/kg	90 min prior to the match simulation	- BLC ↑ - Tat ↑ - RPE ↔
Gough et al. 2019	7 male elite professional boxers, age: 27.1 ± 5.1	3 × HIIR protocol, followed by 2 ×T_{LIM}(1&2), (75 min rest) as well as a specific protocol of boxing punching combinations	0.3 g/kg	10 min after the treatment	- Lactate threshold ↑ - time to fatigue: ↑
Razaei et al. 2019	8 karatekas age: 20.5 ± 2.4	A karate-specific aerobic test	SB+ caffeine SB: 0.3g/kg caffeine: 6g/kg	Three-day intake, 120 to 60 min prior to the treatment	-TTE: ↑ -RPE ↔ -BLC ↑
Ragone et al., 2020	10 male jiujitsukas age: 22.2 ± 3.9	Test of maximum voluntary contraction, intermittent isometric test of contraction	0.3 g/kg	60 min prior to the test	- BLC ↑ - Overall number of contractions ↔ - Duration of the performance ↔

Study	Participants	Implemented physical treatment	Dose of $NaHCO_3$	Timing of intake	Main findings
Durkalec-Michalski et al. 2020	18 female and 33 male wrestlers age: 19.2 ± 3.1;	2 × Wingate test with throwing a testing dummy during the rest period of the SJFT	0.025 to 0.150 mg/kg	10 days prior to testing	- PP: ↔ - MP: ↔ - Power drop: ↔ - Average power ↑ - Power during the performance of the Wingate test: ↑ - Number of throws: ↑ only among the males
Koozehchian et al., 2020	40 well-trained male taekwondo athletes, age: 21.4 ± 1	3 × Taekwondo anaerobic intermittent leg kick test, 60 sed rest between bouts	CR+SB, SB SB: 0.5g·kg CR: 20g	5 days prior to testing	- PP↑ - MP↑ - Fatigue index ↔ - BLC ↓
Sarshin et al. 2021	40 male taekwondo athletes age: 21 ± 1	Taekwondo anaerobic intermittent leg kick test	0.5 g/kg	during 5 days prior to testing	- PP: ↑ - MP↑ - BLC ↑ - RPE ↑ - Overall number of leg kicks ↑

Legend: EG: experimental group; **CG:** control group; **n:** number of participants; **HR**: heart rate; **BA**: beta-alanine; **SB**: sodium bicarbonate; **CAF:** caffeine; **CK**: creatine kinase; **CR**: creatine monohydrate; **HIIR**: high intensity interval run; **SJFT**: Special Judo Fitness Test; **JMS**: Judo match simulation; **T_{LIM}**: high-intensity treadmill running; **RPE**: rates of perceived exertion; **PP**: Pick Power; **MP**: Mean Power; **Tat**: total attack time; **TTE**: Time to exhaustion; **TMW**: Total mechanical work; **WT:** Wingate test; **BLC:** blood lactate concentration; **LD**: Lactate dehydrogenase; ↑:significant increase**;** ↓:significant decrease**;** ↔**:** no significant difference**;** †: significant difference.

By searching the electronic databases, a total of 669 significant studies were identified. In addition, based on other data sources, a further 8 studies were noted. After the removal of duplicates, 49 studies remained. The abstracts of 57 articles were analyzed. Based on the exclusion criteria, 20 articles were removed. According to the clearly defined inclusion criteria, 17 studies were included in the final analysis. The total number of participants included in the review is 423. Of them, 383 participants were male, and only 40 female. The articles were published from 2006 to 2021.

DISCUSSION

The aim of this systematic review was to analyze the effects of $NaHCO_3$ supplementation among combat sports athletes. The analysis of the results provides compelling evidence in favor of acute or chronic $NaHCO_3$ supplementation as an ergogenic substance which could have an impact on several aspects in the performance of athletes in judo [23, 31, 32], taekwondo [17, 20], karate [17, 33] [28, 29], wrestling [18, 19], jiu-jitsu [32] and boxing [16].

The effects of $NaHCO_3$ on physical performance

Considering that combat sports are characterized by occasional bouts of high-intensity activity for which energy is obtained predominantly from anaerobic sources – the breakdown of phosphocreatine (PCr) and through the glycolytic metabolism [17, 19], $NaHCO_3$ as an ergogenic substance can impact the enhancement of anaerobic capacity, and thus have an effect on adequate performance. Dukralec et al. [18] determined that supplementation of 100 mg · kg− over a period of 10 days led to an increase in the maximum level of power on the Wingate test (WT) among a group of elite wrestlers who used supplementation. However, a reduction in the time needed to achieve maximum power was noted only during the second WT attempt. The possible reasons for the deviation in the obtained results could be explained by the fact that the implemented protocols cannot be applied, considering that the test required 30 seconds of high intensity work, while the recovery period lasted one minute. Similarly, Artioli et al. [31] studied the acute effects of $NaHCO_3$ on a sample of 23 professional judokas, using different WT protocols for the upper extremities, and concluded that there were no improvements in their performance during the first two matches, while an improvement was noted for matches 3 and 4. These findings are in agreement with the work of Oliveire et al. [24] who indicated that an improvement among judo and jiu-jitsu athletes who consumed 0,125 g/kg over a period of 5 days, was noticeable during the third and fourth performance of the WT. These results should be considered more relevant since a specific and more adequate protocol was implemented. In each of these studies it was claimed that the $NaHCO_3$ is an ergogenic substance which can improve anaerobic performance, but only after the first onset of fatigue. While previous studies [18, 24, 31] focused on the effects of $NaHCO_3$ during dynamic tests, only one study [34] focused on how $NaHCO_3$ affected the isometric hand grip strength among jiu-jitsu athletes, whereby no statistically significant changes in the experimental group were noted. According to the authors [34], a possible explanation is that no desired effects occurred since the flexor muscles of the fingers have a very small amount of muscle mass compared to larger muscle groups. Therefore, there is no increased distribution of lactates which would lead to fatigue and the potential effects of $NaHCO_3$.

After that, the data available on the effects of $NaHCO_3$ supplementation on the specific match performances were contradictory. While the first study of Artioli et al. [21] suggests that the number of attacks did not increase following the intake of 0,3 g/kg $NaHCO_3$ during a simulated judo match, a later study [31] indicated that an improvement occurred in the number of throws when this test was used (SJFT) during the second and third attempt. Furthermore, the same dose led to an increase in the number of punches thrown during a boxing match [16]. In this field of research, some studies were not able to analyze the ergogenic effects of $NaHCO_3$ on specific performances among combat sports athletes [18, 23, 25]. The use of 0,3 g/kg $NaHCO_3$ did not lead to an increase in the number of throws among junior and senior cadet judokas [25]. Durkalec-Michalski et al. [18] determined that there were no statistically significant differences between the group that took the supplement and the placebo group, in terms of the number of throws during a specific sparring test in wrestling. Similarly, Durkalec-Michalski et al. [19] also determined that $NaHCO_3$ supplementation over a period of 10 days increases the total number of throws, but only among male judokas, while female athletes were not susceptible to changes under the influence of $NaHCO_3$ consumption. A possible reason for these results could be found in the heterogeneity of the sample of participants. Specifically, Sančić et al. [25] worked with a sample of young judokas, while Durkalec-Mihalski et al. [19] noticed that female athletes showed no sign of being affected by $NaHCO_3$ consumption, since the pH value among female athletes is more susceptible to smaller changes compared to male athletes during high intensity activities. However, it should be mentioned that enhancements in these studies were achieved by consuming only $NaHCO_3$. More recent studies [23, 32, 35, 36] focused on the effects of consuming $NaHCO_3$ in combination with other stimulants which could impact specific performances. Felippe et al. [23] studied the isolated effects of caffeine and $NaHCO_3$ and their combination in judo, and determined an improvement in the overall performance only when the caffeine and $NaHCO_3$ were combined. In support of these findings, Tobias et al. [32] reported the effectiveness of a combined supplementation of $NaHCO_3$ and beta-alanine (BA) on the overall performance of judo and jiu-jitsu athletes. Individual intake of $NaHCO_3$ and BA increased power during the second and fourth performance, but the difference lies in the fact that the effect of improvement in the total performed work was greater when these two supplements were taken in combination, compared to when they were taken individually.

In addition to physical performances, numerous scientists [17, 20-22, 25, 32, 33] drew parallels between the use of $NaHCO_3$ and increased levels of lactates following testing. More precisely, the sarcolemma is impenetrable for the bicarbonate [37] which leads to higher concentrations of H+ ions in the blood, but not in the intramuscular area [38]. This process leads to a decrease in acidosis within the muscle cells and indicates a high level of performances without the negative effects of fatigue [39].

Dosing, timing, and the unwanted effects of $NaHCO_3$

The most frequent dose of $NaHCO_3$ was 0,3 g/kg during acute intake [16, 20-23, 25, 31, 40], while during chronic intake it ranged from approximately 0,025 mg/kg [18] to 0,5 g/kg [17], which suggests that smaller doses [41] might not have an effective enough impact on performance in combat sports. Taking into consideration the consumption of $NaHCO_3$ or a suitable placebo, it was determined that the intake took place 60 to 120 minutes prior to the experimental program, , since the claim was that $NaHCO_3$ requires at least 60 minutes to be absorbed and to achieve maximum concentration in the plasma [42]. In addition, acute use of $NaHCO_3$ can lead to various negative effects such as stomach pain or vomiting [43]. In order to avoid any discomfort, in some studies [17-19, 24, 32] the participants took lower doses over a period of 5 to 10 days. However, when analyzing the negative effects of supplementation, several factors should be taken

into consideration. The training status [10] and the form in which $NaHCO_3$ was ingested [43] were defined as mediators which can impact the effect of $NaHCO_3$. This systematic review does have certain limitations regarding the variety of performance tests and the used variables. However, combat sports are complex activities in which physical work is one of several factors needed for success. In that sense, further studies are needed to determine the effects of $NaHCO_3$ on sport-specific situations during simulated matches, especially in situations when decision making is involved. That is why these findings are incomplete and are still insufficient to help determine whether and how $NaHCO_3$ improves overall performance in combat sports.

CONCLUSION AND PRACTICAL IMPLEMENTATION

Acute or chronic $NaHCO_3$ supplementation is effective in improving several variables of physical performance in combat sports during tests and simulated matches. Enhanced performance resulted in an increased capacity of the glycolytic system. When it comes to the physiological mechanism of action of $NaHCO_3$, it is based on an increase in buffer capacity and an increase in bicarbonate ions, i.e. raising the pH value in the fluid of the extracellular part. In this way, it is possible to maintain an alkaline environment, increasing the gradient of extracellular H+ ions, which in turn stimulate lactate/H+ cotransporter. This gradually leads to a higher flux of H+ ions from the intracellular to the extracellular fluid, which then bind to the circulating HCO_3^- ion and as a consequence have an increase in pH value, i.e. reduction of acidity leading to fatigue.

Reducing the accumulation of H+ in the active muscles would enable the process of muscle contraction to be maintained for a longer time and to continue the process of ATP synthesis through the glycolysis process, which delays the onset of muscle fatigue during high-intensity exercises. This mechanism is in line with the Cori cycle, as well as with the alanine cycle. The importance of the Cori cycle lies in the prevention of acidosis caused by the activity of anaerobic muscle capacities. Due to the accumulation of lactate, muscle spasms could occur, and the only way to chemically utilize lactate in the body is with the help of lactate dehydrogenase. It has long been believed that during cell recovery after exercise, 85% of lactate is eliminated by transport to the liver where it is then converted to glucose or glycogen [46,47]. Recent studies on rats as a model of the organism reveal that the common fate of lactate is oxidation. In addition, various authors suggest that the role of the Cori cycle is not so high, but that its share is only 10 to 20% [47]. Contrary to popular belief that lactates are the main cause of muscle fatigue, a decrease in pH value within muscle caused by an increase in H+ is the main cause of fatigue [48]. This is also applicable to the alanine cycle where amino acids are precursors of gluconeogenesis, i.e. alanine takes the place of glucose (reference). Studies examining the synergistic effect of NaHCO3 and caffeine supplementation have shown that in addition to the already described effects of $NaHCO_3$, caffeine has a positive effect on delaying fatigue through mechanisms that include its contribution to lowering extracellular potassium and increasing plasma catecholamine [44]. In the case of synergistic effects of beta-alanine and sodium bicarbonate supplementation, there is an increase in muscle carnosine and plasma HCO_3, thus strengthening the intra- and extracellular buffer system and trying to improve sports performance or delay or reduce muscle fatigue in high-intensity sports in whom intramuscular acidosis occurs [45]. Consuming sodium bicarbonate can also raise your blood sodium levels, which may increase blood pressure in some people. In addition, large amounts of sodium can make your body retain water. While increased hydration could be useful for those exercising in the heat, it may be disadvantageous for those competing in weight-category sports. All this leads to the conclusion that a diet low in sodium should be kept during sodium bicarbonate supplementation. High consumption of sodium bicarbonate can increase potassium excretion, which in some cases can cause potassium deficiency, so during the supplementation period, a diet rich in potassium is recommended (diet rich in vegetables especially green such as lettuce and cabbage). [49].

However, the positive effects of use are usually visible after the onset of fatigue. Also, the use of $NaHCO_3$ is associated with an increased concentration of blood lactates. Contradictory results exist when it comes to sports-specific activities during simulated matches, RPE and the negative effects in the form of nausea, which clearly indicate that it is necessary to synchronize the timing, amount, form of intake, and the individual characteristics of athletes as well as the characteristics of the combat sport in order for the overall effect to be optimal. It is very important to mention that sodium bicarbonate is a very quick-acting antacid. It is used as an antacid to treat heartburn, indigestion, and upset stomach. However, it should be used only for temporary relief. When sodium bicarbonate mixes with stomach acid, it produces gas. This may cause abdominal pain, bloating, nausea, diarrhea, and vomiting. These side effects appear to be dose-dependent, meaning higher doses may lead to worsened stomach issues. Further, not everyone will experience these side effects. The severity of symptoms can vary based on the amount taken and personal sensitivity. To decrease side effects, try taking sodium bicarbonate with a carbohydrate-rich meal, spreading your doses throughout the day, taking the supplement 180 minutes before exercise, and/or taking enteric-coated capsules, which are easier on the stomach. In the cases with hypochlorhydria consumption of additional $NaHCO_3$ could cause serios health issues and maldigestion.

This systematic review provides data relevant for sports professionals and athletes regarding the use of $NaHCO_3$ as a stimulant prior to or during training and matches. In addition, it would be useful for future studies to continue analyzing the effects of $NaHCO_3$ in situations similar to matches, and to take into consideration cognitive abilities, and not only the individual differences among athletes, but also the differences between the very combat sports themselves.

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ELECTROMECHANICAL ASSOCIATION AS A STEMI MIMICRY - CASE REPORT

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ABSTRACT

Electromechanical association is a very unique physiological type of ECG artifact caused by the radial artery pulse tapping. ECG presentation of this artifact may imitate primary repolarization changes characteristic for acute coronary syndrome or electrolyte abnormalities. The peculiarity of electromechanical association is the synchronization with regular cardiac cycles, making a diagnostic challenge even for experience physicians. The present case is a rare example of an ECG artifact localized in a specific pattern mimics ECG changes of acute myocardial infarction with ST elevation (STEMI), including reciprocal ST depression. Moreover, serial prehospital ECGs verify evolution from hyperacute T waves to true ST elevation, as we seen in acute myocardial infarction. The key for artefact recognition was the knowledge about ECG leads derivation and identification the affected electrode. Electromechanical association frequency is unknown: it may be common, but often unrecognized finding. So far, electromechanical association in serial ECGs wasn't reported, as per our knowledge.

Keywords: *Electromechanical association, STEMI, artifact.*

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INTRODUCTION

Electrocardiogram (ECG) artifacts are abnormalities that have origin others than electrical activity of the heart. They may have two different sources: physiological (in patient's body) and non-physiological (out of patient's body) (1). Both of these ECG artifacts are common findings in everyday practice, especially in outpatients, during transportation and in emergency departments (2). Careful reading and correlations with clinical findings are usually sufficient for artifacts uncovering. However, some rare patterns can confuse and led toserious misdiagnosis or wrong and unnecessary procedures and interventions.

Electromechanical association is recognized as physiological type of ECG artifact caused by radial artery pulse tapping (3). Many physicians are not familiar with this phenomenon and its ECG presentation. Also, literature data are limited to only few case reports letting many questions remain opened. Cause of its appearance in particular patients along with the precise generating mechanism is still unknown. The contribution of arterial wall elasticity seems to be minor, being this artifact was described both in patient with arteriovenous fistula for dialysis and in patients with normal anatomy of radial artery (3,4). Tobe more confused, it is reported in ECG recording with both self-adhesive and clip-cuff electrodes, but also with limb electrode applied to the chest (3-5). Most likely, some specific relationship between artery movement, patient's skin and the electrode is necessary, although details are not fully understood.

CASE REPORT

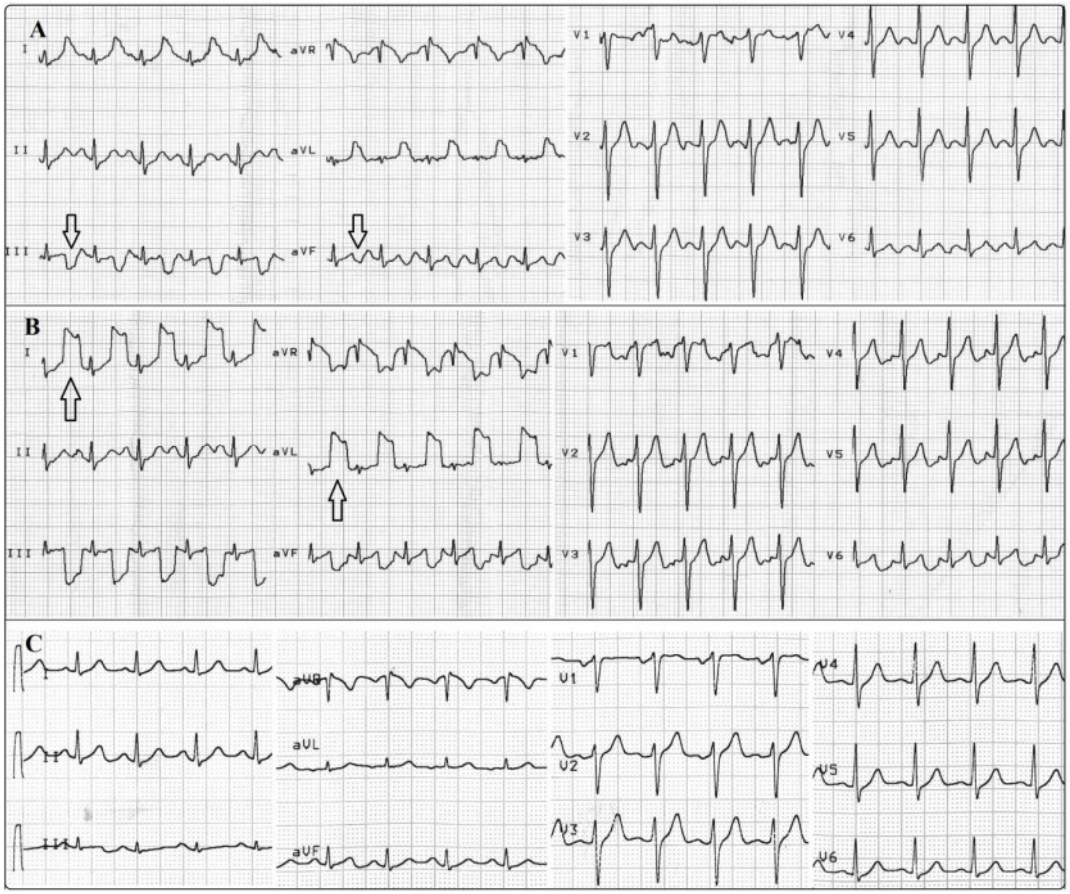
Previously healthy 33-year-old man was examined by the Emergency Medical Service, Clinical center of Serbia, Belgrade. 2 hours after the onset of weakness, dizziness and fever. The patient denied any chest discomfort. His vitals were: BP 120/80mmHg; HR 140/min; RR 18/min; Sat O2 97% on the room air; T 37,8º C.

The first electrocardiogram (ECG) showed tall T waves in leads I and aVL, along with widely inverted T waves in leads III and aVF, highly suspected on early stage of high lateral heart wall ischemia (Figure 1 A, arrows). Subsequent ECG (made immediately after the first one, without moving the patient) seemingly present STEMI evolution: huge, bizarre ST elevations in leads I and aVL, with inverted T waves in leads III and aVF associated with odd-looking ST depression (Figure 1 B, arrows). The patient was transferred to the ED, as suspected acute coronary syndrome. However, in ED ECG was completely normal (Figure 1 C) along with all requested laboratory results. Accordingly, the pre-hospital ECGs were recognized as artifacts and the patient was discharged home.

ECG presentations of this artifact are usually bizarre T/ST changes, but sometimes it could mimic primary repolarization changes similar to acute coronary syndrome (ACS), electrolyte disturbance or even arrhythmias (3,6). The peculiarity of electromechanical association is being present in every cardiac cycle, as the regular deflections with a fixed coupling-interval between QRS complex and that bizarre T/ST change in every affected lead (6). There is no precise data about this artefact frequency, but most likely it is not so rare, rather it's unrecognized. If misunderstood for pathological conditions, may lead to unnecessary therapies and interventions (7,8).

It is very uncommon to be localized in a specific pattern mimicking ECG changes of acute STEMI, including 'reciprocal changes'. Moreover, electromechanical association as an imitation of STEMI evolution in serial ECGs wasn't reported in literature, so far.

Figure 1. A) Initial prehospital ECG; B) Subsequent ECG; C) Hospital ECG.



DISCUSSION

ECG artifacts are typically unrelated to any of regular ECG waves (P, QRS and T). The lack of synchronization with cardiac cycle is the major difference between artifacts and ECG findings they mimic (1). In our knowledge, electromechanical association is the only one ECG artifact that doesn't fit in previous, and therefore could be the serious problem even for experience ECG reader.

This artifact is relatively recently discovered with few literature data on this topic. Actually, we find two papers with ECGs showing electromechanical association that was misread for other conditions. One was published in 2000. by Hung et al. (9) as ECG changes mimic acute myocardial infarction in acute pancreatitis. In 2005, Özhan et al. (10) reported a "bizarre electrocardiogram" attributing it to transient silent ischemia, associated with abnormal left ventricular motion.

Aslanger was the first who recognized the source of these ECG changes as radial arterial pulse tapping. The reason for this unusual artifact is overlapping between electric signal on limb electrode and radial pulse tapping just under the electrode (3,4,11).

ECG findings are presented like regular deflections in every cardiac cycle, sometimes mimicking acute repolarization changes. This relationship is best seen in simultaneously recorded a photoplethysmogram (PPG) pulse wave on wrist with an ECG (Figure 2). Each QRS complex precedes an arterial pulse occurring, what explain fixed coupling-interval between QRS complex and artefact in every affected leads.

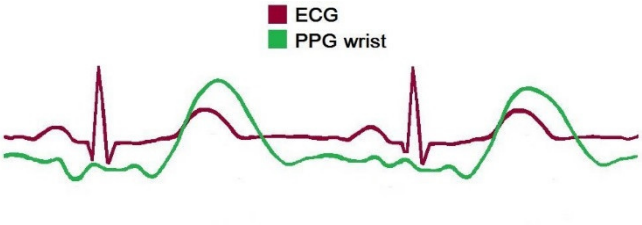


Figure 2. Electrocardiogram (ECG) and photoplethysmogram (PPG) pulse wave.

Aslanger also noticed that one of limb leads remains normal and changes-free, suggesting that it's not deriving from affected electrode (3,8). That has very simple explanation from the way that ECG leads are constructed (13).

In Einthoven's triangle, right arm (RA) is always negative pole (-), left leg (LL) is always positive pole (+) and left arm (LA) could be positive or negative (14).

Depend on bipolar leads electric voltage differences, limb leads will be derivate:

- Lead I: RA (+) and LA (-);
- Lead II: RA (+) and LL (-);
- Lead III: LA (+) and LL (-).

Augmented leads are constructed using The Goldberger Central Terminal (GCT), which compare electric potential between two limb leads as one electrode with the other electrode presenting the remain limb lead. The GTC inputs are always negative pole (-).

- Lead aVR: comparing potential between LA and LL (GTC/aVR) with RA (+);
- Lead aVL: comparing potential between RA and LL (GTC/aVL) with LA (+);
- Lead aVF: comparing potential between RA and LA (GTC/aVF) with LL (+).

The GTC will also constitute the negative pole of unipolar precordial electrodes (14).

Therefore, any disturbance in one arm electrode will affect all leads that include that culprit electrode, either directly or indirectly via the GCT (11).

A closer look at the ECG in our case reveals key features typical for LA electrode disturbance. The leads constructed from LA electrode (I, III and aVL) (14) show the most pronounced ECG artifacts. Being almost spatially opposite, leads III and aVL normally have "mirror picture' from each other. That is the reason for opposite polarity of artifacts in these two leads. In favor of previous, only unaffected limb lead is II and that lead doesn't involve LA electrode. Therefore, the reason for electromechanical association in our case was synchronization of left radial artery pulse tapping and LA electrode.

Interesting findings in this case were ST segment elevations organized in specific pattern for true high lateral wall ischemia and suspicious 'evolution' of ST/T changes. That is confusing, but just random finding, meaning only more pronounced artifacts on second prehospital ECG.

Newest data suggests some interesting explanation about this artifact. The authors propose that electromechanical association have a relationship between QRS complex and artifact deflection in a fixed coupling-interval, generating from the clip-cuff electrode motion with each blood flow pulsation. According to them, electromechanical association is more likely to be generated in heart diastole, than systole (15).

CONCLUSIONS

Electromechanical association is an ECG artefact caused by overlapping of the artery pulse tapping with the electrical signal generated in the ECG electrode placed over the same limb. The phenomenon often remains unrecognized or unexplained in the form of a bizarre ECG, rarely mimics acute coronary syndrome. A key finding for resolving the dilemma are changes typical of signal disturbances generated by the electrode from the affected limb, while the disorder has no effect on signals that do not involve the affected limb.

It is very important to recognize this artifact, in aim to avoid unnecessary, sometimes invasive interventions.

The exact mechanism of electromechanical association still remains unexplained. We will notice that in our case the patient has artifacts on both of prehospital ECGs. During recording, electrodes were removed and put again by the same medical technician approximately in the same place on the patient's wrists. However, patient himself didn't make any movement and remained lying still during that time. It is obvious that some specific relationship between electrode on patient's wrist, skin and radial artery itself is required. But all contributing factors necessary to produce this artifact remain unknown.

INFORMED CONSENT STATEMENT

All subjects gave their informed consent for inclusion before they participated in the study. This study is carried out in accordance with relevant ethical international guidelines. All authors have approved the manuscript and agree with submission.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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TUBEROUS SCLEROSIS AND KIDNEY FAILURE - A CASE REPORT

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ABSTRACT

Tuberous sclerosis (TS) is a rare systemic autosomal-dominant genetic disease in which multiple hamartomatous lesions occur in many organs and tissues. Kidney involvement of TS usually includes angiomyolipomas, cysts, rarely kidney tumors, but there is not much description of other kidney involvement (proteinuria, hematuria, advanced kidney failure). We present a patient with preterminal renal failure who is diagnosed with tuberous sclerosis. A 52-year-old female was admitted to the hospital, due to decreased kidney function with worsening of the general condition and crises of consciousness. During the examination, cutaneous lesions -facial angiofibrolipomas were noticed, the patient was dehydrated and pale. Laboratory analyzes showed elevated parameters of nonspecific inflammation, anemic syndrome, azotemia (Cr 578umol / l, urea 50.1mmol / l, CKD e GFR 10 ml / min / 73m^2), leukocyturia, microerythrocyturia, proteinuria 1.5 g / 24h. MSCT of the abdomen indicated hemangiomas of the liver, enlarged kidneys with several hypodense and hyperdense changes - angiomyolipomas. Due to the crisis of consciousness, MSCT of the head was performed, on which cysts and calcifications of the brain were observed. During the treatment, there was a confusing state, agitation, and epilepsy seizure. MRI brain showed multi locular brain cysts with ependymal and subependymal calcified nodules in the lateral ventricles.The applied treatment resulted in a partial recovery of kidney function (CKDeGFR 17 mil / min / 1.73 m2, and further approach in the monitoring and treatment of this patient was multidisciplinary. Chronic renal failure of 4 stages was maintained for a further six-month follow-up. We underline the importance of a multidisciplinary approach in the treatment of patients, bearing in mind that, as in our patient, a rare genetic disease - tuberous sclerosis can be manifested by kidney failure of preterminal rank.

***Keywords**: Tuberous sclerosis, angiomyolipomas, renal failure.*

INTRODUCTION

Tuberous sclerosis (TS), or tuberous sclerosis complex as it is also called, is a multisystem genetic disease in which multiple tumors are formed in many organs and tissues (brain, skin, kidneys, liver, heart, lungs, eyes). The frequency of this disease is 1: 6000-1: 10/000 newborns, in the general population 1: 20.000 (1, 2). The underlying disorder is thought to be a mutation in the TSC1 gene on chromosome 9 or the TSC2 gene on chromosome 16, which encode hamartin and tuberin and represent tumor suppressor genes (3,4). Renal lesions are described in a large number of cases (57.5%) and manifest as angiomyolipoma (85.4%), cyst (44.8%), rarely renal cell carcinoma (4.2%), however, a small number of authors describe the occurrence of renal failure of preterminal rank and proteinuria (5, 6).

CASE REPORT

Due to the worsening of the general condition with the development of renal failure and previous data on the disorder of consciousness, a 52-year-old patient was admitted to the Nephrology Clinic. During the examination, cutaneous lesions in the form of facial angiofibrolipomas were noticed and the patient was dehydrated and pale. The other finding was normal. In laboratory analyzes with elevated parameters of nonspecific inflammation (ESR 115 mm/h, CRP 41.45mg/l, anemic syndrome (Hb 79g / l), azotemia (Cre. 578umol / l, urea 50.1mmol / l, hyperkalemia 5.3, CKD and GFR 10 mil/min/73m^2), were observed in the urine leukocyturia, microerythrocyturia, proteinuria 1.5g / 24h. Due to the crisis of consciousness, the patient was examined by a neurologist, and MSCT of the head was performed, on which cysts and calcifications of the brain were observed. Abdominal ultrasound showed hemangiomas of the liver, renal cysts (up to 21 mm) with numerous lipomatous changes. MSCT of the abdomen indicated hemangiomas of the liver, kidneys about 16.5 cm in diameter, irregular structure without corticomedullary border with multiple hypodense and hyperdense changes (Figure 1). During the treatment, confusion, agitation, and then epilepsy seizures were treated in consultation with a neurologist and psychiatrist. Neurological examinations, EEG, which was within physiological limits, and MR of the head having confirmed multilocular cystic changes in the brain with ependymal and subependymal calcified nodules in the lateral ventricles (Figures 2 and 3). Examination by an ophthalmologist revealed a whitish irregular change in the fundus which may correspond to a typical change by tuberous sclerosis. The diagnosis of tuberous sclerosis was made based on typical brain changes, skin changes of the face, renal lesions (amgiomyolipomas and cysts). The applied rehydration, diuretic antibiotic, and other symptomatic therapy resulted in partial recovery of kidney function (Cre 305 umol / l) CKDeGFR 17 mil/min / 1.73 m^2, and a further approach in monitoring and treatment of this patient was multidisciplinary.

Figure 1. CT image demonstrates irregular kidney structure without corticomedullary border with multiple hypodense and hyperdense changes - angiomyolipomas

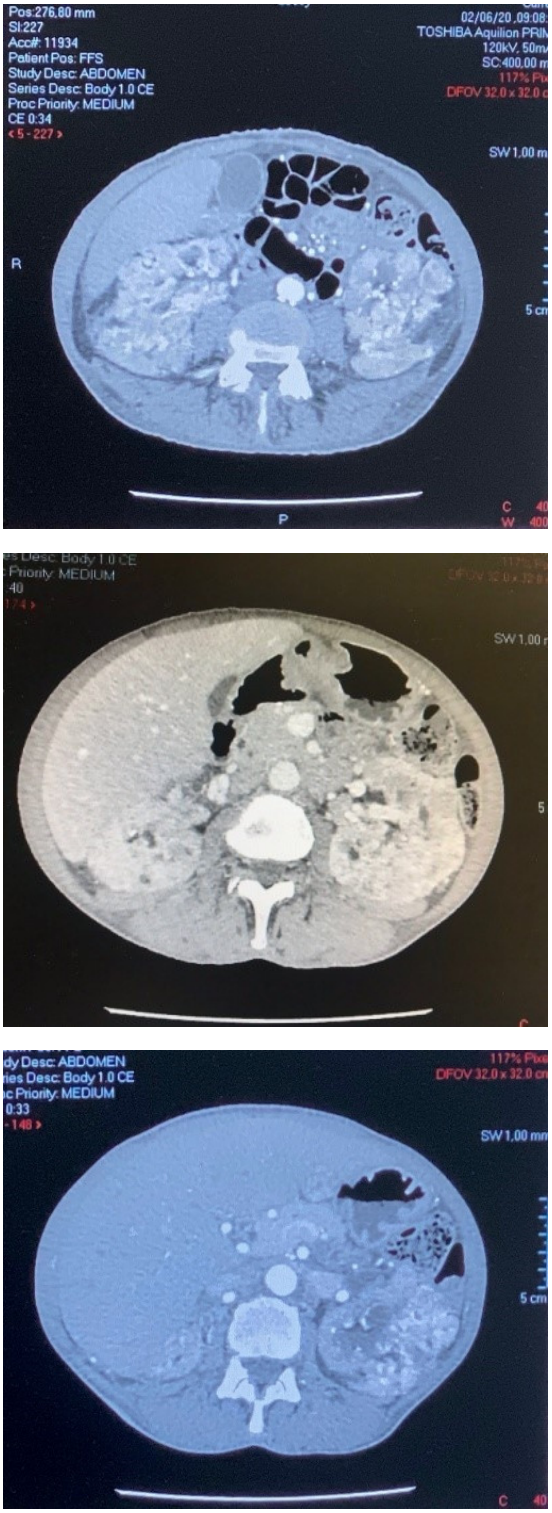
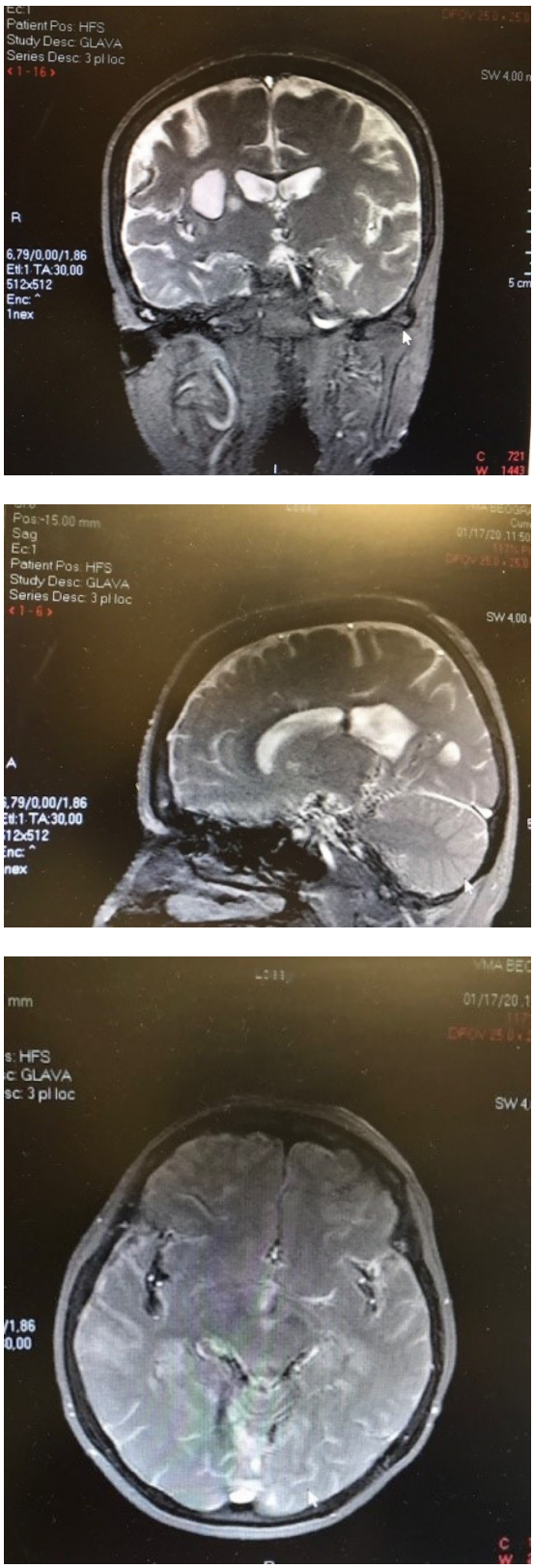


Figure 2. MR of the head having confirmed multilocular cystic changes in the brain with ependymal and subependymal calcified nodules in the lateral ventricles



DISCUSSION

Tuberous sclerosis is a genetic disease that usually manifests itself in the pediatric population, most often with disorders at the central nervous system (1-5). This is why these patients are usually diagnosed by neuropediatricians (53%), neurologists (17%), less often by other specialties among which nephrologists are represented in 4% of cases (6).

Bearing in mind that TS is characterized by disorders of many organs and systems for easier diagnosis, the recommendations of the International Tuberous Sclerosis Complex Consensus Group were adopted in 2012. when the recommendation for genetic tests was introduced (7). Diagnostic criteria are genetic and clinical, which are divided into major (hypopigmented macules ≥3, at least 5 mm in diameter, angiofibromas ≥3 or fibrous plaques, nail fibromas ≥2, shagreen skin, multiple retinal hamartomas, cortical dysplasias) subependymal astrocytomas of giant cells, cardiac rhabdomyomas, lymph angioleomiomas, angiomyolipomas ≥2. And in minor criteria: skin lesions "confetti", tooth erosions> 3, intraoral fibroids ≥2, retinal achromatic changes, multiple kidney cysts, non-renal hamartomas (7). Our patient had cortical tubers, subependymal nodules, renal angiomyolipomas, multiple renal cysts, and facial skin changes - multiple angiofibromas,

According to a large study that included 2093 patients with TS who ranged in age from 0-71 years (average age was 13 years), the most common manifestations were cortical tubers (82.2%) subependymal nodules (78.2%) hypomelanotic macules 66.8%, facial angiofibromas (57.3%), renal angiomyolipomas (47.2%) while other manifestations had a lower frequency (6).

Changes in the brain, skin, and kidneys dominated also in our case report, and in addition to these, changes were observed in the liver (hemangiomas) and the fundus. Epileptic seizures also occurred on a couple of occasions, with the control EEG being within the reference range.

According to the literature, the frequency of epileptic seizures in patients with TS is described in about 80% of cases, but this frequency is lower in the age group over 50 years (8,9). In a Japanese study of 166 patients with TS, 80% of patients were younger than 50 years, and in the group older than 50 years the most common brain lesions were subependymal nodules and autism, refractory epilepsy and subependymal astrocytomas were not observed (9). The frequency of epilepsy in general, refractory epilepsy, and intellectual disabilities in this study decrease with the age of the patients, i.e. they are more pronounced at a younger age, somewhat more common in male (9).

In addition to brain lesions, kidney lesions are often described within TS. Most studies have described the presence of kidney angiomyolipomas and cysts, a significantly lower percentage of cancers. Other kidney disorders, especially the progression of kidney failure, pathological urine sediment

(proteinuria, erythrocyturia) and arterial hypertension are described very rarely and are very serious complications whose early detection and treatment would be very important, especially in children (8). A study by Janssens et al, described the occurrence of arterial hypertension in 32% of cases, proteinuria in 16%, hyperfiltration in 25%, and terminal kidney failure in 7.5% (8). In our patient, kidney failure was initially observed (preterminal grade GFR 10-17mil / min / 1.73m2), and that was the reason she was admitted to the Nephrology Clinic. Among other disorders in the laboratory analysis, a pathological finding of urine in the form of proteinuria 1.5g / 24h and microerythrocyturia was observed. A favorable response to the applied treatment was manifested in a slight recovery of kidney function (Cre 305 umol / l , CKDeGFR 17 mil/min / 1.73 m2) , which was then maintained in a further six-month follow-up.

A study by Malaga-Dieguez et al, indicated that kidney disorders in the form of arterial hypertension (36% of cases) and lower GFR in 20% of patients were more pronounced in children and young adolescents with the TSC2 gene mutation (10). Similar data are confirmed by the Tuberous Sclerosis registry, which describes a statistically significantly higher prevalence of angiomyolipomas in TSC2 compared to patients with TSC1 mutation (59.2%; 33.3%) as well as a higher prevalence in females (11). The prevalence of multiple renal cysts is also linked to the TSC2 gene mutation and the ratio is 33.4% to 13.7% in the TSC1 mutation (11). In most patients, angiomyolipomas were asymptomatic (82%), and pain (6.1%), hematuria (5.0%), microscopic hematuria (4.3%), arterial hypertension (5.7%), and decreased kidney function were reported rarely (9%) (11). Our patient had bilateral angiomyoliposis and multiple renal cysts that were about 21 mm in size, with microerythrocyturia, proteinuria, and arterial hypertension, and kidney failure.

CONCLUSION

We underline the importance of a multidisciplinary approach in the treatment of patients, bearing in mind that, as in our patient, a rare genetic disease - tuberous sclerosis can be manifested by the kidney failure of preterminal rank.

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RESEARCH AND PUBLICATION ETHICS

Research Involving Human Subjects

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

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

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

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

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

Research involving Animals

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

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


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