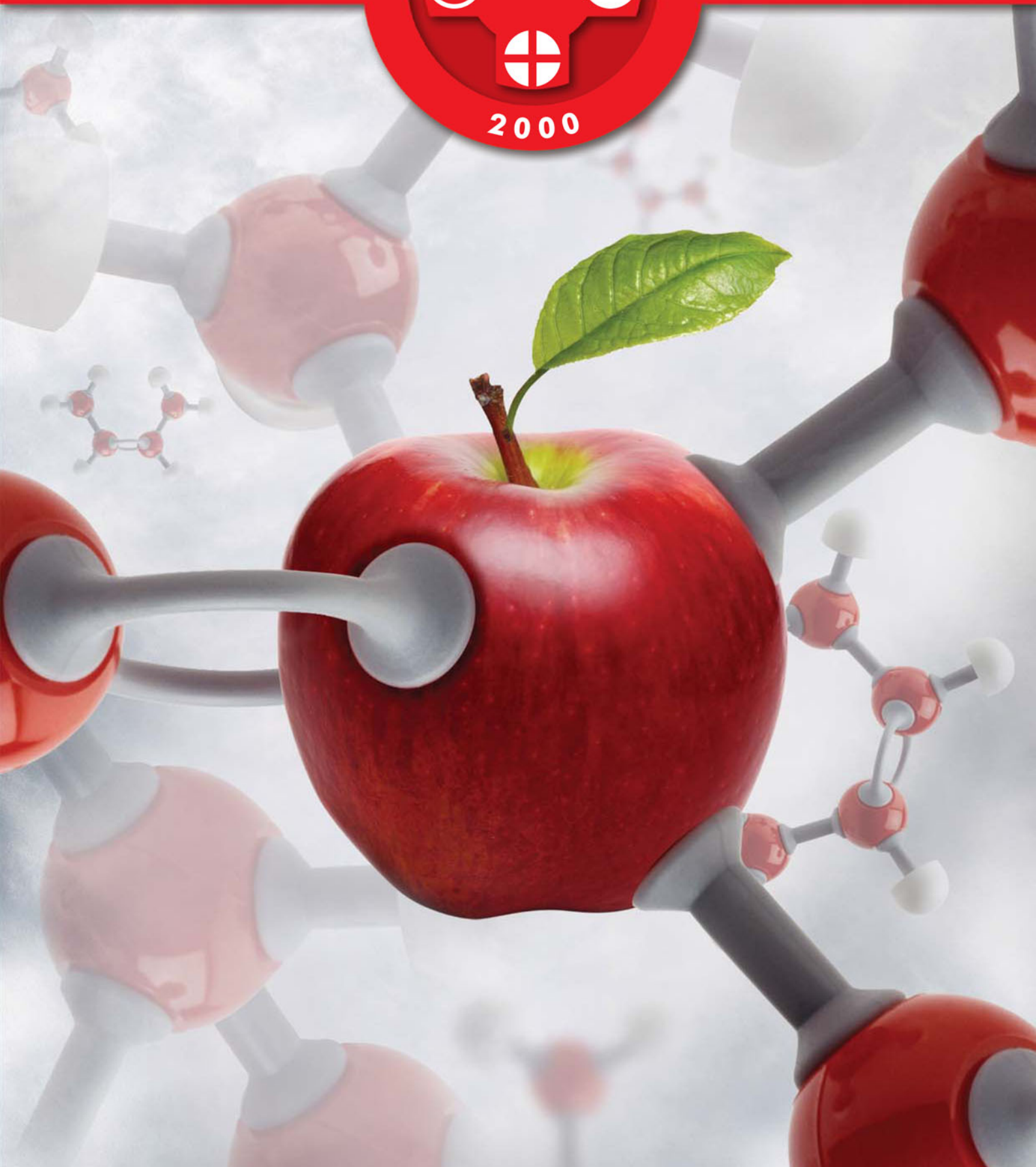
DE GRUYTER
OPEN

ISSN 1820-8665

Vol. 15•No4 • DECEMBER 2014.

Serbian Journal of Experimental and Clinical Research

2000



Serbian Journal of Experimental and Clinical Research

2000

Print
Faculty of Medical Sciences,
University of Kragujevac

Indexed in
EMBASE/Excerpta Medica, Index Copernicus, BioMedWorld, KoBSON, SCIndeks, Chemical Abstracts Service, Cabell's Directory, Celdes, CNKI Scholar (China National Knowledge Infrastructure), CNPIEC, EBSCO Discovery Service, Elsevier - SCOPUS, Google Scholar, J-Gate, Naviga (Softweco), Primo Central (ExLibris), ReadCube, SCImago (SJR), Summon (Serials Solutions/ProQuest), TDOne (TDNet), WorldCat (OCLC)

Address:
Serbian Journal of Experimental and Clinical Research, Faculty of Medical Sciences, University of Kragujevac
Svetozara Markovica 69, 34000 Kragujevac, PO Box 124
Serbia
http://www.medf.kg.ac.rs/sjecr/index.php

SJECR is a member of WAME and COPE. SJECR is published four times circulation 250 issues
The Journal is financially supported by Ministry for Science and Technological Development, Republic of Serbia
ISSN 1820 – 8665

Table Of Contents

HEALTH FINANCING CONSTRAINED BY POPULATION AGING – AN OPPORTUNITY TO LEARN FROM JAPANESE EXPERIENCE

Seiritsu Ogura[1], Mihajlo Jakovljevic[2]
[1]Institute of Aging, Graduate School, Department of Economics, Hosei University, Tokyo, Japan
[2]Department of Pharmacology and Toxicology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia

FINANSIRANJE ZDRAVSTVENE ZAŠTITE U USLOVIMA STARENJA POPUALCIJE - PRILIKA DA UČIMO NA JAPANSKOM ISKUSTVU

Seiritsu Ogura[1], Mihajlo Jakovljević[2]
[1]Nacionalni Institut za Starenje Populacije, Odsek Postdiplomskih studija, Hosei Univerzitet, Tokyo, Japan
[2]Katedra za farmakologiju i toksikologiju, Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija

Received / Primljen: 1.12.2014. Accepted / Prihvaćen: 1.12.2014.

The Global Aging of Nations

Population aging is a rather new phenomenon in historical demography (1). It initially began with increased life expectancy at birth followed by decreasing female fertility rates among the most developed countries. While its earliest shy roots were visible more than century ago (2), in most high income regions of the world, the phenomena started to attract academic and public attention only in the 1980's. Clearly one driving factor was the technological revolution in medicine, which succeeded in saving lives from many acute illnesses and controlling many chronic illnesses. The other factor is the changing social role of women; after decades of economic growth after the WWII, the economy started to absorb women from home to work places, giving them higher incentives to work, get education and have a job career, or, paying them to have less children.

Although the increased life expectancy and falling fertility had been observed in almost all the high income countries in the last quarter of XX century, some countries have been affected more than the others. Japan was one of those countries; it has the combination highest life expectancy and one of the lowest fertilities. Due to the success of its universal health insurance and aggressive public health policies, since 1980, the country has been consistently at the top of the life expectancy list of the world. Its fertility has started to dip noticeably in the second half of 1980's, and its TFR has been around 1.3 for the last several years. As a result, it has the most aged population that has been declining for almost a decade. During this period, it has been struggling to find ways to restore its fertility rates, and to pay for the mounting public pension costs and the health care costs.

Population aging among today's fast developing and emerging markets came with many decades delay mostly during last quarter of XX century (3). Up to date it is broadly accepted that this demographic change is common even among middle income developing nations (4). By far the most typical pattern of policy induced rapid aging among the communist countries belong to China and its one child policy (5). One of the picturesque sayings says that some of the Third World nations actually succeeded to become old even before they became fully developed and mature economies.

The case of population aging in Eastern Europe and the Balkans traces its causes in socialist policies of Cold War era. In this region total fertility threshold fell beneath 2.1 (children per female) mostly after the 1980`s (6). Socioeconomic transition taking place since 1989 actually worsened negative demographic trends in the region (7). Some recent public health successes of strategies designed to combat aging happened among the leading emerging BRICs economies mostly during the past decade (8).

Age Profile of Health Care Costs

There has been some strong disagreements particularly in the US regarding the practical importance of aging in explaining the increase in health care costs (9), but there is little doubt that aging contributes to increase health care costs; "In developed countries, where acute care and institutional long-term care services are widely available, the use of medical care services by adults rises with age, and per-capita expenditures on health care are relatively high among older age groups. Accordingly, the rising proportion of older people is placing upward pressure on overall health care spending in the developed world, although other factors such as income growth and advances in the technological capabilities of medicine generally play a much larger role." (10).

The per-capita health care costs by age-groups

Unlike the US which has a significant private health care market, 99% of Japanese health care goods and services are produced and consumed within the public health insurance framework. Prices have been tightly controlled,

DE GRUYTER OPEN
UDK: 614.2:314(520); 614.2:314(497.11); 364.69/ Ser J Exp Clin Res 2014; 15 (4): 175-181
DOI: 10.2478/SJECR-2014-0022

Corresponding author: Seiritsu Ogura, PhD Professor Emeritus Hosei University Faculty of Economics
2-17-1 Fujimi, Chiyoda-ku, Tokyo 102-8160, Tel & FAX: +81-3-5937-4102

and introduction of new technologies have been regulated until the government is sure that public insurance can pay for them. The health care costs of a few months preceding death, for example, is a fraction of what they are in US. Moreover, for almost for two decades, there has been little income growth. In the ten year period between 2002 and 2012, however, the national health care costs increased from 30.95 trillion yen to 39.21 trillion yen, or almost 27 % increase. Clearly, it has been the population aging that drove the costs in Japan.

Let us first look at the empirical relationship between the per-capita health care costs and the age of a population. Presumably it is affected by a number of factors; the underlying health capital stock at different ages, relative costs of health care services, and access to health care services. Figure 1 shows the annual per-capita health care costs by five-year age group in 2012 Japan below age 65 (11). Since these costs are calculated from the public health care insurance benefits data, they are very precise, although they exclude two important items of the OECD accounting base; maternity related services and long-term care services.

It is hard to find age-profile data for per capital health care costs in other countries; in fact, the only one we could find was Yamamoto data for the US (12). Since the effect excluding long-term care services can be very significant beyond age 65, we limit the comparison below the age 65. The dashed line in Figure 1 represent the age-class profile for the US. In spite of the huge difference in the structure of the health care systems, we find the age-profiles of the two countries are surprisingly similar, once we control for the difference in the levels of health care costs. Moreover, part of the difference between the two countries may be due to the exclusion of maternity costs in Japanese data. This probably means that while the prices of medical goods and services may be much higher in the US, the underlying medical technologies or knowledge governing demand side and the supply side are still common. For example, parents take their children to the doctors in similar situations, seek medical help for themselves in response to similar health shocks, and do not encounter significant rationing in health care services in these age groups.

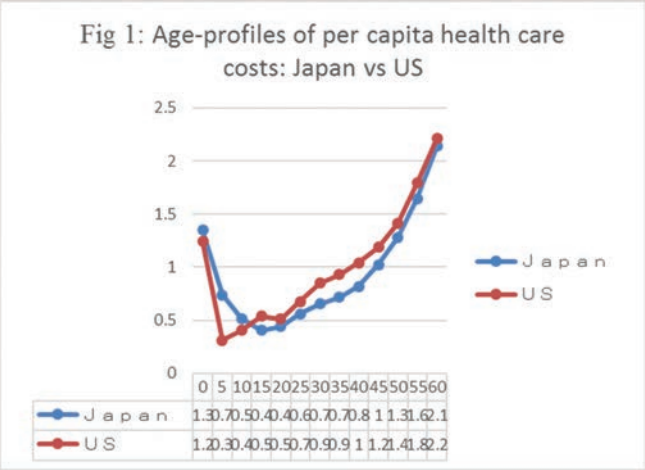


Figure 1. Age-profiles of per capita health care costs: Japan vs US (the annual per-capita health care costs by five-year age group in 2012 in Japan and the US below age 65)

Unfortunately, it is much harder to compare the per-capita health care costs beyond age 65, as long term care costs no longer can be ignored. Moreover, the line between the medical care and long-term care is very fuzzy, varying from one country to another. Also in the long-term care, since the role of public services may be vary greatly from one country to another, we will stay away from the comparison of the two countries beyond age 65.

The Survival Probability and Age Profile of Lifetime Healthcare Costs

Suppose two countries have the same average health care costs and the age profiles of the per-capita healthcare costs. It still could happen that the two countries could have a different size distribution of health care costs across age-groups, if the life expectancies in the two countries are different. Particularly, given the U shaped age-profile of average health care costs, the country with a longer life expectancy will have more elderly population who consume more healthcare, and hence will consume more proportion of total health care. For this reason, in computing age profile of lifetime healthcare costs, we have to take into account the survival probabilities to given ages.

The second column of Table 1 represents the per-capita average health care costs in thousand yen (or about 10 dollars). The third column represents the unisex survival probability of an average Japanese at each age class. For simplicity, we have computed the square root of the product of male and female survival probabilities at the midpoint age of each age-class in the 2012 Life Tables (13) as our unisex survival probability of the age-class. The fourth column shows the product of the second and third columns, or their expected values for an individual of a given age in the group, multiplied by a factor of 5. Notice that as each age-class represents 5 different ages, and an individual is going to stay in a given age-class cell for five years, the expected value the product of health care costs and survival probability must be multiplied by 5. By summing the entries of our fourth column, we the average lifetime health care expenditure of 25,253 thousand yen.

Of particular interest for us is the age distribution of the lifetime health care costs, for which we have computed the cumulative costs and the proportion in the fifth and sixth column of the table. From the sixth column, we can see that an individual has spent only 42% of the lifetime costs before he/she reaches age 65. In other words, he/she is yet to spend the remaining 58% after the age 65, almost half of which he/she will spend in the ten-year period of ages 65-74. Clearly this heavy spending in the last two or three decades is the core of our financing problem in the health care of the elderly, particularly in view of the age distribution of lifetime income.

Table 1. Lifetime Health Care Costs and Lifetime Income (per capita average health care costs in 1000 ¥ (∞10 $))

	Per Capita Health Care Costs (2012)	Unisex Survival Probability	Five Year Period Health Care Costs	Cumulative Health Care Costs	Cumulative Health Care Costs (%)	Per Capita Income (2013)	Five Year Period Income	Cumulative Income	Cumulative Income (%)
0 ~ 4	¥236	0.997	¥1,178	¥1,178	0.05				
5 ~ 9	¥129	0.997	¥644	¥1,822	0.07				
10 ~ 14	¥92	0.997	¥457	¥2,279	0.09				
15 ~ 19	¥73	0.995	¥362	¥2,640	0.10				
20 ~ 24	¥79	0.994	¥393	¥3,034	0.12	¥170	¥8,444	¥8,444	0.06
25 ~ 29	¥102	0.992	¥503	¥3,537	0.14	¥170	¥8,427	¥16,871	0.13
30 ~ 34	¥119	0.989	¥587	¥4,124	0.16	¥173	¥8,559	¥25,430	0.20
35 ~ 39	¥130	0.987	¥642	¥4,766	0.19	¥173	¥8,538	¥33,968	0.27
40 ~ 44	¥148	0.982	¥727	¥5,493	0.22	¥198	¥9,741	¥43,709	0.35
45 ~ 49	¥181	0.976	¥882	¥6,375	0.25	¥198	¥9,677	¥53,386	0.43
50 ~ 54	¥229	0.966	¥1,104	¥7,479	0.30	¥247	¥11,934	¥65,320	0.52
55 ~ 59	¥292	0.950	¥1,385	¥8,864	0.35	¥247	¥11,742	¥77,062	0.62
60 ~ 64	¥379	0.927	¥1,757	¥10,621	0.42	¥212	¥9,847	¥86,910	0.70
65 ~ 69	¥477	0.892	¥2,126	¥12,746	0.50	¥212	¥9,470	¥96,380	0.78
70 ~ 74	¥625	0.840	¥2,623	¥15,369	0.61	¥187	¥7,842	¥104,222	0.84
75 ~ 79	¥776	0.761	¥2,953	¥18,323	0.73	¥187	¥7,107	¥111,329	0.89
80 ~ 84	¥914	0.634	¥2,899	¥21,221	0.84	¥187	¥5,924	¥117,254	0.94
85	¥1,037	0.450	¥2,333	¥23,555	0.93	¥187	¥4,204	¥121,458	0.98
90	¥1,037	0.238	¥1,234	¥24,789	0.98	¥187	¥2,223	¥123,682	0.99
95	¥1,037	0.078	¥402	¥25,191	1.00	¥187	¥725	¥124,407	1.00
100	¥1,037	0.012	¥62	¥25,254	1.00	¥187	¥112	¥124,519	1.00

Size of Out-of-Pocket Payments

In spite of the universal public health insurance coverage, the government has controlled the access to health care of particular groups in the population by changing their out-of-pocket payments. For most Japanese, the standard out-of-pocket payment is 30% of the cost of treatment at clinics/hospitals and 30% of the cost of drugs at the pharmacy. Two groups are exceptions to this rule; the first group are children; the out-of-pocket rate for children under the age 6 is 0.2, or 20% of the cost of treatment or drugs. However, most municipalities offer programs to relieve all or most of the co-payments for infants and children under the age 16. The second group are the elderly; the out-of-pocket payment of the elderly between the ages 70-74 are now set at 0.2, and the out-of-pocket payment of the elderly above the age 75 is 0.1.

For the last three decades, the government has been increasing the co-payments of the elderly. The elderly whose income are above certain levels are now subject to the standard co-payment rate.

Distribution of economic well-being across age-groups

The distribution of income across different age-groups is primarily determined in the labor market, and then modified by the tax system and the transfer programs of the government. Compared with other developed nations, the age profile of wages/salaries in Japanese firms has been known to be steeper; it starts lower, keeps on increasing until early 50' s , much later than American or European firms, and then falls in the latter part of 50's. Most firms terminate labor contracts with a worker when he/she reaches the age 60, but offer some form of continued employment up to the age 65 at reduced wages/salaries. As a result, the labor force participation rate is generally higher, and the proportion of elderly households that have labor income is much higher, than in the other developed countries. For example, in 2012, 86% of households whose heads are between age 60-64 have some labor income, and the proportions are 65% for 65-69, 47% for 70-74, 35% for 75-79, and 29% for age 80 or over, although some of which are the earnings of younger family members (14). Once retired, the public pension programs

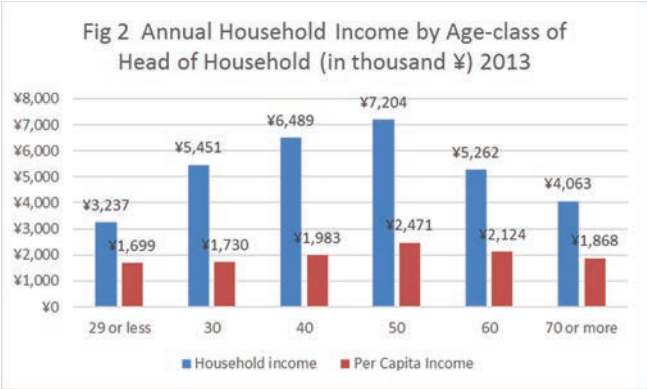


Figure 2. Household Income by Age-class of Head of Household (the total income and per-capita income by 10 year age-class in 2013)

of retired workers have been fairly generous; for example, the replacement rate for employee's pension has been set at 60%. In contrast, the basic pension benefit for self-employed workers is rather modest. Most of public pension benefits are exempt from income taxation.

It is very difficult to find reliable statistics on age distribution of income, and, in spite of its relatively small sample size for measuring income distribution, the income questionnaire of Comprehensive Survey of MHLW is almost the only source of the information. Specifically, the Survey provides total household income and per-capita income by the age-class of head of household. Our Figure 2 shows the total income and per-capita income by 10 year age-class in 2013; as expected, household income falls substantially with the age of the head of household moves from the 50's to the 60's, and then to the 70's or over. On the other hand, the fall in the per-capita income is surprisingly modest; it drops by 14% from as the head age goes from the 50's to 60's, but only by 8% from the 60's to 70's or over.

Using the per-capita income information in a similar manner to the per-capita health care costs, in Table 1, we have added the age-class distribution of income the cumulative income prior to reaching each age-class, and the distribution of lifetime income (%) respectively in columns 8, 9, and 10 of the table. Given the age-class income data of *Comprehensive Survey 2013*, we came up with a figure of 124.5 million yen as our per capita lifetime income. From the 10th column, we can see that an individual has already received 70% of lifetime income before he/she reaches age 65. In other words, even with the relatively generous public pension programs and high rate of labor force participation of the elderly, he/she can expect to receive only 30% of lifetime income to finance consumption after the age 65.

Thus before the age 65, since an individual incurs 40 percent of lifetime costs but receives 70% of lifetime income, the ratio of costs to income, which is a measure of the economic burden of health care costs is 4/7, or 0.57. After the age 65, an individual incurs 60% of lifetime costs but receives only 30% of lifetime income, the ratio of income to costs is 6/3, or 2.0. Thus if we divide our population into two insurance groups, one group consisting of individuals less than age 65, the other group consisting of individuals at age 65 or older, the first group's economic burden is only 60% of the lifetime average, while the second group's economic burden is 200% of the lifetime average.

Japanese Health Care Financing for the Elderly and Retired

Now we earn most of labor income before age 65 but, unfortunately, we need most of health care after age 65. For any country providing public health care insurance for workers and their families, this means that it is not difficult to provide health insurance for workers and their family members, but it is extremely difficult to continue to provide health insurance after they retire. The retirees program will be always running deficits, as the cost of its benefits will be much higher while the revenue will be much lower. In the beginning, the government will make up the difference by subsidies. But as the population ages, and the number of retired workers swells, the government will no longer be able to pay the entire deficit from the tax revenue. Thus, the government will start collecting more money from the workers than they need to pay for the cost of their benefits, and use the surplus to make up the shortage. As we will explain below, Japan is an example of such a mixture of government subsidies and cross-subsidization.

In 2006, Ogura et al. wrote, "Japan's current public medical insurance can be compared to an unstable two-story building whose second floor is becoming heavier each day while its first floor is losing strength. There are three pillars in the first floor that support the weight of the whole building." "The second floor of our building consists of the health care insurance for the elderly, which provides medical care benefits to those over age seventy for very little cost" (15).

After almost a decade, this structure has added another floor between the first and the second, a mezzanine floor, changed some rules in accommodating people between the floors, but it's not clear if it has become less unstable. At the moment, the first floor of this public health insurance building accommodates everyone under the age 75, and the second floor accommodates everyone over the age 75. Between the first and the second, there is a mezzanine that accommodates everyone over the age 65, accessible only from the first floor.

In the first floor, we still see three pillars supporting the weight of the whole building; employees insurance programs, national health insurance programs, and government subsidies. The first pillar is the strongest of the three, and consists of (a) more than 14 hundred firm- specific health insurance associations covering 29 million employees and dependents (Health Insurance Managed by Associations), (b) the single Health Insurance Managed by Government (HIMG), covering 35 million employees and dependents of smaller firms, and (c) less than 50 programs known as the Health Insurance for Government Employees (HIGEs), covering 9 million public sector employees and dependents. These programs collect different premiums from the employees and their employers in proportion to their

wages/salaries. In fact, they collect more than twice the costs of their own benefits, and provide an important support for the health care costs of the elderly. Their financial strength comes from (a) almost perfect withholding at the source of income, (b) sharing of the tax with the employers.

The second pillar represents more than 18 hundred "National Health Insurance" programs (NHIs) run by municipal governments, covering 33 million self-employed, retired, or unemployed workers and their family members. In short, they insure everyone under the age 75 who are not covered by the employees programs. On closer examination, this pillar is actually not standing on its own, leaning heavily on the third pillar. There are three reasons for their structural weakness; first, on average, these individuals have limited financial means; the average per-capita income, in fact, is 830 thousand yen, about one-half of the employees programs. Secondly, unlike the employees, they do not have employers to share the cost of the premium.

Thirdly, they are much older because NHIs accept the retired workers; their mean age is 50.4 years old, compared with 34.3 for HIMA, or 36.4 for HIMG. As a result, while the per-capita cost of the benefits (316 thousand yen) is roughly twice of the employees programs, they manage to collect only 3.2 trillion yen, less than one-third of the costs of their benefits (10.1 trillion yen), from their premiums.

The third pillar then represents the subsidies of the governments that supports the second (NHIs), and the first (HIMG) to a lesser degree. Because of NHIs financial weakness, for decades, the national government had been subsidizing half of the costs of their benefits. With the introduction of reinsurance schemes for the elderly in 1985, the subsidies also have covered the contributions for reinsurance programs; at the moment there are two such programs; contributions for the young-old (age 65-74), and the contribution for the old-old (age 75 plus). In addition to NHIs, HIMG is also subsidized currently at the rate of 16.4% for its benefits and contributions, to compensate the income differential with HIMA.

The new mezzanine of our building represents the reinsurance program for the young-old, or those above the age 65 but below age 75. Since it is a pure reinsurance program involving all the first floor programs, we will call it a mezzanine floor. As we have seen, since NHIs no longer can pay for all the benefits of everyone under the age 75, starting in 2009, everyone between the age 65 to age 74 are asked to go up to the mezzanine. The reinsurance program then computes the total costs of their benefits, 6.5 trillion yen in 2014, which are to be collected from all the insurance programs in the first floor, according to their shares in the number of insured individuals under the age 75.

Not surprisingly, most of them in the mezzanine are already retired, and NHIs insure almost 80% of them, but nationally, NHIs account only 32% of the individuals under the age 75. Instead of paying 80% of the costs, NHIs now have to pay only 32%. Hence this mezzanine scheme transfers almost 50% (= 80% - 32%) of the cost of benefits for young-old, or 3.0 trillion yen, from NHIs to the employees programs. Another smaller transfer program for the retired workers under the age 65 transfers 0.7 trillion yen from the NHIs to the employees programs. These reinsurance schemes leave a shortfall of 3.2 trillion yen in NHIs still to pay for the individuals in the first floor. Although the financial transactions are very complex, this is what governments pay to NHI under various subsidy schemes.

The second floor, which is actually the third story of this building, supports the health care costs of the old-old, or those age 75 or older. Currently, there are 15 million elderly above the age, and the costs of their benefits amount to 14.4 trillion yen per year, or more than 40% of the entire costs of public health insurance benefits. The elderly themselves are asked to contribute 10%, or more if their income is high enough, and hence, in effect, they pay 1.6 trillion yen. Governments contribute 6.8 trillion yen, in statutory 50% direct contributions, and 1.0 trillion yen in indirect subsidies for NHI and HIMG's contributions. The employees programs contribute 5.0 trillion yen, or 40% of the costs of benefits for the elderly.

Why is the system so complex? First, Japan started with two-kinds of employees programs; in addition to self-sustaining, firm-specific employees programs, the government was running and subsidizing a huge program for the employees of small firms. Secondly, Japan started also with programs for the rest of population; for farmers, the self-employed, the unemployed or the retired that needed heavy subsidy from the very beginning. Thus as aging started, and cross-subsidization from the non-elderly was needed, the government had to subsidy the weaker ones to help them pay the cross-subsidy. It is this subsidy-on-subsidy that makes the financing system of the elderly's health care costs extremely complex, and non-transparent.

The Example of Aging Serbia

Serbia as the largest country of Western Balkans falls within the same distinct population shrinking trend common throughout the surrounding region (16). This trends becomes particularly concerning while keeping in mind uneven distribution of sexes across the country. Due to half a century long village-to-town migration pattern substantial geographic heterogeneity has been created with excess of healthy young men (aged 20-39) in remote, rural areas and excess of healthy young women (aged 20-39) in urban cores (17). Fertility rates were steadily decreasing and life expectancy exhibited modest rise since 1991. Nevertheless serious population shrinking continued because of net reproduction rate lower than one since 1955 (18). The process was likely substantially slowed down by an influx of over 600,000 refuges during the civil wars and dissolution of Yugoslavia (19). Important part of the complex landscape is the long term migration of young people in their most productive age towards the rich economies of Western Europe and North America (20). This emigration, geographically uneven, was most intense for underdeveloped Eastern and Southern regions of the country, ironically the ones that accepted the least portion of permanently inhabited refu-

gees during the 1990`s. All of aforementioned facts make the population aging issue even more peculiar and difficult to reach by common policies. After years of substantial efforts by policy makers and experts in the field, Government of Serbia has adopted and implemented its National Strategy in Aging 2006 – 2015 whose outcomes are yet to be seen (21).

Consequences of Aging for the National Health System of Serbia

Serbia's health system is funded as a mixed Bismarck system with elements of former Yugoslavia's municipally funded health care (22). Bismarck's social insurance financing pattern relies on mandatory contributions for social and health insurance by the employers and employees. Essential feature of increased portion of the elderly within society and decreased portion of youth is shrinking labour force of the country. This dwindling taxpayer's base will inevitable contribute less revenues in the long run and thus lead to weakened health care funding. Such phenomenon has already been clearly described in locally published evidence (23). Another issues presents rapidly increasing number of retired senior citizens who are about to be supported by shrinking actively employed national work force (24). Ultimate impact to the overall capacities of health system is significantly greater demand for medical care by the elderly (25). Economic burden of some prosperity illnesses has been assessed in cost-of-illness studies and turned out to be substantial (26-28). Particularly sensitive issue is very expensive terminal and palliative care for the aged patients in their last year of life with incurable cancer (29). Among many of the vulnerabilities of the aged comes under development of long term home care supportive network in Serbia (30). Many of the retired senior citizens after their spouse's death are left alone by their families sunk in poverty with income insufficient to cover basic medical and nutrition needs (31).

The true size of work load for medical facilities and an overall economic burden imposed by steady aging of Serbian community is yet to be assessed in the upcoming years. As elsewhere across Eastern Europe and Balkans there is significant lag in development of electronic patient records. This fact is limiting our ability to properly assess resource utilization patterns and establish demand based provision of medical services (32). Therefore responsiveness of the system remains unsatisfactory with long waiting lists in some therapeutic areas such as orthopaedic, cardiovascular surgery and interventional radiology (33). Implementation of cost-effective solutions to cope with problems is still far from being common practice among policy makers while Health Technology Assessment agencies are absent in most of the Western Balkans region (34).

Health expenditure in the country has been recording almost steady growth since 2000 with modes, global recession induced temporary shortcomings (35). Large part of Serbian health market value increase was attributed to the approved reimbursement of novel medical technologies by the authorities such as high-tech pharmaceuticals (36). In some therapeutic areas the National Health Insurance Fund's polices have contributed to the growing public debt due to Increased civil expectations for cutting edge medical care. Few other issue such as overregulation, informal payments and unequal access to medical services among the poor citizens and in rural areas, due to their joint complexity, are unlikely to be met soon (37). Strategic determination by the national authorities to adjust undergoing health reforms to dominant population aging trend shall be urgently needed. As we have witnessed from the examples from Asia as well as Europe, responsiveness of the health systems and social services to the sensible needs of massive population of senior citizens will remain one of the key challenges in future.

Acknowledgement – The Ministry of Education Science and Technological Development of the Republic of Serbia has funded the underlying studies behind Serbian part of this contribution through Grant OI 175014. Publication of results was not contingent to Ministry's censorship or approval.

Conflict of Interest: There are no conflict of interest.

References

1. Kinsella, K., & Velkoff, A.V. (2001). U.S. Census Bureau, Series P95/01-1. *An Aging World: 2001*. U.S. Government Printing Office, Washington, DC.
2. Rowland, T.D. (2009). *Global Population Aging: History and Prospects*. International Handbooks of Population Aging, 1, 37-65.
3. Preston, S.H., Himes, C., & Eggers, M. (1989). Demographic conditions responsible for population aging. *Demography, 26*(4), 691-704.
4. Tucker, K.L., & Buranapin, S. (2001). Nutrition and aging in developing countries. *J Nutr, 131*(9), 2417S-2423S.
5. Liang, J., Tu, E.J., & Chen, X.M. (1986). Population aging in the People's Republic of China. *Soc Sci Med, 23*(12), 1353-1362.
6. Chawla, M., Betcherman, G., & Banerji, A. (2007). From red to gray: the" third transition" of aging populations in Eastern Europe and the former Soviet Union. World Bank Publications. Retrieved September 15, 2014, from http://siteresources.worldbank.org/ECAEXT/Resources/publications/454763-1181939083693/full_report.pdf
7. Adeyi, O., Chellaraj, G., Goldstein, E., Preker, A., & Ringold, D. (1997). Health status during the transition in Central and Eastern Europe: development in reverse?. *Health Policy and Planning, 12*(2), 132-145.
8. Jakovljevic, M. (2014). The key role of leading emerging BRIC markets for the future of global health care. *SJECR, 15*(3), 139- 143.

9. Newhouse, J.P. (1992). "Medical care costs; how much welfare loss?". *Journal of Economic Perspectives, 6*(3), 3-21.
10. World Health Organization. National Institute of Aging. (2011). National Institute of Health. Global Health and Aging. U.S. Department of Health and Human Services.
11. Ministry of Health, Labor and Welfare. (2014, October 8). *Outline of National Health Care Costs*, Table 5. Retrieved October 15, 2014, from http://www.mhlw.go.jp/english/new-info/.
12. Yamamoto, D.H. (2013). *Health Care Costs-From Birth to Death*. Health Care Cost Institute's Independent Report Series- Report 2013-1. Retrieved October 15, 2014, from http://www.healthcostinstitute.org/files/Age-Curve-Study_0.pdf.
13. Ministry of Health, Labor and Welfare. (2013, July 25). *Outline of Simple Life Tables 2012.* Retrieved October 15, 2014, from http://www.mhlw.go.jp/english/new-info/.
14. Ministry of Health, Labor and Welfare.(2013). *Comprehensive Survey of Living Conditions. Income Section, Table 13.* Retrieved October 15, 2014, from http://www.mhlw.go.jp/english.
15. Ogura,S., T.Kadoda, & Kawamura, M. (2006). Removing the Instability and Inequity in the Japanese Health Insurance System. In Wise, D.A., & Yashiro, N. (Eds), *Health Care Issues in the United States and Japan* (pp.83-111). University of Chicago Press.
16. Ševo, G., Despotovic, N., Erceg, P., Jankelic, S., Milosevic, D. P., & Davidovic, M. (2009). Aging in Serbia. *УСПЕХИ ГЕРОНТОЛОГИИ, 22*(4), 553-557.
17. Nikitovic, V. (2010). *"Frozen" Demographic Potentials of Serbia – The Limit to Sustainable Population Development, Spatial demography of the Balkans: trends and challenges*. Budva, Monténégro: IVth International Conference of Balkans Demography.
18. Rašević, M. (2010). On ageing and old age in Serbia. *Zbornik Matice srpske za društvene nauke*, (131), 199-209.
19. Nikitović, V., & Lukić, V. (2010). Could refugees have a significant impact on the future demographic change of Serbia?. *International Migration, 48*(1), 106-128.
20. Vuković, D. (2005). Migrations of the labour force from Serbia. *SEER-South-East Europe Review for Labour and Social Affairs*, (04), 139-150.
21. Government of Serbia. (2005). *National Strategy in Aging 2006 – 2015.* "Official Gazette of the Republic of Serbia", No. 55/05 and 71/05 – correction. Retrieved October 15, 2014, from http://www.erisee.org/sites/default/files/Adult%20education%20strategy-%20endorsed%20Serbia%20ENG.pdf.
22. Jakovljević, M., Jovanović, M., Lazić, Z., Jakovljević, V., Đukić, A., Velicković, R., & Antunović, M. (2011). Current efforts and proposals to reduce healthcare costs in Serbia. *SJECR, 12*(4), 161-163.
23. Milivojevic, S., & Filimonovic, D. (2012). Ageing of Population and Demographic Consequences in Future Labour Market Trends. *Institute of Economic Sciences Book Chapters, 1*, 427-443.
24. Stojilković, J. (2011). Growing number of pensioners and population aging in Serbia. *Zbornik radova Geografskog instituta" Jovan Cvijić", SANU, 61*(2), 69-84.
25. Biorac, N., Jakovljević, M.B., Stefanović, D., Perović, S., & Janković, S. (2009). Assessment of diabetes mellitus type 2 treatment costs in the Republic of Serbia. *Vojnosanit pregl, 66*(4), 271-276.
26. Jakovljevic, M.B., Lazic, Z., Gajovıc, O., Tanaskovic, I., Milovanovic, D., & Atanasijevic, D. (2012). GOLD Stage Impact on COPD Direct Medical Costs in Elderly. *J Health Behav Pub Health, 2*(3), 1-7.
27. Dong, H. J. (2014). *Health Maintenance in Very Old Age: Medical Conditions, Functional Outcome and Nutritional Status. LINKÖPING UNIVERSITY MEDICAL DISSERTATIONS No. 1398,* Sweden.
28. Jakovljevic, M., Cupurdija, V., & Lazic, Z. (2013). Cost of illness of community-acquired pneumonia. Review of the literature and possible strategies in the Serbian health care setting, SEEd Medical Publishers, Farmeconomia. *Health economics and therapeutic pathways, 14*(4), 133-139.
29. Kovacevic, A., Dragojevic-Simic, V., Rancic, N., Jurisevic, M., Gutzwiller, F., Matter-Walstra, K., & Jakovljevic M. (2015). End-of-life costs of medical care for advanced stage cancer patients. *Vojnosani Pregl, 72* (4): in press.
30. Mihic, M.M., Todorovic, M.L., & Obradovic, V.L. (2014). Economic analysis of social services for the elderly in Serbia: Two sides of the same coin. *Eval Program Plann, 45*, 9-21.
31. Arsenijevic, J., Pavlova, M., & Groot, W. (2014). Out-of-pocket payments for public healthcare services by selected exempted groups in Serbia during the period of post-war healthcare reforms. *Int J Health Plann Manage, 29*(4), 373-398.
32. Jakovljevic, M., Ranković, A., Rančić, N., Jovanović, M., Ivanović, M., Gajović, O., & Lazić, Z. (2013). Radiology Services Costs and Utilization Patterns Estimates in Southeastern Europe— A Retrospective Analysis from Serbia. *Value in Health Regional Issues, 2*(2), 218-225.
33. Ranković, A., Rancić, N., Jovanovic, M., Ivanović, M., Gajović, O., Lazić, Z., & Jakovljević, M. Impact of Imaging Diagnostics on the Budget–are We Spending Too Much? *Vojnosanit Pregl, 70*(7), 709–711.
34. Jakovljevic, M. B. (2013). Resource allocation strategies in Southeastern European health policy. *The European Journal of Health Economics, 14*(2), 153-159.
35. Jakovljevic, M. (2014). Health Expenditure Dynamics in Serbia 1995 – 2012. Hospital Pharmacology – International Multidisciplinary Journal, 1(3), in press.
36. Jakovljevic, M.B. (2014). Oncology monoclonal antibodies expenditure trends and reimbursement projections in the emerging Balkan market, Farmeconomia. *Health economics and therapeutic pathways, 15*(1), 27-32.
37. Dickov, V. (2012). Inadequacy the Health System in Serbia and Corrupt Institutions. *Materia socio-medica, 24*(4), 262.

LATENT MURINE CYTOMEGALOVIRUS INFECTION CONTRIBUTES TO EAE PATHOGENESIS

Jelena Milovanovic[1], Aleksandar Arsenijevic[1], Bojana Stojanovic[1], Marija Milovanovic1, Stipan Jonjic[2], Branka Popovic[2], Nebojsa Arsenijevic[1], Miodrag L. Lukic[1]

[1]Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Serbia

[2]Department of Histology and Embryology, Faculty of Medicine, University of Rijeka, 51000 Rijeka, Croatia

LATENTNA INFEKCIJA MIŠJIM CITOMEGALOVIRUSOM IMA ULOGU U PATOGENEZI EKSPERIMENTALNOG AUTOIMUNSKOG ENCEFALOMIJELITISA

Jelena Milovanović[1], Aleksandar Arsenijević[1], Bojana Stojanović[1], Marija Milovanović[1], Stipan Jonjić[2], Branka Popović[2], Nebojša Arsenijević[1], Miodrag L. Lukić[1]

[1]Centar za molekulsku medicinu i istrazivanje matičnih ćelija, Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Srbija

[2]Katedra za histologiju i embriologiju, Medicinski fakultet, Univerzitet u Rijeci, 51000 Rijeka, Republika Hrvatska

Received / Primljen: 15.06.2014. Accepted / Prihvaćen: 24.06.2014.

ABSTRACT

Viral infection has been identified as the most likely environmental trigger of multiple sclerosis (MS). There are conflicting data regarding the role of cytomegalovirus (CMV) in MS pathogenesis.

We utilised experimental autoimmune encephalomyelitis (EAE)-resistant BALB/c mice and murine cytomegalovirus (MCMV), the murine homolog of CMV, to examine the mechanism by which viral infection enhances autoimmune neuroinflammation. Mice subjected to latent neonatal MCMV infection developed the typical characteristics of EAE. Similar to MS, the MCMV-infected EAE-induced mice developed infiltrates in the central nervous system (CNS) composed of similar percentages of CD4+ and CD8+ T cells. The influx of both Th1 and Th17 cells into the CNS of MCMV-infected EAE-induced mice was observed. Interestingly, the development of autoimmune neuroinflammation after latent MCMV infection was accompanied by a significant influx of Tc17 cells (CD8+IL-17+ and CD8+RoRγt+) but not Tc1, cells. Our results suggest that latent MCMV infection affects the development of inflammatory lymphocytes that exhibit encephalitogenic potential, thereby mediating increased CNS pathology following EAE induction, and that CMV represents a possible environmental factor in the pathogenesis of MS and other autoimmune diseases.

Key words: *EAE, viral infection, CMV, BALB/c mice*

SAŽETAK

Virusna infekcija se navodi kao najverovatniji faktor okoline koji utiče na razvoj multiple skleroze (MS). Postoje konfliktni podaci o ulozi infekcije citomegalovirusom (CMV) u patogenezi multiple skleroze. Koristili smo BALB/c miševe, rezistentne na indukciju eksperimentalnog autoimunskog encefalomijelitisa (EAE), i mišji citomegalovirus (MCMV), mišji homolog humanom citomegalovirusu da ispitamo kako virusna infekcija može da utiče na razvoj autoimunske neuroinflamacije. Miševi sa latentnom neonatalnom infekcijom mišjim citomegalovirusom su razvili tipičan EAE. Slično kao u MS, MCMV EAE miševi su razvili infiltrate u centralnom nervnom sistemu (CNS) sa sličnom zastupljenošću CD4+ i CD8+ T limfocita. Uočen je influks i Th1 i Th17 ćelija u CNS MCMV EAE miševa. Interesantno je da razvoj autoimunske inflamacije nakon latentne MCMV infekcije prati značajan influks samo Tc17 (CD8+IL-17+ i CD8+RoRγt+), a ne i Tc1 ćelija. Naši rezultati ukazuju da latentna MCMV infekcija verovatno utiče na razvoj inflamatornih limfocita koji mogu da indukuju autoimunski proces u CNS-u, direktno pojačava razvoj patoloških procesa u CNS-u nakon indukcije EAE i ukazuje na CMV kao na mogući faktor okoline koji utiče na razvoj multiple skleroze i drugih autoimunskih bolesti.

Ključne reči: *EAE, virusna infekcija, CMV, BALB/c miševi*

DE GRUYTER OPEN

UDK: 616.832-004.2-02; 616.98 / Ser J Exp Clin Res 2014; 15 (4): 183-190
DOI: 10.2478/SJECR-2014-0023

Correspondence to:
Marija Milovanovic, MD, PhD; Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac , Svetozara Markovica 69, 34000 Kragujevac, Serbia; Phone: +38134306800; Fax: +38134306800112; E-mail: marijaposta@gmail.com

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) that is characterised by varied clinical courses, pathologies, and inflammatory patterns (1). Experimental autoimmune encephalomyelitis (EAE) is an experimental model of multiple sclerosis that is induced in susceptible animals via active immunisation with myelin antigens mixed with an adjuvant. MS is a multifactorial disease that develops in susceptible hosts after introduction to environmental factors which trigger MS by promoting the activation of myelin-specific T cells that typically circulate in the periphery (2). The most important environmental factor considered to play a causal role in MS pathogenesis is infection (2). It has been postulated that in susceptible individuals, alterations in the mechanisms regulating the immune response to viruses may contribute to MS pathogenesis. Among the various infective agents, Epstein–Barr virus (EBV) has been the most strongly associated with increased MS risk (2). It was shown that EBV infection polarises the adaptive immune response and heightens CNS pathology following EAE induction and likely influences MS pathogenesis (3). Additionally, an elevated CD8+ T cell response to EBV lytic antigens has recently been detected in active MS and during relapses (4).

Cytomegalovirus (CMV), which is classified into the Betherpesvirinae subfamily, is a species-specific herpes virus that establishes a life-long infection in its hosts. The CMV virion contains a double-stranded DNA viral genome whose latent and lytic types of genes encode approximately 230–250 proteins, many of which play immune-regulatory roles (5). CMV initially infects epithelial cells, and after cell-associated viremia, the virus infects different cells, including fibroblasts, epithelial cells, endothelial cells, and smooth muscle cells (6). CMV persists in myeloid precursor cells, from CD34+ pluripotent stem cells to CD14+ monocytes, resulting in the latent infection of these cells (7). When these cells subsequently enter the visceral parenchyma and differentiate into macrophages or myeloid dendritic cells, the latent virus reactivates into the lytic phase, which activates T cell-mediated immunity to suppress the infection, indicating that CMV infection modulates the immune response of the host.

However, studies investigating the association between CMV and MS have been inconclusive due to conflicting findings of both a protective and harmful influence of CMV on MS, likely in part as a consequence of small sample sizes. A recent well-powered meta-analysis found no significant difference in the rate of CMV seropositivity between MS patients and healthy controls based on pooled samples from all studies to date (8). However, some evidence for a protective effect of CMV infection on MS risk was found when only prospective studies were included in the analysis. However, all of these studies contain limitations, as they did not assess the temporal relationship between CMV infection and MS onset or the influence of CMV infection at specific time points on the MS risk. In all of these studies, CMV infection was confirmed only by detecting anti-CMV antibodies without any data demonstrating the presence of CMV DNA in the cells, the expression of lytic or latent viral genes or the temporal changes in their expression during different phases of MS.

We have recently reported that BALB/c mice, which are widely accepted as resistant to EAE induction using the peptide $MOG_{35-55,}$ developed EAE when ST2 signalling was blocked (9, 10, 11, 12). In this study, we used newborn BALB/c mice subjected to infection with MCMV to explore possible effect of latent CMV infection on EAE development.

MATERIALS AND METHODS

Infection, Induction and Scoring of EAE

Female 6- to 8-week-old BALB/c mice were used throughout this study. New-born mice 6 to 12 h postpartum were inoculated i. p. with 200 PFU of wild-type MCMV (MW97. 01 strain) or 200 µl of phosphate buffered saline (PBS) as a control. EAE was induced via subcutaneous administration of 200 µL of a suspension at 2 sites above the hind flanks. The suspension consisted of 300 µg of the peptide $MOG_{35–55}$ (Sigma Aldrich, Germany) in 100 µL of PBS emulsified with 100 µL of complete Freund`s adjuvant (Sigma Aldrich, Germany) containing 0.7 mg of heat-inactivated *Mycobacterium tuberculosis* (strain H37 RA; Difco Laboratories, Detroit, MI). Each mouse was immediately injected intraperitoneally and 48 hours later with 300 ng of pertussis toxin (List Biological Laboratories, Campbell, USA) in 100 µL of 0.9% NaCl. Clinical signs of EAE were assessed daily using the following scoring system as previously described: grade 0, no signs; grade 1, paralysed tail; grade 2, ataxic; grade 2.5, one hind leg paralysed; grade 3, both hind legs paralysed; grade 3.5, 3 legs paralysed; grade 4, both hind legs completely paralysed and mild front limb paralysis; and grade 5, moribund (13). The mice were monitored daily and provided with administered fluids and mashed chow at the base of the cage for all mice displaying a clinical score of 3. The mice were maintained at our animal facilities in a temperature-controlled environment under a 12-hour light/12-hour dark cycle and were provided with standard laboratory food and water *ad libitum.* All experiments were approved by and conducted in accordance with the guidelines of the Animal Ethics Committee of the Faculty of Medicine at the University of Kragujevac in Serbia.

Isolation of Mononuclear Cells from the CNS

At day 15 post-EAE induction (mean clinical score of 3 for the MCMV-infected EAE-induced mice), the mice were perfused with PBS, and the brain and the spinal cord were carefully removed. The mononuclear cells from the CNS were isolated as described previously (14). Briefly, the brains and spinal cords were separately homogenised

in RPMI 1640 (Sigma Aldrich) containing 10% FBS and 1 mg/ml collagenase type I (Sigma-Aldrich) and incubated at 37°C for 60 min. After digestion, the tissue was passed through a 70 mm mesh filter, pelleted, resuspended in 10 ml of 30% Percoll (Sigma-Aldrich), overlaid onto 5 ml of 70% Percoll and centrifuged at 390 g for 20 min. The myelin layer was removed, and the mononuclear cells, which accumulated in the intermediate phase, were collected, washed twice in PBS and resuspended in medium. The total cell numbers were determined by counting using a haemocytometer, and cell viability was assessed based on Trypan blue exclusion.

Flow Cytometry

For cytofluorometry, fluorochrome-conjugated antibodies against the following proteins were used: CD4, CD8, CD45, CCR6, CXCR3, T-bet, RoRγt, IL-17, IFN-γ, and TNF-α (BD Biosciences). The cells were incubated in the antibodies in PBS containing 2% FBS for 30 min at 4°C, followed by analysis. For intracellular staining of cytokines, the cells were stimulated for 4 hours in RPMI 1640 containing 10% FBS (Gibco), GolgiPlug (BD Biosciences), 10 ng/ml PMA and 500 ng/ml ionomycin. The antibodies for the cell surface markers were added to the cells in PBS containing 2% FBS for 30 min on ice. After washing, the cells were resuspended in Fix/Perm buffer (eBiosciences) for 30–45 min on ice, washed twice and incubated in the Abs for the intracellular antigens (cytokines) in Perm buffer (for 30 min on ice). For staining of transcription factors, unstimulated cells were used. The data were acquired using a FACSCalibur (BD Biosciences) and were analysed using FlowJo software (Tree Star).

Statistical Analysis

All statistical calculations were performed using SPSS 13. 0 for Windows software. The results were analysed using Student's t and the Mann-Whitney U test. The data in this study were expressed as the means+SD or the means+SEM. Values of $p<0.05$ were considered to be significant.

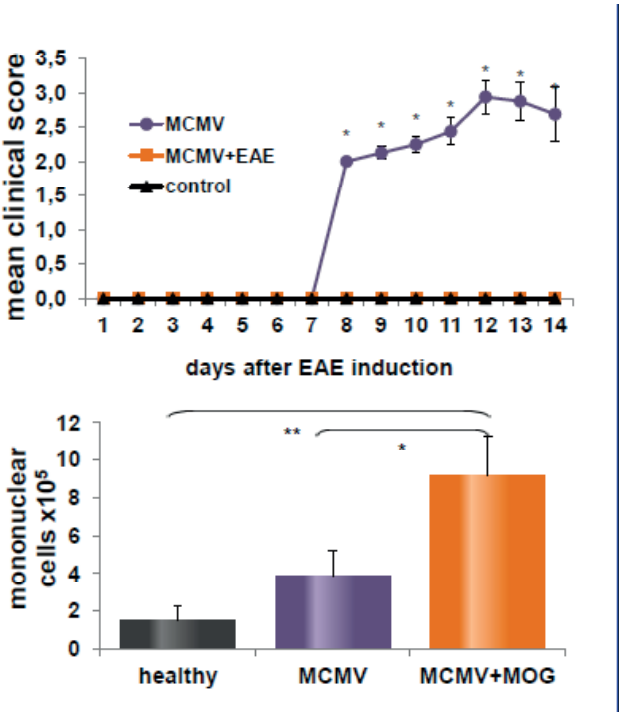


Figure 1. BALB/c mice subjected to neonatal MCMV infection are susceptible to EAE.
BALB/c mice and BALB/c mice subjected to neonatal MCMV infection were immunised with MOG_{35-55}/CFA and were monitored daily for clinical signs of EAE. The data presented were obtained from representative experiment that included eight mice per group (means ± SEM). At the peak of EAE mononuclear cells from the spinal cord and the brain tissues were isolated, and the cells were counted after exclusion of dead cells, which were stained with Trypan blue. The total number of isolated mononuclear cells is presented as the mean ± SD per group, *P<0. 05; **P<0. 005. Significance was assessed using Student's t-test.

RESULTS

MCMV Infection of Neonatal BALB/c Mice Facilitates the Development of EAE

We have previously shown that BALB/c mice, which are resistant to EAE induction using the peptide MOG_{35-55} (11, 12), develop EAE in the absence of ST2 signalling. Because it was shown that altering the immune response modulates the susceptibility to EAE, we aimed to explore the role of latent viral infection in EAE pathogenesis. To study the possible effect of viral infection on EAE, we analysed the clinical characteristics of EAE in BALB/c mice neonatally infected with MCMV (Fig. 1). The absence of clinical signs of EAE in these BALB/c mice was consistent with the minimal total cell number isolated from the CNS of these mice, in contrast to significant cell infiltration into the CNS of MCMV-infected BALB/c mice. The BALB/c mice subjected to neonatal MCMV infection but not to MOG35-55 immunisation did not exhibit any signs of EAE but displayed a higher cell number isolated from the CNS than the BALB/c mice subjected to neonatal MCMV infection without MOG_{35-55} immunisation.

BALB/c Mice Subjected to Neonatal MCMV Infection and EAE Display a Similar Percentage of Infiltrating CD4+ and CD8+ Lymphocytes in the CNS

To characterise the event at the level of the target tissue, we compared the cellular composition of the mononuclear cells in the three groups of mice (Fig. 2). Flow cytometric analysis revealed a significantly higher percentage of infiltrating CD45+CD4+ and CD45+CD8+ T lymphocytes in the CNS of MCMV-infected EAE-induced BALB/c mice compared with untreated BALB/c mice and with BALB/c mice subjected to neonatal CMV infection alone. Analysis of the expression of Th1- and Th17-related chemokine receptors (CXCR3 and CCR6) revealed significantly increased influx of CD4+CXCR3+ and CD4+CCR6+ T lymphocytes in the CNS of MCMV-infected EAE-induced

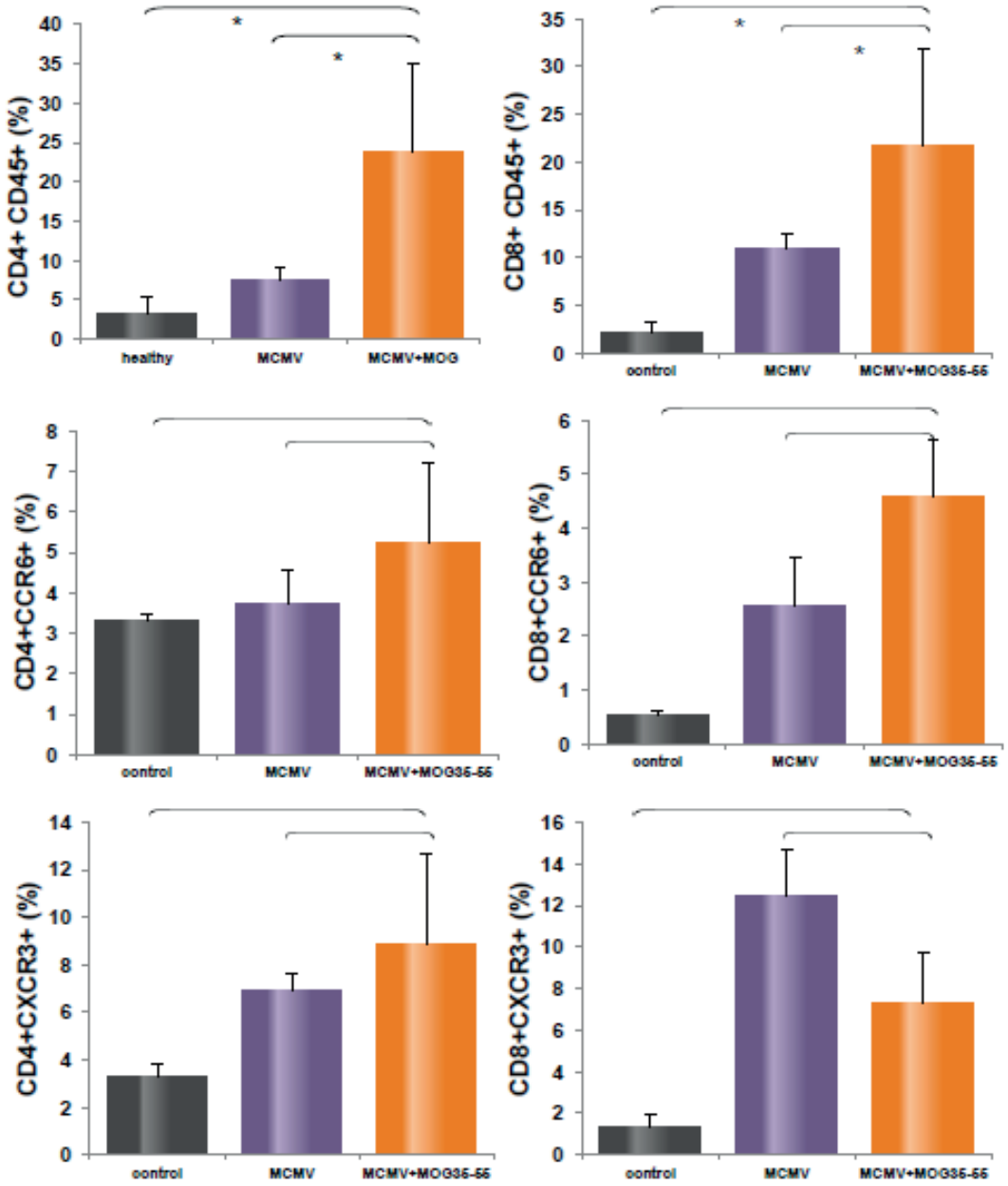


Figure 2. MCMV-infected EAE-induced mice display a similar percentage of CD4+ and CD8+ cells in the CNS. Fifteen days after immunisation, mononuclear cells were isolated from the spinal cords and the brains and were used for flow cytometric analysis of the percentages of CD45+CD4+, CD45+CD8+, CD4+CCR6+, CD4+CXCR3+, CD8+CCR6+ and CD8+CXCR3+ cells. The data presented were obtained from a representative experiment (means+SD; *P<0. 05; **P<0. 005). Significance was assessed using Student's t-test.

mice compared with untreated and MCMV-infected mice. However, the influx of CD8+ T lymphocytes expressing Th1-related chemokine receptors was significantly higher in the MCMV-infected mice than in the MCMV-infected EAE-induced mice, whereas the percentage of CD8+ T cells expressing the Th17-related chemokine receptors was significantly higher in the EAE-induced MCMV-infected mice (Fig. 2).

EAE Mice Subjected to Neonatal MCMV Infection Display an Increased Percentage of Infiltrating Tc17 Cells in the CNS

Further, we assessed the effects of viral infection on the influx of proinflammatory CD4+ and CD8+ T cells into the nervous tissue. Therefore, we quantified the number of Tbet- and RoRγt-expressing and IL-17-, IFN-γ-, and TNF-α-producing CD4+ T and CD8+ T cells that infiltrated

into the CNS at the peak of the disease. Flow cytometric analysis of the CNS mononuclear cells revealed that inflammatory CD4+ and CD8+ cells were nearly absent from the CNS of the BALB/c mice. Influx of Th1 and Th17 cells was significant only in the EAE-induced MCMV-infected BALB/c mice. The percentage of CD4+ T cells expressing Th1- (IFN-γ and TNF-α) and Th17-related cytokines (IL-17) and expressing Th1- (Tbet) and Th17-related transcription factors (RoRγt) was significantly higher in the MCMV-infected EAE-induced mice compared with the MCMV-infected and untreated mice. The influx of Tc1 cells (CD8+ cells expressing IFN-γ, TNF-α and Tbet) into the CNS was significant in the MCMV-infected mice . The percentages of Tc1 cells were slightly lower in the MCMV-infected EAE-induced mice than in the MCMV-infected mice. However, the percentages of Tc17 cells (CD8+ cells expressing RoRγt and IL-17) in the CNS were significantly higher in the MCMV-infected EAE-induced mice than in the MCMV-infected and untreated mice. Moreover, there were hardly very few Tc17 cells in the CNS of the MCMV-infected mice. Collectively, our results indicate significant influx of CD8+IL-17+ and CD8+RoRγt+ cells into the CNS of new-born MCMV-infected BALB/c mice after MOG_{35-55} immunisation.

DISSCUSION

A very important difference between MS and EAE is the equivalent level of CD8+ and CD4+ T cell infiltration in MS plaques in contrast to the predominance of infiltrating CD4+ T cells in the CNS of mice with EAE (15). Analysis of the antigen receptor expression patterns of CD8+ T cells in the CNS infiltrates in MS suggests the local activation of antigen-induced immune responses (16). There is the lack of experimental models that are suitable to study the role of CD8+ cells in CNS autoimmunity. Recently, it was shown that latent EBV infection exacerbates EAE signs in mice and induces the infiltration of CD8+IFN-γ+granzyme+ cells into the brain parenchyma (3). In this model of EAE, no viral DNA was detected in the CNS (based on PCR) during EAE, and the virus was hypothesised to indirectly influence the autoimmune response. CMV shares homology with EBV and induces latent infections that can subsequently become reactivated, resulting in different consequences.

We demonstrated that latent neonatal infection with MCMV alters the susceptibility of BALB/c mice to EAE, leading to the equivalent infiltration of CD4+ and CD8+ T cells into the CNS after EAE induction, which activates a significant Tc17-mediated immune response in the CNS that is accompanied by typical EAE paralysis.

In our model of neonatal MCMV infection, the mice develop histopathological lesions that are characteristic of meningoencephalitis. Histopathological lesions are composed of infected cells associated with infiltrating mono- and polymorpho-nuclear leukocytes or activated resident microglia (17, 18). This cell infiltration persists in the brain even after the termination of productive viral infection, when the replicating virus is no longer detected in the brain (19). The predominant immune cell population in MCMV-infected new-born brains is CD8+ T lymphocytes, and these cells persist in the CNS after the resolution of acute infection (20). Viral DNA persists in the mononuclear cells outside the CNS.

However, the MCMV-infected EAE-induced mice displayed significantly higher cell infiltration in the CNS than age-matched mice subjected to neonatal MCMV infection alone. EAE induction in the MCMV-infected mice was followed by the significant influx of CD4+ T cells and, importantly, CD8+ T cells. We detected the influx of CD4+ T cells expressing CXCR3, the chemokine receptor for CXCL10, and CD4+ T cells expressing CCR6, a receptor that is known to play a key role in the initial development of autoimmune infiltration into the CNS (21, 22). On the other hand, EAE induction in mice subjected to latent MCMV infection increased the influx of CD8+CCR6+ T cells and decreased the influx CD8+CXCR3+ T cells compared with MCMV infection alone. MCMV infection is known to attract CD8+CXCR3+ cells, and it appears that the newly developing autoimmune process attracts a different population of CD8+ cells (23). This result indicates that latent MCMV infection of BALB/c mice in the periphery affects the immune response to the myelin antigen, driving the immune cells towards an inflammatory phenotype that facilitates the entry of these cells into the CNS and the induction of autoimmune processes. Recently, it was reported that MCMV infection of murine fibroblasts altered the expression of 10748 genes (24). Among the most strongly induced genes were those corresponding to Interferon-β, the transcription factor T-bet, and the chemokine CXCL10. The expression levels of all proteins examined correlated with their transcript levels. The role of the chemokine CXCL10 and the transcription factor Tbet, which are markers of Th1 cells, in EAE pathogenesis is well known (25, 26, 27). Immune challenge (immunisation with MOG_{35-55}) is assumed to reactivate MCMV infection, leading to the expression of genes whose products affect the polarisation of the adaptive immune response.

We also found that the CNS infiltrates of mice subjected to latent MCMV infection followed by EAE induction contained a significantly higher percentage of Th1, Th17, and Tc17 cells compared with mice subjected to MCMV infection alone. No increase was detected in the percentage of CD8+ cells expressing Tbet and the Th1-related cytokines TNF-α and IFN-γ in the CNS with autoimmune process developed after viral infection compared with mice with new-born MCMV infection. Autoimmune neuroinflammation clearly developed after previous MCMV infection altered the dominant population of CD8+ cells in the CNS. Tc17 cells (IL-17- and RoRγt-expressing CD8+ cells) are required for Th17 accumulation and for the development of EAE (28). Patients with

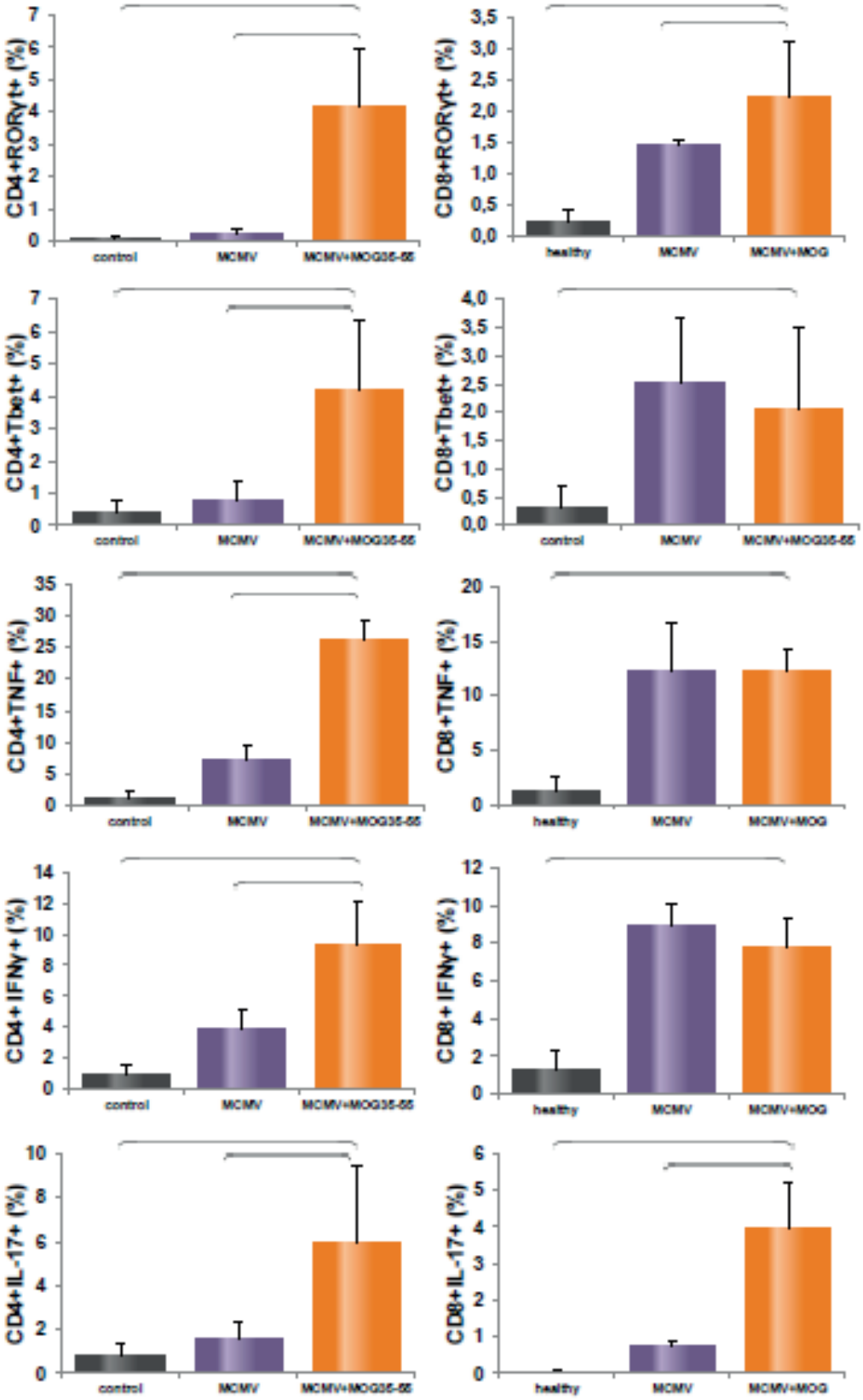


Figure 3. Influx of Tc17 cells following the induction of neuroinflammation in MCMV-infected mice.
Fifteen days after immunisation, mononuclear cells were isolated from the spinal cords and the brains and were used for flow cytometric analysis of the percentages of CD4+Tbet+, CD4+CDRoRγt+ , CD4+TNF-α+, CD4+IFN-γ+, CD4+IL-17+, CD8+Tbet+, CD8+CDRoRγt+, CD8+TNF-α+, CD8+IFN-γ+, and CD8+IL-17+ cells. The data presented were from a representative experiment (means+SD; *P<0. 05; **P<0. 005). Significance was assessed using Student's t-test.

early-stage MS harbour a greater number of Tc17 cells in the cerebrospinal fluid than in the peripheral blood. Tc17 cells contribute to the initiation of CNS autoimmunity by supporting Th17 cell pathogenicity. Our results indicate that latent MCMV infection contributes to the expansion of this subpopulation of CD8+ cells, which is known to play a role in autoimmune neuroinflammation, and the influx of these cells into the CNS.

CONCLUSION

Our findings suggest that latent CMV infection affects EAE development and, possibly, MS pathogenesis, likely via its influence on the initial development of inflammatory lymphocytes that display encephalitogenic potential. Furthermore, this model of EAE may be useful for the examination of the role of CD8+ T cells in CNS autoimmunity.

ACKNOWLEDGMENTS

This work was funded by grants from the Serbian Ministry of Science and Technological Development (Grant Nos. ON175071, ON175069, and ON175103) and the Faculty of Medical Sciences at the University of Kragujevac in Serbia (MP 01/14). No potential conflicts of interest relevant to this article were reported.

REFERENCES

1. Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG. Multiple sclerosis. N Engl J Med. 2000; 343 (13): 938-952.
2. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: the role of infection. Ann Neurol. 2007; 61(4): 288-299.
3. Casiraghi C, Shanina I, Cho S, Freeman ML, Blackman MA, Horwitz MS. Gammaherpesvirus latency accentuates EAE pathogenesis: relevance to Epstein-Barr virus and multiple sclerosis. PLoS Pathog. 2012; 8(5): e1002715.
4. Angelini DF, Serafini B, Piras E, et al. Increased CD8+ T Cell Response to Epstein-Barr Virus Lytic Antigens in the Active Phase of Multiple Sclerosis. PLoS Pathog. 2013; 9(4): e1003220.
5. Boeckh M, Geballe AP. Cytomegalovirus: pathogen, paradigm, and puzzle. J Clin Invest. 2011; 121(5): 1673-1680.
6. Bentz GL, Jarquin-Pardo M, Chan G, Smith MS, Sinzger C, Yurochko AD. Human cytomegalovirus (HCMV) infection of endothelial cells promotes naive monocyte extravasation and transfer of productive virus to enhance hematogenous dissemination of HCMV. J Virol. 2006; 80(23): 11539-11555.
7. Kondo K, Kaneshima H, Mocarski ES. Human cytomegalovirus latent infection of granulocyte-macrophage progenitors. Proc Natl Acad Sci U S A. 1994; 91(25): 11879-11883.
8. Pakpoor J, Pakpoor J, Disanto G, Giovannoni G, Ramagopalan SV. Cytomegalovirus and multiple sclerosis risk. J Neurol. 2013; 260(6):1658-1660.
9. Bernard CC. Experimental autoimmune encephalomyelitis in mice: genetic control of susceptibility. J Immunogenet. 1976; 3(4): 263-274.
10. Hurwitz AA, Sullivan TJ, Sobel RA, Allison JP. Cytotoxic T lymphocyte antigen-4 (CTLA-4) limits the expansion of encephalitogenic T cells in experimental autoimmune encephalomyelitis (EAE)-resistant BALB/c mice. Proc Natl Acad Sci U S A. 2002; 99(5): 3013-3017.
11. Milovanovic M, Volarevic V, Ljujic B, et al. Deletion of IL-33R (ST2) abrogates resistance to EAE in BALB/C mice by enhancing polarization of APC to inflammatory phenotype. PLoS One. 2012; 7(9): e45225.
12. Jiang HR, Milovanović M, Allan D, et al. IL-33 attenuates EAE by suppressing IL-17 and IFN-γ production and inducing alternatively activated macrophages. Eur J Immunol. 2012; 42(7): 1804-1814.
13. Stromnes IM, Goverman JM. Active induction of experimental allergic encephalomyelitis. Nat Protoc. 2006; 1(4): 1810–1819.
14. Ponomarev ED, Shriver LP, Maresz K, et al. GM-CSF production by autoreactive T cells is required for the activation of microglial cells and the onset of experimental autoimmune encephalomyelitis. J Immunol. 2007; 178(1): 39–48.
15. Babbe H, Roers A, Waisman A, et al. Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. J Exp Med. 2000; 192(3): 393-404.
16. Junker A, Ivanidze J, Malotka J, et al. Multiple sclerosis: T-cell receptor expression in distinct brain regions. Brain. 2007; 130(Pt 11): 2789-2799.
17. Kosugi I, Kawasaki H, Arai Y, Tsutsui Y. Innate immune responses to cytomegalovirus infection in the developing mouse brain and their evasion by virus-infected neurons. Am J Pathol. 2002; 161(3): 919-928.
18. van den Pol AN, Reuter JD, Santarelli JG. Enhanced cytomegalovirus infection of developing brain independent of the adaptive immune system. J Virol. 2002; 76(17): 8842-8854.
19. Kosmac K, Bantug GR, Pugel EP, Cekinovic D, Jonjic S, Britt WJ. Glucocorticoid treatment of MCMV infected newborn mice attenuates CNS inflammation and limits deficits in cerebellar development. PLoS Pathog. 2013; 9(3): e1003200.
20. Bantug GR, Cekinovic D, Bradford R, Koontz T, Jonjic S, Britt WJ. CD8+ T lymphocytes control murine cytomegalovirus replication in the central nervous system of newborn animals. J Immunol. 2008; 181(3): 2111-2123.

21. Reboldi A, Coisne C, Baumjohann D, et al. C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. Nat Immunol. 2009; 10(5): 514–523.
22. Liston A, Kohler RE, Townley S, et al. Inhibition of CCR6 function reduces the severity of experimental autoimmune encephalomyelitis via effects on the priming phase of the immune response. J Immunol. 2009; 182(5): 3121–3130.
23. Hokeness KL, Deweerd ES, Munks MW, Lewis CA, Gladue RP, Salazar-Mather TP. CXCR3-dependent recruitment of antigen-specific T lymphocytes to the liver during murine cytomegalovirus infection. J Virol. 2007; 81(3): 1241-1250.
24. Juranic Lisnic V, Babic Cac M, Lisnic B, et al. Dual Analysis of the Murine Cytomegalovirus and Host Cell Transcriptomes Reveal New Aspects of the Virus-Host Cell Interface. PLoS Pathog. 2013; 9(9): e1003611.
25. Yang Y, Weiner J, Liu Y, al. T-bet is essential for encephalitogenicity of both Th1 and Th17 cells. J Exp Med. 2009; 206(7): 1549–1564.
26. Lalor SJ, Segal BM. Th1-mediated experimental autoimmune encephalomyelitis is CXCR3 independent. Eur J Immunol. 2013; 43(11): 2866-2874.
27. Carter SL, Müller M, Manders PM, Campbell IL. Induction of the genes for Cxcl9 and Cxcl10 is dependent on IFN-gamma but shows differential cellular expression in experimental autoimmune encephalomyelitis and by astrocytes and microglia in vitro. Glia. 2007; 55(16): 1728-1739.
28. Huber M, Heink S, Pagenstecher A, et al. IL-17A secretion by CD8+ T cells supports Th17-mediated autoimmune encephalomyelitis. J Clin Invest. 2013; 123(1): 247-60.

THE EFFECTS OF ACUTE VIBROACOUSTIC MICROVIBRATIONS ON THE RAT HEART RATE, RHYTHM AND STRUCTURE

Dusko Kornjaca[1], Vladimir Jakovljevic[2], Stefan Zivkovic[3], Marko Djuric[3], Nela Puskas[4], Dragan Djuric[3]
[1]Independent Medical Practice, Novi Sad
[2]Department of Physiology, Faculty of Medical Sciences, University of Kragujevac
[3]Institute of Medical Physiology "Richard Burian", Faculty of Medicine, University of Belgrade, Serbia
[4]Institute of Histology and Embriology "Aleksandar Kostic", Faculty of Medicine, University of Belgrade

EFEKTI AKUTNE PRIMENE VIBROAKUSTIČKIH MIKROVIBRACIJA NA FREKVENCIJU, RITAM I STRUKTURU SRCA PACOVA

Duško Kornjača[1], Vladimir Jakovljević[2], Stefan Živković[3], Marko Đurić[3], Nela Puškaš[4], Dragan Đurić[3]
[1]Nezavisna medicinska praksa, Novi Sad
[2]Katedra za fiziologiju, Fakultet medicinskih nauka Univerziteta u Kragujevcu
[3]Institut za medicinsku fiziologiju "Rihard Burijan", Medicinski fakultet Univerziteta u Beogradu
[4]Institut za histologiju i embriologiju "Aleksandar Kostić", Medicinski fakultet Univerziteta u Beogradu

Received / Primljen: 27.09.2014. Accepted / Prihvaćen: 12.11.2014.

ABSTRACT

The body surface of homeothermic organisms produces constant microvibrations. In the past, many studies were conducted on this topic, and the amplitude of the microvibrations was described as a sensitive marker of muscle tension and body activity. Subsequent studies indicated that the frequency of the microvibrations is an important variable affecting the body. The aim of this research was to examine the effects of the vibroacoustic microvibrations on the rate, rhythm and structure of the rat heart during physiological conditions. Microvibrations of specific frequency and amplitude were induced by a Vitafon-T, four different modes were used, and the effects of the microvibrations on ECG characteristics and the wall structure of the rat heart were examined. After the application of microvibrations (lasting 10–60 min), no statistically significant changes occurred in the heart rate, but the amplitudes significantly increased after 10, 20 and 30 minutes, and increased even more after 60 minutes. No changes in the heart wall structure were found. Acute in vivo application of vibroacoustic microvibrations in the rats did not produce significant effects on the heart rate and rhythm; however, it increased the amplitude of the R wave by 25–32% in the second standard ECG lead but did not lead to structural changes in the rat heart wall.

Key words: *microvibrations, heart rate, rhythm, structure, heart*

SAŽETAK

Površina tela homeoterma konstantno proizvodi vibracije. U prošlosti su sprovedena mnoga istraživanja na temu mikrovibracija, i opisala amplitudu mikrovibracija kao osetljiv parametar mišićne tenzije i telesne aktivnosti. Kasnija istraživanja su frekvenciju mikrovibracija navela kao varijablu od centralnog značaja za njihove efekte na organizam. Cilj istraživanja je bio da ispitamo efekte vibroakustičkih mikrovibracija na frekvenciju, ritam i strukturu srca pacova u fiziološkim uslovima. U istraživanju je korišćen aparat koji proizvodi mikrovibracije, Vitafon-T određene frekvencije i amplitude, i u skladu sa tim koristili smo četiri režima rada u kojima smo ispitivali efekte mikrovibracija na EKG karakteristike i strukturu zida srca pacova. Utvrdili smo da nakon primene mikrovibracija (u trajanju od 10-60 minuta) nije došlo do statistički značajnih promena u broju otkucaja, vrednosti amplitude R talasa su statistički značajno povećane posle 10, 20 i 30 minuta, a visoko statistički značajno povećane posle 60 minuta. Na preparatu zida srca nema promena u odnosu na normalnu strukturu. Akutna primena vibroakustičkih mikrovibracija kod pacova in vivo ne utiče značajno na frekvenciju i ritam srčanog rada, povećava amplitudu R talasa u drugom standardnom EKG odvodu za 25-32 % i ne dovodi do promena u strukturi zida srca pacova.

Ključne reči: *mikrovibracije, frekvencija, ritam, struktura, srce*

INTRODUCTION

Microvibrations were described as a phenomenon by Rohracher (1) who found that the body surface of humans and other homeothermic organisms, in general, constantly produce vibrations. The amplitude of the microvibrations appeared to be a sensitive marker of muscle tension and total body activity. Later, other researchers (2-7) supported the arguments presented by Rohracher. In a healthy organism, either human or animal, the amplitude of these microvibrations is 1–5 international units with a frequency of 6–12 Hz/sec in a state of maximal relaxation. Basic conclusions of the research done by Rohracher et al. are as follows: microvibrations are possible to record on

DE GRUYTER OPEN
UDK: 612.17.014.42 / Ser J Exp Clin Res 2014; 15 (4): 191-196
DOI: 10.2478/SJECR-2014-0024

Corresponding author:
Professor Dragan M. Djuric, MD, PhD, FIACS; Institute of Medical Physiology "Richard Burian"
Faculty of Medicine, University of Belgrade, Visegradska 26/II, 11000 Belgrade, Serbia; Tel: +381 11 3607 112; Fax: +381 11 3611 261; E-mail: drdjuric@eunet.rs

the whole surface of the body; microvibrations originate in striated muscle; and the amplitude of the microvibrations is multiplied (several times) by contraction of striated muscles, but muscle contractions do not influence the frequency of the microvibrations (which is constant during the given recording). Rohracher (8) identified two significant functions of body microvibrations, which are evident with the emission of quite a small amount of energy. Namely, the body of homeothermic animals has a constant temperature, and the muscles are in a state of permanent readiness. Due to these capacities, fast and positive motor functions are generated (4). Further analysis (5) defined the similarity between alpha waves in an ECG and the wave shapes of frequencies of microvibrations, which pointed to the causality of the phenomenon. These findings were criticised (9) and (10); they concluded that the amplitude of the microvibrations varied depending on the quantity of energy and was a sensitive parameter of muscle tension. Out of the two dependent variables that were investigated (amplitude and frequency), the findings on frequency were of central importance for the origins of the theory. This was due to previous knowledge that muscles produced movements of various amplitudes, but the statement that they showed constant periodical movements with constant frequency was especially important. The established significant facts related to the frequency of the microvibrations are that the frequency is always within an approximate scope of 6–12 cycles per second and that the frequency is constant regardless of the method by which it is measured. The aim of our research was to examine the effects of acutely applied vibroacoustic microvibrations on the frequency, rhythm and wall structure of the rat heart during physiological conditions by using a commercial devise that produces microvibrations of a specific amplitude and frequency. Then, the effects of the microvibrations on ECG characteristics in the second standard lead in rats and the histological wall structure of the rat heart were analysed.

MATERIALS AND METHODS

To investigate the acute effects of the vibroacoustic microvibrations on the rat cardiovascular system during physiological conditions, a Vitafon-T apparatus with defined amplitudes (2.8–12.3 μm) and frequencies (30–18K Hz) was used. With respect to the amplitudes and frequencies, four various modes were used and applied to experimental animals in order to examine the effects of the microvibrations on the frequency, rhythm and histological wall structure of the rat heart. Mode 1 had a frequency of 30–60 Hz and an amplitude of 2.8 μm; Mode 2 had a frequency of 1200–4500 Hz and an amplitude of 6.0 μm; Mode 3 had a frequency of 200–1000 Hz and an amplitude of 5.4 μm; Mode 4 had a frequency of 900–18000 Hz and an amplitude of 12.3 μm. The period of impulse modulation was 0.5, 1 and 2 seconds.

Experimental model for investigating the acute effects of vibroacoustic microvibrations of a certain amplitude and frequency on ECG characteristics, heart rate, heart rhythm and amplitude of waves in the second standard lead in rats

The performed experimental work in rats was aimed at defining ECG characteristics, heart rate, heart rhythm and amplitude of waves in the second standard lead after acute exposure to vibroacoustic microvibrations of a certain amplitude and frequency. In this part of the experiment, the investigation was performed on 4 plus 6 rats (this protocol included a total of 10 Wistar albino rats) that served as a control group at the same time. The rats were 6–8 weeks-old males with a body mass of 160–250 g. They were kept in standard laboratory conditions (air temperature 22 ± 1° C, relative humidity 50%, light-dark cycle 12 : 12 hours, and free access to food and water). The study was performed in stages, and the outlined issues were defined by the experimental protocol. The experiments began with individual animal checkups and measurement of weight. After the conditions for the experimental work were fulfilled, the rats were anaesthetised by applying easy evaporating ether as an anaesthetic. Then, the rats were immobilised. This was followed by connection of the rats to a standard one-channel electrocardiograph. The connection was performed with the help of specially modified electrodes. In continuation, emitters of a Vitafon-T apparatus (*Saint Petersburg, Russian Federation*) were applied directly to the rat skin in the region of the heart and kidneys. The vibroacoustic microvibrations of specific amplitudes were emitted, and the frequencies were distributed in four modes. Before the microvibrations began, an initial ECG was recorded, which also served as a control. Exposure to the microvibrations lasted 10 minutes in each mode. The first four rats were additionally phonated until 60 minutes, recording their ECG at 10, 20, 30 and 60 minutes. After each mode was completed, 5- minute rests were introduced. After exposure to the microvibrations was completed, the results were carefully classified, technically processed and analysed.

Experimental model for investigating the acute effects of the vibroacoustic microvibrations of various amplitudes and frequencies on the rat heart wall structure (hematoxylin and eosin staining method)

In this part of the experiment, the effects of the acute vibroacoustic microvibrations on the heart wall structure of the rat were examined by histological analysis. The experimental animals that were previously exposed to the acute vibroacoustic microvibrations were sacrificed, and the heart was extracted. The extraction was performed by surgical opening of the abdomen, accessing the diaphragm and performing a thoracotomy. The diaphragm was incised in an arc from left to right, and then the thorax was opened laterally along the mammillary line. When the thorax was opened, the pericardium on top of the heart was cut, and thus, prepared for isolation. The blood vessels at the heart

base were resected, and the organ was isolated from the thorax and put into an icy physiological solution (-4° C to -10° C) to stop all physiological processes in the myocardium. Then, the heart was placed in the formalin solution and further processed histologically.

Statistical data analysis

In the statistical data processing, the basic methods of descriptive statistics were used (mean value (X), frequencies, percentages, sample mean value, standard deviation (SD) and standard error (SE)). To test the hypothesis and significance, Student's t-test for dependent and independent samples and two-factor analysis of variance (ANOVA) were used. The qualification of connection between variables was determined using Pearson's linear correlation, which defined the proportions between the two variables. It was expressed as a coefficient of correlation. The coefficient of correlation was high if the coordinated interdependence of activities existed. The probability (a) and significance (r) were established using a standard formula. A p of <0.05 was considered statistically significant. Database and analysis of the results were performed using SPSS 10.0 (*SPSS lnc, Chicago, IL, USA*). The research was performed in accordance with the *Helsinki Declaration* and further amendments, and approval of the experimental protocols was obtained by the Ethical Committee of Faculty of Medicine.

RESULTS

Acute effects of vibroacoustic microvibrations of a certain amplitude and frequency on ECG characteristics, heart rate, heart rhythm and wave amplitudes in the second standard lead in rats

After applying the vibroacoustic microvibrations (lasting 10 minutes) in the heart region, no statistically significant changes occurred in the heart rate. The most marked changes occurred after applying Mode 3, but even these changes were not statistically significant (Appendix 1). When analysing the frequency of rhythm disorder in the second standard ECG lead during the application of the vibroacoustic microvibrations, slight disturbances in the cardiac rhythm were recorded in 25% of the rats for Modes 3 and 4 (Modes 3 and 4 each had one rat with arrhythmias). During Modes 1 and 2, no arrhythmias were recorded (Appendix 2). After analysing the heart frequency after application of the vibroacoustic microvibrations (60 minutes long), no statistically significant changes in the heartbeat were found in Mode 1. The most significant changes occurred after 20 minutes, but they were not statistically significant. Analysis of the frequency of the registered disturbances of the heart rhythm in the second standard ECG lead after 60 minutes of microvibrations revealed that the R wave amplitude in Mode 1 had a statistically significant increase after 10, 20 and 30 minutes (p<0.5), and after 60 minutes, the amplitude further increased with greater statistical significance (p<0.01), (Appendix 3). Figure 4 shows anan ECG recording before and after application of the an microvibrations directly on the rat heart.

Acute effects of the vibroacoustic microvibrations of certain amplitudes and frequency on the rat heart wall structure using hematoxylin and eosin staining

Histological processing of the rat heart wall by hematoxylin and eosin staining showed that the various modes of the vibroacoustic microvibrations did not change the normal structure of the heart (Appendices 5 and 6).

Appendix 1

Table 1. The heart rate measured in the second standard ECG lead after a 10- minute application of the vibroacoustic microvibrations in four different modes (n=4 rats).

Heart rate (beats/min, x±SE)		
Mode 1		
Control	422.50±2.50	Student's t-test p=0.229
Experimental group	445.00±14.43	
Mode 2		
Control	443.33±18.55	Student's t-test p=0.742
Experimental group	440.00±26.55	
Mode 3		
Control	444.33±24.03	Student's t-test p=0.667
Experimental group	446.66±26.66	
Mode 4		
Control	433.66±27.28	Student's t-test p=0.423
Experimental group	440.00±30.55	

Appendix 2

Table 2. The frequency of cardiac rhythm disturbances measured in the second standard ECG lead after a 10- minute application of the vibroacoustic microvibrations in four different modes (n=4 rats).

Rhythm	Disturbance	No disturbance	Chi-squared test
Mode 1			
Control	0 (0%)	4 (100%)	p>0.05
Experimental group	0 (0%)	4 (100%)	
Mode 2			
Control	0 (0%)	4 (100%)	p>0.05
Experimental group	0 (0%)	4 (100%)	
Mode 3			
Control	0 (0%)	4 (100%)	p>0.05
Experimental group	1 (25%)	3 (75%)	
Mode 4			
Control	0 (0%)	4 (100%)	p>0.05
Experimental group	1 (25%)	3 (75%)	

Appendix 3

Table 3. The heart rate, frequency of cardiac rhythm disturbances, and amplitude of R waves measured in the second standard ECG lead after a 60 minute application of the vibroacoustic microvibrations in Mode 1 (n=6 rats).

Mode 1		
Heart rate (beats/min)		
Control	443.33±25.90	Student's t-test K:10' p=0.620 K:20' p=0.394 K:30' p=0.384 K:60' p=0.833
10'	451.66±33.30	
20'	456.66±27.52	
30'	455.00±27.04	
60'	448.33±35.34	
	Repeated measurements ANOVA P=0.646	
Rhythm (yes/no disturbance)		
Control	0/6	Chi-squared test p=0.604
10'	2/4	
20'	2/4	
30'	2/4	
60'	2/4	
R wave amplitude (mm)		
Control	5.50±1.04	**Student's t-test** **K:10' p=0.020** **K:20' p=0.020** **K:30' p=0.020** **K:60' p=0.005**
10'	7.26±1.16	
20'	7.45±1.31	
30'	7.63±1.45	
60'	8.05±1.55	
	Repeated measurements ANOVA p=0.001**	

DISCUSSION

Microvibrations exist in every living organism. The origins of the microvibrations include the pulsating heart activity (infrasound range), vascular activity and muscle activity (sound range). Muscle cells provide microvibrations that are necessary for tissues to maintain their function even in the resting state, which demands considerable energy consumption. The work of Soviet and Russian scientists clarified the knowledge of the existence and effects of the microvibrations on a whole organism. The effect of the so-called hydrodynamic pump was discovered by A. I. Arinchinim who, in his book "Peripheral Heart of Man", claims that muscle fibres tremble along with sound oscillations. His conception is that microvibrations are a physical element by which an organism reduces peripheral resistance in the capillary network and increases venous outflow of blood. The role of the microvibrations in pumping blood and lymph through the blood and lymph vessels in one direction was proven. The frequency of oscillatory motion of smooth muscles in the walls of these vessels improves the efficiency of the venous and lymphatic pumps, and the optimum amplitude of movement-oscillation of the muscles seems to correspond to the diameter of the lumen. As these vessels have different diameters, vibroacoustic vibrations of various shapes, frequencies, amplitudes and length are used to achieve synchronised stimulation of the various vessel types. Consequently, for each diameter of blood and lymphatic vessel, there is not only an optimum frequency, but also optimum characteristics of the waves (energy). Another important characteristic is a reduction in resistance due to blood flow. Vibroacoustic microvibrations reduce the friction between the blood layers at a certain frequency, thus, reducing the viscosity and vascular resistance and leading to an increase in the fractional force, which is a biological stimulus for the production

Appendix 4

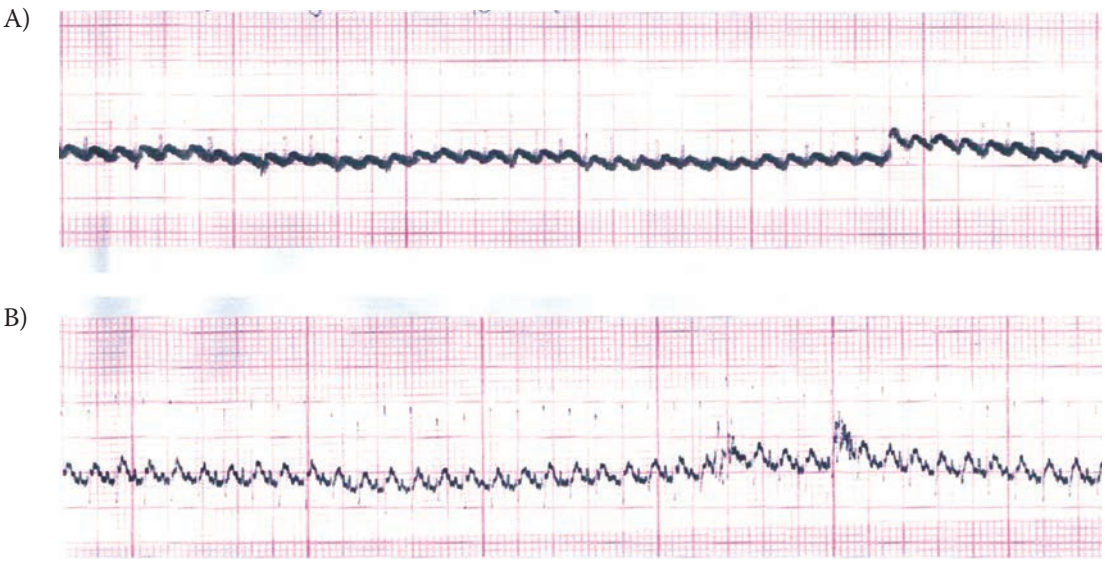
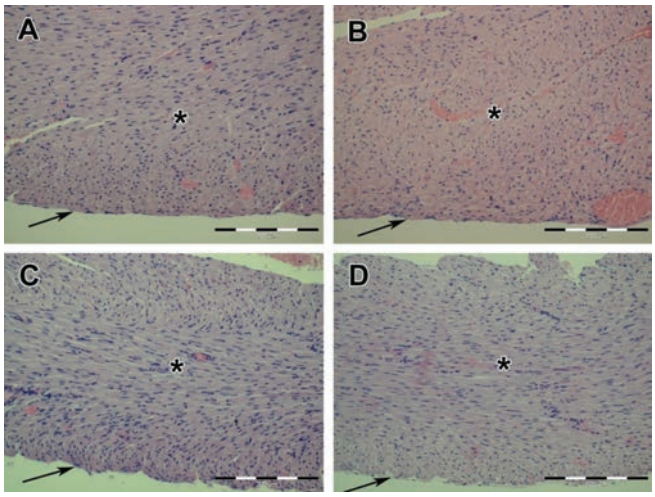
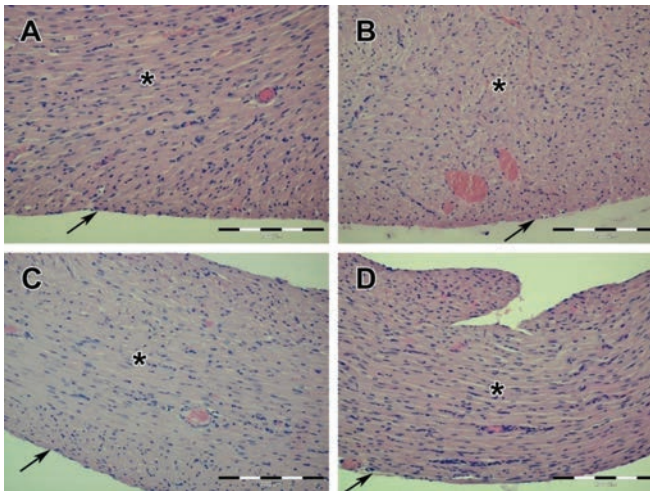


Figure 1. Changes in the R wave amplitude on ECG recordings before (A) and after (B) the application of vibroacoustic microvibrations directly to the rat heart.



Appendix 5

Figure 2. Normal wall structure of the rat heart (before the application of vibroacoustic microvibrations). A) left ventricle, B) left atrium, C) right ventricle, D) right atrium; arrow=epicardium; asterisk=myocardium.



Appendix 6

Figure 3. Wall structure of the rat heart after the application of vibroacoustic microvibrations in Mode 1. A) left ventricle, B) left atrium, C) right ventricle, D) right atrium; arrow=pericardium; asterisk=myocardium.

of nitric oxide. Certain effects of applying low frequency sound on the human cardiovascular system have already been indicated. In a study that applied infrasound (below 20 Hz) to astronauts on the Apollo mission, 21 males aged 21–33 years old were stimulated with sounds (between 2 Hz and 12 Hz) between 119–144 decibels in a stimulation chamber. No electrocardiographic disturbances were recorded during the stimulation. The heart rate was higher in 6 people by more than 6 beats per minute during maximum stimulation, while in 5 people, a decreased heart rate was recorded (11). This investigation did not observe any discomforts such as disorientation, mental confusion, or tiredness or decreased sensory abilities in any person. However, the effects of the vibrations and noise on the human cardiovascular and respiratory systems were also investigated in aeronautical workers and helicopter pilots (12, 13). The biological effects of infrasound and low-frequency noise explained by mechanotransduction cellular signalling and its connection with vibroacoustic disease were intensively studied during the last 15 years (14-18). Furthermore, the effects of chronic exposure to low frequency noise on rat pleural mesothelial cells and rat tracheal epithelia were confirmed (19). This attention to low intensity and frequency of vibroacoustics were further studied in the effects of low intensity pulsed ultrasound on integrin-FAK-PI3K/Akt mechanochemical transduction in chondrocytes of osteoarthritic rabbits (20).

In our research, vibroacoustic microvibrations (up to 60 minutes, frequency 30–18 Hz and amplitude 2.8–12.3 μm) were acutely induced in order to evaluate their effects on the cardiovascular system in rats. Analysis of the heart rates in response to the vibroacoustic microvibrations (10 minutes long) did not show statistically significant changes. The most noticeable changes occurred after the application of the microvibrations in Mode 3, but the changes were not statistically significant. When analysing the frequency of disturbances in the heart rhythm in the second standard ECG lead during the vibroacoustic microvibrations, slight changes in the cardiac rhythm were noticed in Modes 3 and 4 in 25 % of the rats (one rat per mode had arrhythmias). No arrhythmias were noted during the application of Modes 1 and 2. When analysing the R wave amplitudes in the second standard lead after the application of the vibroacoustic microvibrations (10 minutes long), a statistically significant increase in the amplitude was present in Modes 3 and 4, while Modes 1 and 2 did not show statistically significant changes. We hypothesise that the increased amplitude of the R waves could be due to vasodilatation induced by the microvibrations on the rat body, which led to increased influx of blood into the heart, i.e., increased cardiac contractions by the Frank-Starling law. Our results could also be due to the direct effect of the microvibrations on cardiac muscle (or tissue), which resulted in the increased contractility. Analysis of the heart rates after application of the vibroacoustic microvibrations (60 minutes long) showed no statistically significant changes in Mode 1. The most prominent changes occurred after 20 minutes of stimulation, but the changes were not statistically significant. Analysis of the frequency of the registered disturbances in the heart rhythm in the second standard ECG lead after application of the microvibrations for 60 minutes showed that the values of the amplitude in Mode 1 were statistically significant after 10, 20 and 30 minutes, and after 60 minutes, the values of the amplitude were highly statistically significant. Consequently, Mode 1 showed that potentially useful effects could appear with the use of low frequencies and amplitudes of microvibrations, although the application of maximum frequencies or amplitudes of microvibrations should be further investigated. Results from the hematoxylin and eosin staining

of the rat heart wall showed that the acute application of the microvibrations did not change the normal histological wall structure. In conclusion, the increase in the amplitudes of the R waves in the second standard ECG lead by 25–32% was not correlated with changes in the heart wall structure. Further research on the detailed physiological mechanisms of the obtained results should be conducted in the future.

ACKNOWLEDGEMENTS.

This work is part of aan MSc thesis by Dr. Duško Kornjaca; the thesis was an defended at the Faculty of Medical Sciences, University of Kragujevac.

REFERENCES

1. Rohracher H: Schwingungen im menchlichen Organismus. Anz. Phil-Hist. Ost Akad. Wiss. 1946; 23
2. Denier A: The microvibrations of the body as an expression of physiological tonus. EEG Clin Neurophysiol, 1957; 9: 362
3. Heller-Jahnel: Die Microvibration bei psychischer Spannung und Entspannung; Z Psychother Med Psychol 1959; 9: 34-38
4. Luhhan W: Die Mikrovibration bei vorgestellen Bewegungen. Unpublished doctoral dissertation, University of Vienna, 1953 manley RG: Waveform analysis. London: Chapman & Hall, 1954
5. Nirrko O: On the intraindividual correspondence between the EEG alpha rythm and muscle vibration. Report No. 2, University of Helsinki Psychological Institute, 1961
6. Sugano H: Studies on the microvibration. Kurume Med J, 1957; 4: 97-113
7. Swarofsky H: Die Microvibration bei Affecten und bei Temperaturaenerungen. Doctoral dissertation, University of Vienna, 1958
8. Rohracher H: Neue Untersuchungen uber biologische Microschwingungen. II Anz Phil-Hist Ost Akad Wist 11-15, 1954
9. Oswald I: On the origin of the EEG alpha rhythm. Psychol. Rev, 1961; 68: 360-362
10. Rosner BS: Alpha rhythm of the EEG and mechanical properties of the brain. Psychol Rev 1961; 68: 259-360
11. Alford BR, Jerger JF, Coats AC, Bilhingham J, French BO, McBrayer RO: Human tolerance to low frequency sound. Transactions of the American Academy of Ophthalmology and Otolaryngology, 1966; 701, 40-47
12. Marciniak W, Rodriguez E, Olszowska K, Atkov O, Botvin I, Araujo A, Pais F, Soares Ribeiro C, Bordalo A, Loureiro J, Prazeres De Sá E, Ferreira D, Castelo Branco MS, Castelo Branco NA: Echocardiographic evaluation in 485 aeronautical workers exposed to different noise environments. Aviat Space Environ Med, 1999: 70(3 Pt 2):A46-53
13. Kåsin JI, Kjellevand TO, Kjekshus J, Nesheim GB, Wagstaff A: CT examination of the pericardium and lungs in helicopter pilots exposed to vibration and noise. Aviat Space Environ Med, 2012: 83(9):858-64
14. Castelo Branco NA: The clinical stages of vibroacoustic disease. Aviat Space Environ Med, 1999: 70(3 Pt 2):A32-9.
15. Castelo Branco NA, Rodriguez E: The vibroacoustic disease--an emerging pathology. Aviat Space Environ Med, 1999: 70(3 Pt 2):A1-6.
16. Branco NA, Alves-Pereira M: Vibroacoustic disease. Noise Health, 2004: 6(23):3-20.
17. Alves-Pereira M, Castelo Branco NA: Vibroacoustic disease: biological effects of infrasound and low-frequency noise explained by mechanotransduction cellular signalling. Prog Biophys Mol Biol, 2007: 93(1-3):256-79.
18. Branco NA, Ferreira JR, Alves-Pereira M: Respiratory pathology in vibroacoustic disease: 25 years of research. Rev Port Pneumol, 2007: 13(1):129-35.
19. De Sousa Pereira A, Grande NR, Monteiro E, Castelo Branco MS, Castelo Branco NA: Morphofunctional study of rat pleural mesothelial cells exposed to low frequency noise. Aviat Space Environ Med, 1999: 70(3 Pt 2):A78-85.
20. Cheng K, Xia P, Lin Q, Shen S, Gao M, Ren S, Li X: Effects of low-intensity pulsed ultrasound on integrin-FAK-PI3K/Akt mechanochemical transduction in rabbit osteoarthritis chondrocytes. Ultrasound Med Biol, 2014: 40(7):1609-18.

PROTEIN AND LIPID CONCENTRATIONS IN PATIENTS WITH DIFFERENTIATED THYROID CANCER TREATED WITH RADIOACTIVE IODINE-131

Olgica Mihaljevic[1], Snezana Radivojevic[2], Svetlana Djukic[1,2], Ljiljana Mijatovic Teodorovic[1,2], Irena Kostic[1], Ilija Jeftic[1], and Snezana Zivancevic Simonovic[1]
[1]Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
[2]Clinical Centre Kragujevac, Kragujevac, Serbia

KONCENTRACIJA PROTEINA I LIPIDA KOD PACIJENATA SA DIFERENTOVANIM KARCINOMOM ŠTITASTE ŽLEZDE KOJI SU LEČENI RADIOAKTIVNIM JODOM-131

Olgica Mihaljević[1], Snežana Radivojević[2], Svetlana Đukić[1,2], Ljiljana Mijatović Teodorović[1,2], Irena Kostić[1], Ilija Jeftić[1], and Snežana Živančević Simonović[1]
[1]Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija
[2]Klinički centar Kragujevac, Kragujevac, Srbija

Received / Primljen: 24.08.2013. Accepted / Prihvaćen: 15.03.2014.

ABSTRACT

Short-term, overt hypothyroidism in patients with differentiated thyroid cancer (DTC) before radioiodine (131-I) therapy might be accompanied by a number of metabolic changes, including altered protein and lipid metabolism. Protein concentrations and their relationship to lipids in the serum of DTC patients have not been fully elucidated. The aim of our study was to evaluate the protein and lipid concentrations in 24 DTC patients before and 3 and 7 days after 131-I therapy compared with those of 20 healthy control subjects. After radioiodine therapy, the mean protein concentration (78.71 ± 6.71 g/L vs. 87.16 ± 6.04 g/L; p = 0.003) and cholesterol level (8.12 ± 2.13 mmol/L vs. 8.84 ± 2.09 mmol/L; p = 0.001) were lower 3 days after therapy; this persisted up to 7 days after therapy, whereas triglyceride concentrations were higher 3 days after therapy (2.44 ± 1.07 mmol/L vs. 2.26 ± 1.08 mmol/L; p = 0.041) and returned towards the pretreatment values at 7 days after 131-I therapy. There was an indirect correlation between the protein and triglyceride concentrations 3 days after 131-I therapy in patients over 50 years old (Spearman's r = - 0.583, p = 0.048) but not in patients under 50 years old (Pearson's r = - 0.277, p = 0.384). Radioiodine therapy of DTC patients led to decreased serum protein and cholesterol concentrations, accompanied by increased triglyceride levels; these changes were especially evident in older subjects with metastases.

Keywords: *cholesterol; differentiated thyroid cancer; proteins; radioiodine therapy; triglycerides*

SAŽETAK

Prolazna, manifestna hipotireoza koja se javlja kod pacijenata sa diferentovanim karcinomom štitaste žlezde (DTC) pre terapije radioaktivnim jodom (131-I) može biti udružena sa brojnim metaboličkim promenama, uključujući i promene u metabolizmu proteina i lipida. Koncentracija proteina i njihov odnos sa lipidima u serumu pacijenata sa DTC nakon terapije 131-I nedovoljno su ispitani. Cilj našeg istraživanja bio je da se ispita serumska koncentracija proteina i lipida kod pacijenata sa DTC pre, kao i tri i sedam dana posle terapije 131-I. Studijom je obuhvaćeno 24 DTC pacijenata i 20 zdravih ispitanika. Pokazano je značajno, progresivno smanjenje koncentracije proteina (78.71±6.71 g/L vs. 87.16±6.04 g/L; p=0.003) i holesterola (8.12±2.13 mmol/L vs. 8.84±2.09 mmol/L; p=0.001) tri dana nakon terapije 131-I, uz statistički značajno povećanje koncentracije triglicerida tri dana nakon terapije (2.44±1.07 mmol/L vs. 2.26±1.08 mmol/L; p=0.041) i povratkom na preterapijske vrednosti 7 dana posle terapije. Pri tom, indirektna korelacija između koncentracije proteina i triglicerida tri dana posle 131-I pokazana je u grupi pacijenata starijih od 50 godina (Spearman r=- 0.583, p=0.048), što nije bio slučaj sa ispitanicima mlađim od 50 godina (Pearson r=- 0.277, p=0.384). U zaključku, terapija radioaktivnim jodom prouzrokuje smanjenje koncentracije serumskih proteina i holesterola, koje je udruženo sa povećanjem koncentracije triglicerida i posebno je izraženo kod starijih pacijenata sa metastazama.

Ključne reči: *holesterol; diferentovani karcinom štitaste žlezde; proteini; radiojodna terapija; trigliceridi*

DE GRUYTER OPEN

UDK: 615.849.2.035.2:546.15; 612.12.015.3 / Ser J Exp Clin Res 2014; 15 (4): 197-203
DOI: 10.2478/SJECR-2014-0025

Correspondence to: Olgica Mihaljevic, Faculty of Medical Sciences, University of Kragujevac
Zara Markovica 69, 34000 Kragujevac, Serbia; Tel. +381 34 306 800; Fax. +381 34 306 800 ext. 112; E-mail. vrndic07@yahoo.com

INTRODUCTION

Differentiated thyroid carcinomas (DTCs), or well-DTCs, are the most common tumours of the endocrine system (1). They represent approximately 85% of all thyroid carcinomas and include papillary and follicular types (2). As DTCs originate from thyroid follicular cells, which have the ability to concentrate iodine, the treatment of DTC patients with radioactive iodine (131-I) following thyroidectomy is the standard procedure for ablating remnant thyroid tissue and for treating iodine-avid metastases (3). The preparation of DTC patients for 131-I therapy involves two possibilities: thyroid hormone withdrawal (4-6 weeks) to increase the endogenous TSH level to above 30 IU/L or stimulation with exogenous, recombinant TSH (4). In the case of stimulating endogenous TSH secretion, short-term, overt hypothyroidism occurs and is manifested by abnormal thyroid tests (low free thyroxine (fT4) level, elevated thyroid stimulating hormone (TSH) level) and more or less pronounced symptoms and signs of hypothyroidism (impaired mental activity, including problems with concentration and memory, depression, chronic fatigue, muscle weakness, weight gain despite a diminished appetite, dry skin, sensitivity to cold, constipation, menstrual irregularities). Because thyroid hormones influence the rates of lipid synthesis, oxidation and mobilisation, as well as the synthesis and breakdown of proteins, overt hypothyroidism in DTC patients might be accompanied by a number of metabolic changes (5).

Iodine-131 administration and incorporation causes protracted and continuous internal whole body irradiation (gamma radiation of 0.38 MeV photons, beta radiation of 0.19 MeV electrons, half-life of 8.04 days). Part of the applied iodine specifically binds to and is retained in the residual thyroid tissue, whereas some persists in other parts of the body for days after 131-I therapy and might cause radiation damage. Therefore, the administration of large 131-I quantities provides ideal conditions for assessing the *in vivo* effects of prolonged irradiation by radionuclide incorporation and systemic exposure to radiation.

Some metabolic effects of short-term hypothyroidism, with a special focus on lipid metabolism, have been described in a number of earlier studies (6, 7, 8, 9), but the changes in protein and lipid concentrations in DTC patients treated with 131-I have not been fully elucidated. Hence, the aim of this study was to determine whether 131-I therapy leads to changes in serum proteins, as well as to analyse the relationship between the level of serum proteins and lipids during the first 7 days after the administration of radioactive 131-I.

MATERIALS AND METHODS

Study population

The study was approved by the Ethical Committee of the Clinical Centre Kragujevac. All patients and control subjects provided written informed consent according to the Declaration of Helsinki.

The study population included 24 well-DTC patients: 17 (70.8 %) females and 7 (29.2 %) males with a mean age of 54.83 ± 15.17 years. Half of the patients were under the age of 50 (≤50 years), whereas the others were older (>50 years). Of the 24 DTC patients, 18 (75 %) had papillary carcinoma, 5 (20.83 %) had the follicular variant of papillary carcinoma, and one (4.17 %) had follicular carcinoma. Thirteen patients had no clinical evidence of metastasis, whereas 11 patients had metastases in the lymph nodes (9 patients) or in the lymph nodes and lungs (2 patients). None of the patients had been exposed to potentially confounding factors such as other ionising radiation (radiographic examination or scintigraphy) within 3 months before therapy. Patients with previously diagnosed or treated primary lipid metabolism disorders and those with type 2 diabetes mellitus, nephrotic syndrome, renal failure, chronic liver disease or obesity were not included in the study. The patients were released from the hospital 3 days after 131-I therapy or later when the residual activity had reached a value below 2 mR/h, measured at the distance of 1 m, which is equivalent to 20 μSv/h, or 400 MBq, in the patient's body.

The period from surgery (total thyroidectomy) to the administration of 131-I was 4-6 weeks. During that interval, the patients did not receive thyroid hormone therapy and thus developed overt hypothyroidism: decreased free thyroxine and elevated TSH (>30 mIU/L) concentrations and more or less pronounced symptoms and signs of hypothyroidism. Ten days after receiving a low-iodine diet, the patients were treated at the Nuclear Medicine Department of the Clinical Centre Kragujevac according to EANM guidelines (10), with fixed nominal activities of 3.7 GBq (100 mCi) (15 patients) or 5.5 GBq (150 mCi) (9 patients) of sodium [131-I] iodide, administered orally.

The control group comprised 20 healthy subjects: 15 (75 %) females and 5 (25 %) males with a mean age of 46.76 ± 12.89 years. They were colleagues and relatives who were willing to participate and who had not been exposed to sources of ionising radiation for a minimum of 3 months before the study. Control subjects with previously diagnosed or treated primary lipid metabolism disorders and those with type 2 diabetes mellitus, nephrotic syndrome, renal failure, chronic liver disease or obesity were not included in the study. All control subjects were evaluated for thyroid function and thyroid antibodies. Their mean TSH concentration was 1.46 ± 0.72 mIU/L (range 0.4 – 3.5 mIU/L), and they tested negatively for thyroid antibodies.

Blood samples from control subjects were taken only once, whereas samples from DTC patients were collected before 131-I therapy, as well as 3 days and 7 days after 131-I therapy. Blood (5 mL) was taken by venepuncture, and the serum was separated out by centrifugation at 2000 rpm for 15 minutes. The sera were stored frozen at -20°C and were then thawed and assayed together.

Patient no.	Age (y)	Sex (F/M)	Stage (TNM)	Histology (P/F)	fT4 (pg/ml)	TSH (mIU/l)	Dose (GBq)
1	44	M	pT2N0M0	P	1.4	282	3.7
2	77	F	pT2N0M0	P	2.7	130	3.7
3	65	F	pT1N0M0	P	1.8	31	3.7
4	43	F	pT1N0M0	P	0.3	57.7	3.7
5	49	M	pT2N0M0	P	0.4	168	3.7
6	72	F	pT2N0M0	P	1.4	155	3.7
7	46	F	pT1N1M0	P/F	0.8	65.7	3.7
8	45	F	pT1N0M0	P	0.6	359	3.7
9	64	F	pT1N0M0	P/F	1.0	262	3.7
10	21	F	pT1N0M0	P/F	1.5	36.8	3.7
11	39	F	pT1N0M0	P/F	1.9	145.1	3.7
12	59	F	pT1N0M0	P	2.6	151	3.7
13	78	F	pT1N0M0	P	2.2	68.6	3.7
14	48	F	pT1N0M0	P	1.9	148	3.7
15	64	M	pT3N1M1	F	1.5	175	5.5
16	78	F	pT3N1M0	P	2.2	39.9	5.5
17	70	F	pT2N0M0	P	0.9	37.2	5.5
18	39	F	pT1N1M0	P/F	0.8	62	5.5
19	41	M	pT1N1M0	P	4.7	71	5.5
20	67	M	pT1N0M0	P	3.9	32.9	5.5
21	44	F	pT1N1M0	P	0.5	170	5.5
22	65	F	pT2N1M0	P	0.9	125.6	5.5
23	57	M	pT3N1M1	P	2.9	306	5.5
24	41	M	pT1N1M0	P	0.5	364	5.5

Table 1. Clinical and pathological characteristics of the DTC patients treated with 131-I.

Measurement of free thyroxine (fT4) and thyroid stimulating hormone (TSH)

The free thyroxine (fT4) concentration was measured by radioimmunoassay (RIA, OCFD03-FT4, *Cis-Biointernational*, France), with a reference range of 7-18 pg/mL. The thyroid stimulating hormone (TSH) concentration was determined immunoradiometrically (IRMA TSH, INEP, Zemun, Serbia), with a reference range of 0.3-5.5 mIU/L. All measurements were made on a Wallac Wizard 1470 Automatic gamma counter (PerkinElmer Life Sciences, Wallac Oy, 2005, Finland).

Determination of biochemical parameters

Serum concentrations of total proteins, albumin, cholesterol and triglycerides were measured using commercially available enzymatic reagents (Makler d.o.o, Belgrade, Serbia) adapted to an autoanalyser (Olympus AU 400). The normal ranges are as follows: proteins: 64 - 83 g/L; albumin: 35 - 52 g/L; cholesterol: 3.10 – 5.20 mmol/L; and triglycerides: 0.10 – 1.70 mmol/L.

Statistical analysis

All values are expressed as the mean ± standard deviation (SD). The commercial software SPSS version 10.0 for Windows was used for the statistical analysis. The significance of the differences in the determined parameters between control subjects and DTC patients before therapy was analysed by the independent samples t-test or U-test (depending on the distribution), whereas differences within the group of DTC patients were evaluated by applying the paired samples t-test or Wilcoxon test in cases of non-normal distribution. Probability values less than 0.05 were considered to be statistically significant, and those less than 0.01 were considered to be highly significant.

RESULTS

The study population comprised 24 DTC patients and 20 control subjects. The clinical and pathological characteristics of the DTC patients treated with 3.7 or 5.5 GBq of 131-I are given in Table 1. The TSH concentration ranged from 31 to 364 mIU/L, with a mean value of 132.9 ± 99.15 mIU/L, whereas the serum fT4 concentration ranged from 0.30 to 4.70 pg/mL, with a mean value of 1.55 ± 1.1 pg/mL.

The circulating protein and lipid concentrations of DTC patients and healthy controls are shown in Table 2.

Table 2: Concentrations of proteins and lipids in healthy controls and DTC patients before (0 day), three days (3 day) and seven days (7 day) after 131-I therapy

parameter	healthy controls	DTC patients		
		0 day	3 day	7 day
proteins (g/l)	81.68 ± 4.71	87.16 ± 6.04*	78.71 ± 6.71 **	82.54 ± 4.54***
albumins (g/l)	55.36 ± 5.25	57.42 ± 5.23	52.54 ± 5.23 **	53.83 ± 3.63 ***
globulins (g/l)	26.32 ± 5.16	29.71 ± 5.48	26.16 ± 6.37 **	28.79 ± 4.23
cholesterol (mmol/l)	6.11 ± 1.56	8.84 ± 2.09*	8.12 ± 2.13 **	8.41 ± 2.13***
triglycerides (mmol/l)	1.30 ± 0.62	2.26 ± 1.08*	2.44 ± 1.07**	2.14 ± 1.11

*Significant difference between DTC patients before therapy and control group
** Significant difference between DTC patients 3 days after therapy and before therapy
*** Significant difference between DTC patients 7 days after therapy and before therapy

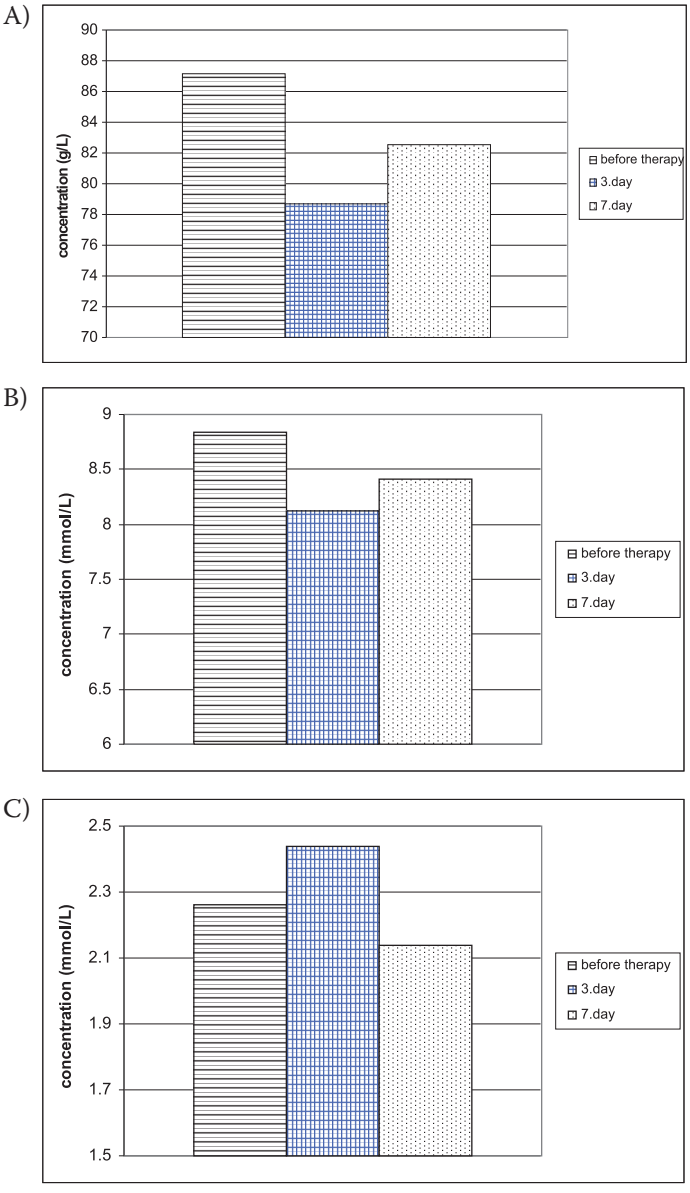


Figure 1. Changes in the concentrations of proteins (A), cholesterol (B) and triglycerides (C) in DTC patients after 131-I therapy.
* Significant difference between DTC patients 3 days after therapy and before therapy.
** Significant difference between DTC patients 7 days after therapy and before therapy.

There were statistically significant differences in the mean total protein concentrations (87.16 ± 6.04 g/L vs. 81.68 ± 4.71 g/L; independent samples t-test, t(42) = 3.453, p = 0.001), cholesterol concentrations (8.84 ± 2.09 mmol/L vs. 6.11 ± 1.56 mmol/L; independent samples t-test, t(42) = 4.642, p<0.001) and triglyceride concentrations (2.26 ± 1.08 mmol/L vs. 1.30 ± 0.62 mmol/L; U test, U = 108.0, z = -3.112; p = 0.002) between the DTC patients before therapy and the control subjects.

After 131-I therapy, the mean total protein concentration decreased from 87.16 ± 6.04 g/L to 78.71 ± 6.71 g/L (paired samples t-test, t(23) = 6.991; p = 0.003) at 3 days; this persisted up to 7 days after therapy (82.54 ± 4.54 g/L vs. 87.16 ± 6.04 g/L; paired samples t-test, t(23) = 4.610; p = 0.002). Similar decreases in the cholesterol concentrations were noted in DTC patients 3 days after therapy (8.12 ± 2.13 mmol/L vs. 8.84 ± 2.09 mmol/L; paired samples t-test; t(23) = 3.865; p = 0.001), followed by a gradual return towards the initial values before therapy. However, the difference between the concentrations 7 days after therapy and those before therapy (8.41 ± 2.13 mmol/L vs. 8.84 ± 2.09 mmol/L; paired samples t-test, t(23) = 2.285; p = 0.032) remained statistically significant. At the same time, the triglyceride concentrations rose after therapy from the initial value of 2.26 ± 1.08 mmol/L to 2.44 ± 1.07 mmol/L at 3 days (Wilcoxon test, z = -2.043, p = 0.041), followed by a return to the pretreatment levels 7 days after 131-I therapy (2.14 ± 1.11 mmol/L vs. 2.26 ± 1.08 mmol/L; Wilcoxon test, z = -1.214, p = 0.225). The serum concentrations of proteins (A), cholesterol (B) and triglycerides (C) in DTC patients before and after 131-I therapy are shown in Figure 1.

Because aging is accompanied by a decline in metabolism, we divided our DTC patients into two groups: the first group comprised patients under the age of 50, and the second comprised patients over the age of 50. There were no significant differences in total protein (87.58 ± 5.36 g/L vs. 86.75 ± 6.86 g/L; independent t-test, t(22) = 0.331, p = 0.744), cholesterol (8.97 ± 0.65 mmol/L vs. 8.72 ± 1.99 mmol/L; independent t-test, t(22) = 0.291, p = 0.774) and triglyceride (2.41 ± 1.29 mmol/L vs. 2.12 ± 0.85 mmol/L; U test, U = 64.0, z = -0.462; p = 0.671) concentrations between the two groups of DTC patients before therapy or

in the reduction rates of these parameters 3 and 7 days after treatment. An indirect correlation between the concentrations of total proteins and triglycerides was noted in patients over the age of 50 at 3 days after 131-I therapy (bivariate correlation test, Spearman's r = -0.583, p = 0.048) (Figure 2), which was not the case in patients under the age of 50 (Pearson's r = - 0.277, p = 0.384). In addition, there was a statistically significant correlation between the rate of cholesterol decline at both 3 and 7 days after therapy and TSH s in older patients (Bivariate correlation test, Pearson's r1 = 0.805, p1 = 0.002; r1 = 0.750, p1 = 0.005). No correlation was observed between the total decrease in protein and TSH in either group of DTC patients.

Because the trapping of 131-I by metastatic tissue is expected, the patients were divided into groups without (13 patients) and with (11 patients) metastases. Statistical analysis indicated no significant differences in the protein concentrations between patients without and with metastases before 131-I therapy (86.12 ± 6.09 g/L vs. 89.25 ± 5.72 g/L, p = 0.240), 3 days after 131-I therapy (78.00 ± 7.62 g/L vs. 80.12 ± 4.45 g/L, p = 0.477) and 7 days after 131-I therapy (83.43 ± 4.84 g/L vs. 80.75 ± 3.45 g/L, p = 0.177) (Table 3). Additionally, no significant differences were found in the triglyceride concentrations between the two groups before 131-I therapy (2.26 ± 1.23 mmol/L vs. 2.25 ± 0.96 mmol/L, p = 0.969), 3 days after 131-I therapy (2.60 ± 1.19 mmol/L vs. 2.13 ± 0.76 mmol/L, p = 0.327) and 7 days after 131-I therapy (2.46 ± 1.15 mmol/L vs. 2.30 ± 1.09 mmol/, p = 0.751). However, looking at the reduction in protein concentrations after 131-I therapy (before – after therapy), a highly significant difference was observed between DTC patients without (2.68 ± 3.32 g/L) and with (8.5 ± 5.47 g/L) metastases (independent samples t-test, t(22) = -3.249, p = 0.004) 7 days after therapy. This indicates a prolonged effect of 131-I in patients with metastases.

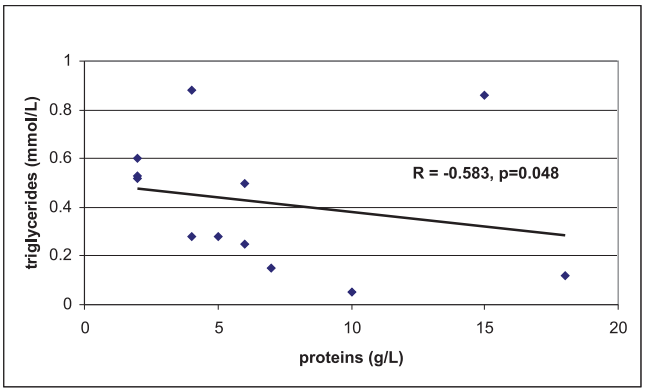


Figure 2. Correlation between the concentrations of proteins and triglycerides 3 days after 131-I therapy in patients over the age of 50.

DISCUSSION

In this study, we analysed the effects of short-term, overt hypothyroidism on the protein and lipid concentrations in DTC patients before and within a week after 131-I therapy. We observed the well-known effect of decreased thyroid function on cholesterol metabolism, but our main finding was the combined decline in serum protein and cholesterol concentrations 3 days after 131-I therapy, which was accompanied by increased serum triglyceride levels. There was an indirect correlation between the concentrations of proteins and triglycerides in patients over the age of 50.

Iodine-131 is used for the ablation of remnant thyroid tissue or for the treatment of iodine-avid metastasis (11). For the optimal accumulation of 131-I in differentiated thyroid tissue, an elevated TSH concentration is required (12). In clinical practice, high TSH levels can be achieved by exogenous TSH administration or by endogenous TSH stimulation (13). To increase the accumulation of 131-I, short-term hypothyroidism was induced in our DTC patients. At the time of 131-I administration, all patients had very high TSH concentrations with significantly elevated levels of cholesterol and triglycerides compared with the control group of healthy subjects. Our results are in agreement with those of previously published studies, in which the associations between the thyroid status and serum lipid concentrations were analysed (14, 15, 16). They are also consistent with the findings of Regalbuto and co-workers (17), who reported an increase in cholesterol levels in DTC patients before therapy. This is not surprising if one takes into account that thyroid hormones affect the synthesis, mobilisation and degradation of lipids (18). It is assumed that the primary mechanism for hypercholesterolemia is the accumulation of LDL cholesterol due to a reduction in the number of its cell surface receptors (19), whereas decreased lipoprotein lipase activity might be responsible for the elevated triglyceride levels (20).

Interestingly, three days after 131-I administration, decreased total serum protein and albumin concentrations and decreased serum cholesterol concentrations were simultaneously recorded in our DTC patients. We assume that this decrease in protein concentrations could be explained by either oxidative stress or decreased liver function. It has been shown that protein oxidation (21) and oxidative damage (22) might be responsible for decreased protein levels in cancer patients. In our DTC patients, the levels of MDA were increased 3 days after 131-I therapy (data not shown). Because liver tests were not performed, we cannot exclude the possibility that radiation damage to the liver led to the decreased serum protein concentrations. Namely, diffuse hepatic uptake of 131-I was shown on post-therapeutic scans in DTC patients (23), and it is well known that the liver synthesises most plasma proteins. The highly significant difference in the reduction of protein concentrations between DTC patients with and without metastases 7 days after the therapy might indicate a prolonged effect of 131-I in patients with metastases.

Unlike cholesterol, triglyceride levels were significantly increased after 131-I therapy, likely as an attempt to compensate for the decline in protein concentrations to preserve the colloidal osmotic pressure. A direct relationship

between hypoalbuminemia and hyperlipidaemia has been reported, but in these studies, more profound decreases in albumin concentration were caused by nephritic syndrome (24, 25, 26), peritoneal dialysis (27) or hepatic dysfunction (28). Because the decline in protein concentration found in our 131-I-treated DTC patients was not as great as in the other specified conditions, the changes in lipid concentrations were also less pronounced.

It is well known that the aging process causes a number of functional and metabolic changes, which are reflected by increased concentrations of serum lipids (29). Slightly impaired thyroid gland function might contribute to the changes in lipid metabolism (30, 31). Therefore, we divided our patients into two groups according to age: one group of patients under the age of 50 and the other over the age of 50. However, there were no significant differences in the serum levels of proteins, cholesterol or triglycerides between these two groups of DTC patients before 131-I therapy. Moreover, hypothyroidism produced similar increases of cholesterol and triglycerides in both groups. Nevertheless, in patients over the age of 50, we found an indirect correlation between the rate of total protein decline and the increase of triglycerides 3 days after therapy.

Despite the limitations of this study (small sample size, the possible existence of clinical conditions not diagnosed or previously treated that might affect lipid metabolism), it is the first study to demonstrate decreased protein and cholesterol concentrations accompanied by increased serum triglyceride levels in DTC patients after radioactive 131-I therapy.

In conclusion, radioiodine therapy in DTC patients leads to decreased serum protein and cholesterol concentrations and increased triglyceride concentrations, which are especially evident in older subjects with metastases.

ACKNOWLEDGEMENTS

The study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant nos. III41010 and ON175069) and by the Faculty of Medical Sciences, University of Kragujevac (Junior Project N° 06/2012).

REFERENCES

1. Kebebew E, Clark OH. Differentiated thyroid cancer: "complete" rational approach. World J Surg 2000; 24: 942–51.
2. Caron NR, Clark OH. Well differentiated thyroid cancer. Scand J Surg 2004; 93: 261–71.
3. Cooper DS, Doherty GM, Haugen BR, et al. Revised American Thyroid Association management guidelines for patients with thyroid nodules and differentiated thyroid cancer. Thyroid 2009; 19: 1167–214.
4. Luster M, Lippi F, Jarzab B, et al. rhTSH-aided radioiodine ablation and treatment of differentiated thyroid carcinoma: a comprehensive review. Endocr Rel Canc 2005; 12: 49-64.
5. Duntas LH, Biondi B. Short-term hypothyroidism after Levothyroxine-withdrawal in patients with differentiated thyroid cancer: clinical and quality of life consequences. Eur J Endocrinol 2007; 156: 13–19.
6. Wu H, Zuo S, Ma C, et al. Short-term overt hypothyroidism affect on lipids after thyroxine-withdrawal in patients with differentiated thyroid carcinoma. Chinese-German J Clin Oncol 2009; 8: 647-9.
7. Gullu S, Sav H, Kamel N. Effects of levothyroxine treatment on biochemical and hemostasis parameters in patients with hypothyroidism. Eur J Endocrinol 2005; 152: 355–61.
8. Prakash A, Lal AK. Serum lipids in hypothyroidism: our experience. Ind J Clin Biochem 2006; 21: 153-5.
9. Sunanda V, Sangeeta S, Prabhakar Rao B. Study of lipid profile in hypothyroidism. Int J Biol Med Res 2012; 3: 1373-6.
10. Luster M, Clarke SE, Dietlein M, et al. Guidelines for radioiodine therapy of differentiated thyroid cancer. Eur J Nucl Med Mol Imaging 2008; 35: 1941-59.
11. Ravishankar U, Pande S, Savita N. I-131 in the management of differentiated thyroid cancer – an update on current recommendations and practices. Apollo Med 2009; 6: 347-54.
12. Woodrum DT, Gauger PG. Role of 131I in the treatment of well differentiated thyroid cancer. J Surg Oncol 2005; 89: 114–21.
13. Vrachimis A, Schober O, Riemann B. Radioiodine remnant ablation in differentiated thyroid cancer after combined endogenous and exogenous TSH stimulation. Nuklearmedizin 2012; 51: 67-72.
14. Canaris GJ, Manowitz NR, Mayor G, Ridqway EC. The Colorado thyroid disease prevalence study. Arch Intern Med 2000; 160: 526–34.
15. Duntas LH. Thyroid disease and lipids. Thyroid 2002; 12: 287–93.
16. Pearce EN. Hypothyroidism and dyslipidemia: modern concepts and approaches. Curr Cardiol Rep 2004; 6: 451–6.
17. Regalbuto C, Alagona C, Maiorana R, et al. Acute changes in clinical parameters and thyroid function peripheral markers following L-T4 withdrawal in patients totally thyroidectomized for thyroid cancer. J Endocrinol Invest 2006; 29: 32-40.
18. Pucci E, Chiovalto L, Pinchera A. Thyroid and lipid metabolism. Int'l J Obesity 2004; 24: 109-12.
19. Saini V, Yadav A, Arora S, Singh R, Bhattacharjee J. Association between different degrees of hypothyroidism and serum lipids. Internet J Med Update 2012; 7: 3-8.
20. Regmi A, Shah B, Rai BR, Pandeya A. Serum lipid profile in patients with thyroid disorders in central Nepal. Nepal Med Coll J 2010; 12: 253-6.
21. Yilmaz IA, Akçay T, Cakatay U, Telci A, Ataus S, Yalçin VR. Relation between bladder and protein oxidation. Int Urol Nephrol 2003; 35: 345–50.

22. Chandran V, Anitha M, Avinash SS, Rao GM, Shetty BV, Sudha K. Protein oxidation: A potential cause of hypoalbuminemia in oral cancer. Biomed Res 2012; 23: 227-30.
23. Omur O, Akgun A, Ozcan Z, Sen C, and OzkiIic H. Clinical implications of diffuse hepatic uptake observed in postablative and post-therapeutic I-131 scans. Clin Nucl Med 2009;34: 11–14.
24. Kaysen GA, Jones H Jr, Joles JA, van Tol A. Effect of plasma oncotic pressure on apolipoprotein levels in rats with Heymann nephritis. Miner Electrolyte Metab. 1996;22:31-8.
25. O'Donnell MP. Mechanisms and clinical importance of hypertriglyceridemia in the nephrotic syndrome. Kidney Int 2001; 59: 380-2.
26. Obrenovic R, Petrovic D, Glišic B, Majkić-Singh N, Trbojevic J, Stojimirović B. Influence of proteinuria on disorders of lipoprotein metabolism. Jugoslov Med Biochem 2005, 24: 259-64.
27. Matsuda I, Maeda T, Takase A, Arashima S. Hyperlipidaemia during persistent peritoneal dialysis. Arch. Dis. Child 1972; 47: 139-40.
28. World Health Organization. Severe falciparum malaria. Trans R Soc Trop Med Hyg 2000; 94: 1 - 90.
29. Gälman C, Matasconi M, Persson L, Parini P, Angelin B, Rudling M. Age-induced hypercholesterolemia in the rat relates to reduced elimination but not increased intestinal absorption of cholesterol. Am J Physiol Endocrinol Metab 2007; 293: E737–E742.
30. Gesing A, Lewiński A, Karbownik-Lewińska M. The thyroid gland and the process of aging; what is new? Thyroid Res 2012; 5: 16.
31. Bremner AP, Feddema P, Leedman PJ, et al. Age-related changes in thyroid function: a longitudinal study of a community-based cohort. J Clin Endocrinol Metab 2012; 97: 1554–62.

CORRELATION BETWEEN CERVICAL CYTOLOGY AND HISTOPATHOLOGICAL CERVICAL BIOPSY FINDINGS ACCORDING TO THE BETHESDA SYSTEM

Gordana M Sosic[1], Goran Babic[1], Aleksandra Dimitrijevic[1], Slobodanka Mitrovic[2], Mirjana Varjacic[1]
[1] Department of Gynecology and Obstetrics, Clinical Centre Kragujevac, Kragujevac, Serbia
[2] Department of Anatomical Pathology

STEPEN KORELACIJE CERVIKALNE CITOLOGIJE PO BETHESDA KLASIFIKACIJI SA PATOHISTOLOŠKIM NALAZIMA CERVIKALNE BIOPSIJE

Gordana M Šošić[1], Goran Babić[1], Aleksandra Dimitrijević[1], Slobodanka Mitrović[2], Mirjana Varjačić[1]
[1]Klinika za ginekologiju i akšerstvo
[2]Služba za patološku anatomsku dijagnostiku

Received / Primljen: 18.06.2014. Accepted / Prihvaćen: 15.09.2014.

ABSTRACT

The Pap test (Pap smear) is a morphological cytodiagnostic test that reveals premorbid and early stages of cervical cancer through the use of cervicovaginal cytology.

The present study was conducted at the Department of Gynecology and Obstetrics, Clinical Center Kragujevac, from January 01, 2013, to December 1, 2013, with patients who were part of the national screening program and who used a secondary cytological examination (Pap test) performed using a conventional method.

The patients were grouped according to the results of a Pap smear and histopathological findings. The classification of cytological smears was performed in accordance with the Bethesda system. The hypotheses established in this study were statistically tested.

The greatest number of cytological findings was NILM. However, the most frequent abnormal cytological findings in terms of percentage were ASC-US. After secondary cervical findings of 8.1% of the total number of women, biopsies were performed. In 68.57% of the biopsies performed, CIN was present in all of them, with the most frequent ones being LSIL (50.6%), HSIL (10.4%) and CA Invasiva (0.5%). This study, using the X^2 test, confirmed that cervical cytology and biopsy results are dependent features (sig. = 0.036), between which there is a medium association (Cramer's V = 0.176). In the ASC-US cytological findings, small percentages of CIN1 and CIN2 were detected. Cervical cytology in this study presented high sensitivity, specificity, positive and negative predictive value. As a relatively inexpensive, painless and easily approachable method, cervical cytology fully substantiates its implementation in diagnostic procedures as well as in organized screening programs.

Keywords: *Cervical cancer, Pap test, Bethesda system*

SAŽETAK

Papa test je morfološki citodijagnostički test kojim se pomoću analize cervikovagnalne citologije otkrivaju predstadijumi i rani stadijumi karcinoma grlića materice.

Studija je sprovedena na Klinici za ginekologiju i akušerstvo, KC Kragujevac u periodu od 01. 01. 2013. god. do 01. 12. 2013. god. na pacijentkinjama kojima je u okviru Nacionalnog skrining programa odrađen sekundarni citološki pregled (PAP test) konvencionalnom metodom.

Pacijentkinje su grupisane prema rezultatima PAP testa i patohistološkim rezultatima. Klasifikacija citoloških briseva je vršena prema Bethesda klasifikaciji. Statističkim testovima su testirane hipoteze postavljene u ovoj studiji.

Najveći broj citoloških nalaza bio je NILM. Procentualno najzastupljeniji abnormalni citološki nalazi su bili ASCUS. Nakon sekundarnog cervikalnog nalaza kod 8,1% od ukupnog broja žena je odrađena biopsija. U 68,57% urađenih biopsija je bio prisutan CIN od kojih je najzastupljeniji bio nalaz LSIL (50,6%), zatim HSIL (10,4%), i CA INVASIVA (0,5%). Ovom studijom, uz primenu Xi^2 testa, potvrđeno je da su cervikalna citologija i rezultati biopsije zavisna obeležja (Sig.=0,036), između kojih postoji veza srednje jačine (Cramer's V=0,176). U citološkom nalazu ASCUS detektovano je mali procenat CIN1 i CIN2. Cervikalna citologija u ovoj studiji pokazuje visoku senzitivnost, specifičnost, pozitivnu i negativnu prediktivnu vrednost. Kao relativno jeftina, bezbolna i lako dostupna metoda u potpunosti potvrđuje svoju primenu, kako u dijagnostičkim procedurama, tako i u organizovanim skrining programima.

Ključne reči: *karcinom grlića materice, PAP test, Bethesda klasifikacija*

DE GRUYTER OPEN

UDK: 618.146-006.6-076.5 / Ser J Exp Clin Res 2014; 15 (4): 205-216
DOI: 10.2478/SJECR-2014-0026

Correspondence: Gordana Sosic, gordana.sosic.2011.02@gmail.com,
Clinical Center "Kragujevac", Kragujevac, Department of Cytogenetic Diagnostic, Department of Gynecology and Obstetrics

INTRODUCTION

Gynaecological cytodiagnostics are relatively quick, inexpensive and minimally invasive methods that can detect precancerous and cancerous conditions of the vagina, vulva and uterus, which promote adequate and timely treatment (1).

According to the Cancer Registry of Central Serbia, in 2011, 882 new cases of cervical cancer occurred within the territories of all districts, which placed it fourth in incidence behind newly discovered cases of breast, colon and rectal, lung and bronchial cancer; of those identified, 879, or 99.7%, were verified using cytological and histological methods (2).

In the Sumadia region, 66 new cases of cervical cancer were identified in 2011, second in incidence to breast cancer.

The 2011 cancer incidence rate per 100 000 individuals in the population of all districts in Central Serbia was 32.3 for primarily localized cervical cancer (with a standardized incidence rate of 22.1).

The total number of deaths from cervical cancer in central Serbia in 2011 numbered 347; in the Šumadia region, 15 women died of this type of cancer in 2011, which is the eighth highest compared to other districts (2).

Cervical intraepithelial neoplasms (CINs) are gross abnormalities of the squamous epithelium comprising two groups of lesions:

- LSILs (low-histological-grade squamous intraepithelial lesions), which include flat warts and CIN1; and
- HSIL (high-histological-grade squamous intraepithelial lesions), which involve CIN2 and CIN3.

Human papillomavirus (HPV) plays a significant role in the development of cervical intraepithelial neoplasia. Clinic data show that the HPV DNA sequence can be found in more than 80% of squamous intraepithelial neoplasms and more than 98% of cervical cancers. Less than 2% of cervical carcinomas are negative for HPV DNA, which can be explained by undetected types of virus in the HPV gene or HPV having been lost in the process of oncogenesis (3).

Persistent HPV infection is a precursor for the development of cervical cancer. HPV types with high oncogenic potential (HPV16, HPV18) characteristically exhibit the tendency to provide persistent infection in comparison to low-oncogenic types (4). The emergence of the pathological process begins under the influence of so-called cocarcinogens: low immunity, smoking, genital infections caused by the influence of other viruses (e.g., HSV type 2, HIV, CMV, *Chlamydia trachomatis*), the effect of drugs (e.g., cytostatics, immunosuppressants), etc. A cocarcinogen causes circular viral DNA interruption, which is then integrated into the host cell's genome, and thus begins mutagenesis (3).

The Pap test is a cervicovaginal cytological smear stained using the Papanicolaou method, which combines in itself an exfoliative cell cytology obtained from the ectocervix and abrasive cell cytology obtained using an endocervical brush from the endocervical canal. The Papanicolaou method and classification in clinical practice was introduced by Dr. Georgios Papanicolaou (1954) (1,5). This morphological test uses a cervicovaginal cytology analysis to reveal pre-stages and early stages of cervical cancer; the Pap smear is actually carried out in order to prevent these malignant disease in women and can be used as a screening test in a population of women who have no symptoms of cervical disease or as a diagnostic test for patients with signs of gynaecological diseases resulting from a positive pre-established Pap test or diagnosed precancerous lesions or cancers of the cervix, vagina, vulva or uterus (6).

The first classification of Pap smears was Papanicolaou numerical cell cytology classification system (1954) that divided cervical (smears) swabs into five groups and had significant drawbacks (7,8,9).

A group of experts in cytopathology and histopathology and clinicians held a meeting in Bethesda (Maryland, USA) in 1988 with the aim of redefining the former classification of cytological findings to achieve uniformity and clearer and more relevant communication between cytopathologists and clinicians, which resulted in the establishment of the new Bethesda system. The original Bethesda system from 1988 was revised twice: first in 1991 and then in 2001 (10).

The interpretation of cervical cytology smears according to Bethesda system (in 1988, 1991, 2001) led to the transition from numerical Papanicolaou classification (1954) to descriptive clarification of the Pap test (Table1).

Table 1 Comparison of cytological classification systems (11).

Papanicolaou System	***Bethesda System***
Inadequate sample	Unsatisfactory result/ inadequate sample
PA I Normal result	Negative for intraepithelial lesion or malignancy, NILM
PA II Present inflammation, benign reactive and reparative changes	Present inflammation, benign reactive and reparative changes Negative for intraepithelial lesion or malignancy (no observed abnormality), NILM
IIIa Atypical cells of undetermined significance •squamous • glandular	ASC-US (in favour of reactive changes)) ASC-H (in favour dysplasia) AGC (atypical glandular cells)
IIIb Dyskariosis of a light degree Dyskaryosis of a medium degree	L-SIL (CIN 1) H-SIL (CIN 2)
IV Dyskariosis of a severe degree	H-SIL (CIN 3) AIS
V malignant cells	invasive carcinoma

The 2001 Bethesda classification system includes the following:

1. First, the adequacy of smear is assessed, which determines whether the smear is satisfactory or unsatisfactory.
2. Satisfactory smears are then classified into negative and pathological categories and interpreted.

A negative swab (negative for intraepithelial lesion or malignancy—NILM) may be a normal smear or swab with the presence of non-neoplastic changes.

Pathological smears involve pathological changes in the plate and/or the glandular epithelia or the presence of other cells (of uncertain significance and other types of cancer cells).

Pathological changes in the platelet-layered epithelium include the following:

1. Atypical squamous cells (atypical squamous cells of undetermined significance—ASC-US—and atypical squamous cells in which HSIL cannot be excluded—ASC-H);
2. Squamous intraepithelial lesions (LSIL and HSIL); and
3. Squamous epithelium carcinoma (PCA).

The pathological changes in the glandular epithelium include the following:

1. Atypical glandular cells not otherwise specified (AGC-NOS);
2. Atypical glandular cells, probably neoplastic (AGC-FN);
3. Endocervical adenocarcinoma in situ (AIS); and
4. Endocervical adenocarcinoma (CA-A) (6, 9, 10, 11, 12).

In this study, we tested the sensitivity, specificity, positive predictive value, negative predictive value, and overall accuracy of cervical cytology based on histological cervical biopsy as the "gold standard" for diagnosis. Based on the data obtained in this study, descriptive and analytical statistics were performed.

METHOD AND PATIENTS

The study was conducted at the Department of Gynecology and Obstetrics, Clinical Center Kragujevac, for a period of eleven months (from January 0 1, 2013, to December 01, 2013) with patients who responded to the national screening program for cervical cancer prevention at the invitation of selected gynaecologist who performed a gynaecological examination and sampled for a Pap test in the health centre in Kragujevac.

Cervical smears were taken by a conventional method according to an expert examination procedure.

Staining was performed according to the Papanicolaou method, in which the standardized manufactured tinctures were used. According to this dyeing method, three colours—the Harris hematoxylin (PA 1 colour) colour engine, which allows the visibility of chromatin, methyl orange (PA colour 2) and polichrome (PA colour 3), which differently colour the cytoplasm of cells, thereby enabling the differentiation of mature epithelial tissues . A composition of 96% alcohol was used for rinsing; xylene was used at the end of the dyeing process.

Coloured and air-dried preparations were placed on Canada balsam and covered glass measuring 24 mm x 50 mm (at least 24 mm x 40 mm) so that the whole smear was covered.

Microscopic analysis of the cervical Pap smear method was performed on a professional binocular microscope with magnifications of 10x, 20x and 100x.

For each cervical smear, a cervical cytological finding was issued and was evaluated with respect to the following factors :

1. Adequacy of the sample;
2. Whether the smear was negative or pathological;
3. Stage of the morphological changes;
4. Vaginal flora; and
5. The gynaecologist's recommendations on further treatment (12).

Satisfactory smears were classified simultaneously according to the Papanicolaou classification and the Bethesda system or the Bethesda system alone.

Within the screening program, a two-way analysis was carried out, wherein all plates were primarily analyzed by cytoscreeners performing a screening of suspected secondary swabs and were then evaluated by a certified clinical cervical cytology supervisor. To ensure the quality of the second cycle, 10% of normal findings, as well as all of the unsatisfactory ones, were also analyzed by the clinical supervisor. The cytological findings were published in the recommended form, with recommendations to the gynaecologist on further action.

After obtaining the cytological findings, the gynaecologists referred women with abnormal cervical cytological findings to colposcopy and/or further diagnostic procedures according to the professional and methodological recommendations provided in the Guide for the treatment and diagnosis of cervical cancer (11).

Cervical biopsies were taken under colposcopic control (i.e., targeted biopsies), with fields that showed the greatest degree of abnormality. Curettage of the cervical canal (endocervical curettage—ECC) was necessary in order to diagnose endocervical lesions that were not observed through colposcopy in cases of squamocolumnar junction (SCJ) that could be visualized and for which cytological findings were abnormal.

The data obtained in this study were statistically analyzed and compared with the help of the program PASW Statistics 18. For the statistical tests, we applied the Kolmogorov-Smirnov test, Kruskal-Wallis test and the chi-square test.

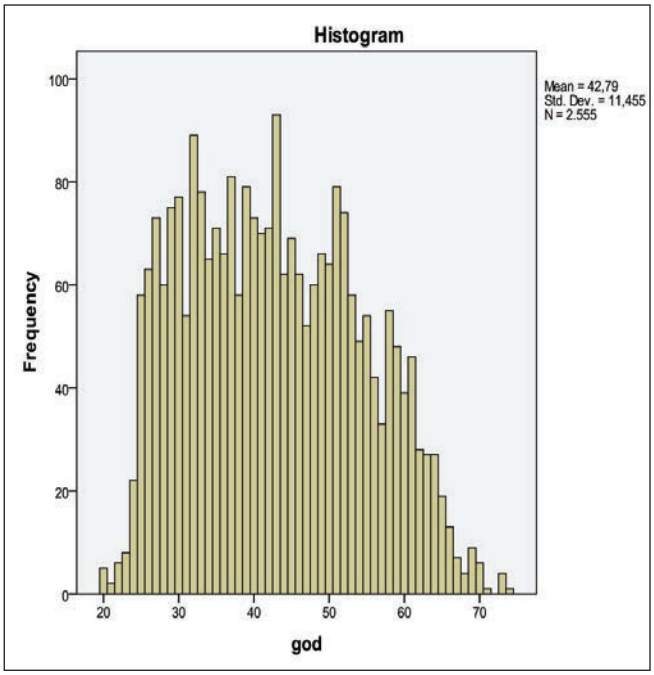


Figure 1. Distribution of the female patients who participated in the screening compared by age

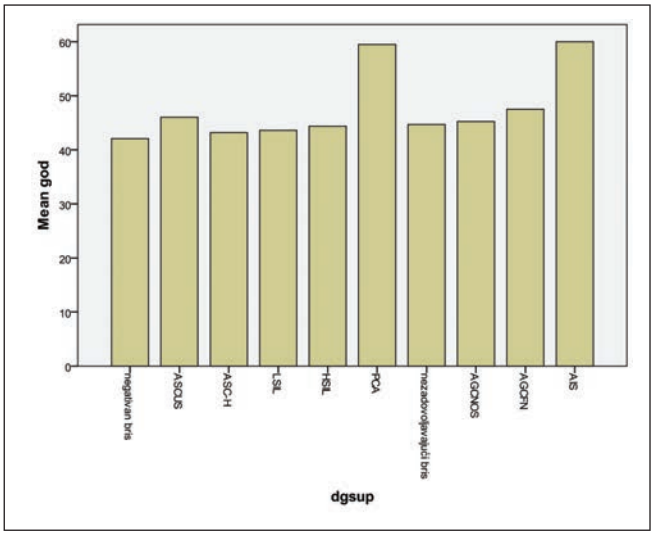


Figure 2. Mean values for the age groups under different cytological results.

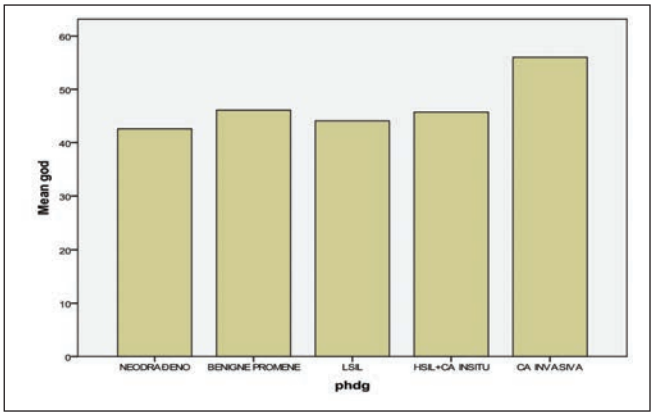


Figure 3. Mean values for the age group according to different biopsy results

For the purpose of testing our previously mentioned hypothesis, we used the following formulae.

Sensitivity = true positives / (true positives + false negatives) x 100

Specificity = true negatives / (true negatives + false positives) x 100

Positive predictive value = true positives / (true positives + false positives) x 100

Negative predictive value = true negatives / (true negatives + false negatives) x 100

Overall accuracy = (true positives + true negatives) / sample size.

RESULTS

The study included 2570 patients from the national screening program. The youngest patient was 20 years old and the oldest was 74 years old. The descriptive statistics for the age of the population in years were as follows : mean of 42.79, median of 42.00, standard deviation of 11, 455.

Cervical cytology findings were grouped into 10 categories (negative cytology—NILM, ASC-US, ASC-H, LSIL, HSIL, PCA, AGC-NOS, AGC-FN, AIS, and unsatisfactory samples).

Biopsies were grouped into 4 groups: negative (benign changes), LSIL, HSIL (HSIL + in situ CA) and invasive CA.

The Kolmogorov-Smirnov test showed that the values for age in this population did not follow a normal distribution (sig. 0.000). The observed histogram showed a slightly asymmetric distribution, shifted to the left (i.e., to younger ages). The largest group (93 of individuals) was that of 43-year-old women (Figure 1).

The largest number of swabs was negative (1930), with an average age of 42.09, and the positive cytological smears were mostly ASC-US (224), with a mean age of 46.02. The second-highest number of findings was LSIL (149), with a mean of 43.61 years. Two squamous cell carcinomas were detected (mean age of 59.50), and one adenocarcinoma in situ was identified (60 years old). In 15 cases, the data on age were missing.

For the Kruskal-Wallis test, we established the null hypothesis that there were no differences in the median age groups formed on the basis of the results of the cytological testing. Since the value of sig. 0,000 is less than 0.5, the null hypothesis was rejected, and significant differences were noted to exist between groups. From the graph, we can see that there is considerable variation with respect to mean age for the PCA and AIS groups (Figure 2).

The greatest number of biopsy findings fell into the LSIL category (106), with a mean value of 44.08 years. Invasive cancer was confirmed in a woman of 56 years (Figure 3).

Based on the Kruskal-Wallis test's (sig. 0.011 < 0.05), the null hypothesis was rejected, meaning that there was a statistically significant difference in median age between the groups with different histopathologies.

Of the total of 2570 analyzed cytological smears evaluated under the supervision of the National Screening Program, the largest group (1930; 75.1%) were negative cytological smears (Figure 4). Of all pathological smears, the most numerous smear finding was ASC-US (224; 8.7% of the total number of samples taken), followed by smears with LSIL findings (149; 5.8% of the total number of samples taken). ASC-H findings numbered 55 (2.1%), 58 (2.3%) findings were HSIL, PCA consisted of 2 cases (0.08%), AGC-NOS was reported for 39 (1.5%), AGC-FN was found in 8 (0.3%), and AIS was found in 1 (0.04%). Unsatisfactory findings were reported for 104 (4%) smears, and these women were once again invited to examination (Table 2).

The total number of detected abnormal cytological smears was 536. Abnormal smears were divided into eight groups of which the largest group was ASC-US, with 224 (41.8% of all abnormal smears), followed by LSIL, with 149 (27.8%),; the smallest number of findings were for AIS, with 1 (0.2%) (Table 2, Figure 5).

Of the total number of women (2570) who had secondary cytological examinations, 2359 (91.8%) did not undergo further diagnostic procedures, and in 211 (8.1%), biopsy and exploratory curettage of the cervical canal were performed. Of all the women who had histological diagnoses in comparison with the total number of women who underwent cytological smear analyses, 82 women (3.2%) had a negative biopsies, 106 (4.2%) had LSIL, 17 (0.9 %) had HSIL (CIN2: 17 (0.7%); CA in situ : (0.2%)), whereas the biopsies detected 1 (0.04%) invasive CA. Results show that after the successfully biopsies, the highest percentage of women had LSIL findings (Table 3).

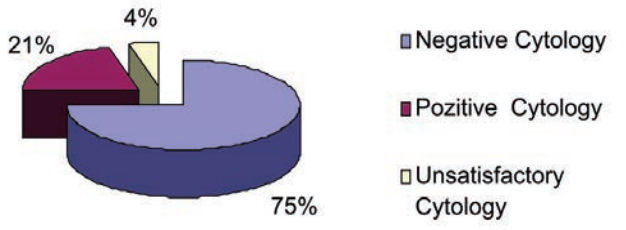


Figure 4. Percentage of negative, positive and unsatisfactory results in the total number of analyzed cytological smears

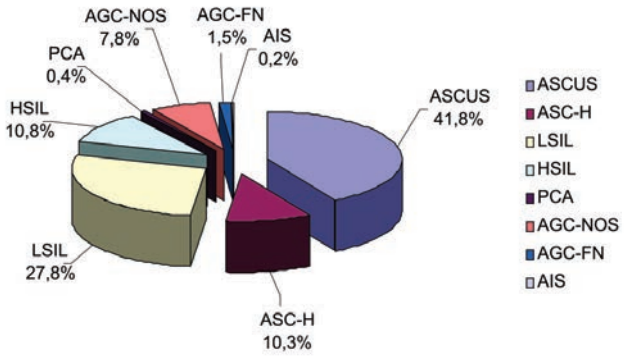


Figure 5. Percentage of the different categories of abnormal cytological smears

Of the total number of successfully completed biopsies, negative results were found for 82 (38.9%), whereas positive histologies were found in 129 (61.1%) (Figure 6). Among the performed biopsies, the largest percentage confirmed LSIL in 106 (50.2%), HSIL (HSIL + in situ CA) in 22 (10.4%) and invasive CA in 1 (0.5%). The histological diagnoses did not detect any of the glandular changes (Figure 7).

Among the positive histological findings, the most frequent finding was LSIL (106 cases; 82.2%) (Figure 8).

After the histopathological test was performed, cytology recorded 62 false-positive results (ASC-US 9, ASC-H 11, LSIL 23 and HSIL 11), and 16 false-negative results (12 LSIL and 4 HSIL that matched CIN2).

From the total of 104 repeated smears, based on dubious colposcopic findings, 9 biopsies were performed (8.56% of repeated smears), of which 6 were negative, and 3 were LSIL (2.89% of the repeated smears).

After colposcopy and/or HPV typing or repeated cytology, biopsy was performed in 12.5% of all cytologies yield-

Table 2 Frequency of cytologic results in the study population

cytological diagnosis of the supervisor	Frequency	Percent	Valid Percent	Cumulative Percent
NILM	1930	75,1	75,1	75,1
ASC-US	224	8,7	8,7	83,8
ASC-H	55	2,1	2,1	86,0
LSIL	149	5,8	5,8	91,8
HSIL	58	2,3	2,3	94,0
PCA	2	0,1	0,1	94,1
Unsatisfactory smear	104	4,0	4,0	98,1
AGC-NOS	39	1,5	1,5	99,6
AGC-FN	8	0,3	0,3	100,0
AIS	1	0,0	0,0	100,0
Total	2570	100,0	100,0	

Table 3 Percentage of biopsies in relation to the total number of women in the study

Histological diagnosis	Frequency	Percent	Valid Percent	Cumulative Percent
UNPROCESSED	2359	91,8	91,8	91,8
BENIGN CHANGES	82	3,2	3,2	95,0
LSIL	106	4,1	4,1	99,1
HSIL+CA INSITU	22	0,9	0,9	100,0
CA INVASIVE	1	0,0	0,0	100,0
Total	2570	100,0	100,0	

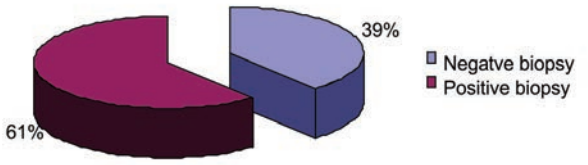


Figure 6. Proportion of the positive and negative biopsy results

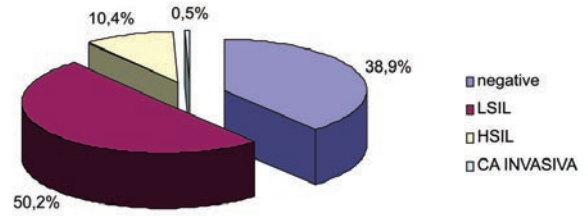


Figure 7. Proportion of histological results after certain biopsies

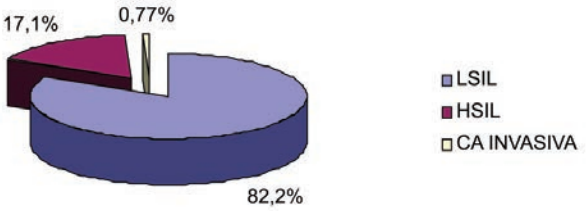


Figure 8. Percentage of categories of histological results in the total number of positive histological results

ing ASC-US findings. Of these, 32.1% of the biopsies had benign findings, and positive biopsies were found in 67.9% (60.7% being LSIL and 7.1% being HSIL). If it is expressed in the total number of cytological diagnoses, 7.6% of the ASC-US diagnoses had LSIL findings, 0.9% had findings of HSIL (of which none were in situ CA), and 8.5% had CIN.

In this study, of the 279 ASC findings, 55 were ASCH (19.71%). Of the total findings of ASCH, 29.1% were confirmed for LSIL and 5.5% were confirmed for HSIL (or from all confirmed CIN in the ASC-H category, 84.21% were LSIL and 15.79% were HSIL).

In our study, in patients with LSIL, cytological findings confirmed LSIL lesions in 41 (59.42%), and 5 (3.4%) were confirmed with HSIL findings. The percentage of benign HP findings in the category of LSIL was 33.33%.

Of all patients who participated in this study, 2.3% had HSIL cytology findings. Among the positive cytology findings, 10.8% were HSIL. By biopsy, 16 (45.7%) LSIL findings were confirmed, as were 7 (20%) HSIL and 1 (2.86%) invasive carcinoma.

In this study, of the six biopsies performed for AGC-NOS, one confirmed LSIL and AGC-FN in 3 cases showed benign characteristics, whereas the AIS cytology findings with clear cytological elements with respect to this cancer were confirmed as HSIL on biopsy (Table 4).

To test the sensitivity, specificity, positive predictive value, negative predictive value and overall accuracy, we did not take into account patients with unsatisfactory smears nor patients with positive cytology findings on whom we did not perform biopsies. We have divided the results by patients, healthy cytology, abnormal cytology and negative cytology. Within the group are the patients who are positive after the negative biopsy findings (16) and abnormal cytologies (110). Within the healthy group are those with negative biopsies and NILM cytologies (a priori groups without disease), within normal cytologies (1914), and those with negative biopsy findings but with abnormal cytologies (62). The total number of patients in this test was 2102 (Table 5, Table 6).

Table 4 Ratio of cytologic results and histopathological results based on different diagnostic categories

Cytological results	Pathohistological diagnosis					Total
	UNPROCESSED	BENIGN CHANGES	LSIL	HSIL+CA INSITU	CA INVASIVE	
NILM	1900	14	12	4	0	1930
ASC-US	196	9	17	2	0	224
ASC-H	25	11	16	3	0	55
LSIL	80	23	41	5	0	149
HSIL	23	11	16	7	1	58
PCA	2	0	0	0	0	2
Unsatisfactory smear	95	6	3	0	0	104
AGC-NOS	33	5	1	0	0	39
AGC-FN	5	3	0	0	0	8
AIS	0	0	0	1	0	1
Total	2359	82	106	22	1	2570

Table 5 The numeric ratio of negative/positive/repeat cytologies in relation to the histological results

		Pathohistology					Total
		UNPROCESSED	BENIGN CHANGES	LSIL	HSIL+CA INSITU	CA INVASIA	
Cytology	negative	1900	14	12	4	0	1930
	positive	364	62	91	18	1	536
	repeat	95	6	3	0	0	104
Total		2359	82	106	22	1	2570

Table 6 Relationship of abnormal/negative cytologies to the number of patients/healthy patients

	ILL	HEALTHY	TOTAL
Abnormal Cytology	110	62	172
Negative Cytology	16	1914	1930
Total	126	1976	2102

The obtained values were as follows.

- Sensitivity = 110/126 x 100 = 87.3%
- Specificity = 1914/1976 x 100 = 96.86%
- Positive predictive value = 110/172 x 100 = 63.95%
- Negative predictive value = 1914/1930 x 100 = 99.17%
- Overall accuracy = (110 +1914) / 2102 x 100 = 96.28%

Using a Chi-square test, we tested the null hypothesis H_0 that there was no correlation between the two variables (the cytological and histological tests).

In order to meet the requirement that at least 80% of the expected frequencies would exceed 5 and that all of the expected frequencies would exceed 1, we grouped findings as follows:

1. Negative and unsatisfactory in one group (neg/unsat.);
2. ASC-US, ASC-H, AGC-NOS, AGC-FN in the group of atypical cells;
3. LSIL in the low-grade group (LOW G); and
4. HSIL, PCA, AIS in the high-grade group (HIGH G).

Histopathological findings were classified into three groups:

1. Negative;
2. LSIL; and
3. HSIL + CA + in situ CA in the invasive group, consisting of the three findings together.

The sig. value for the Pearson chi-square test was 0.036, which is less than 0.05, so the null hypothesis was rejected, and this confirms that cervical cytology and histopathological diagnosis are dependent features.

Based on Cramer's indicators (Cramer's V) for 2 degrees of freedom, the range of influence 0,179 was considered a medium-strength correlation between the variables (cervical cytology and cervical histology).

DISCUSSION

Diagnostic cytology is the art and science of analyzing human cells. The significance of cervical cytology is based on the premise that most cervical cancer lesions develop gradually such that cancer cell precursors and localized intraepithelial carcinomas can be detected by this method, making it is possible to treat a disease and fully cure it. A definite cytological diagnosis must be supported with relevant clinical history. Only when a smear is adequate and all clinical data are in support can you then offer a definite cytological evaluation. Of the utmost importance for the maintenance of satisfactory cytological results is uniformity in the technical preparation of products between laboratories (13,14).

In our study, preparations were decentralized in several primary cytology laboratories within the hospital, and the minimum of variances in the quality of smears was respected. Each technically unsatisfactory smear was returned with a recommendation on the required repetition. The largest number of unsatisfactory smears was attributed to technical reasons. The results of our study showed that, among unsatisfactory smears, low-grade lesions (LSIL) were detected in fewer than 3%, which is in accordance with the results of other authors (8).

It is known that in a certain percentage of unsatisfactory smears, significant cervical lesions can be present, and the cervical cytology can detect or lower the degree of cervical lesions' weight (15). In some cases, highly invasive tumours, known as tumour diathesis, appear (granular amorphous precipitate with nuclear waste and red blood cells), in which cancer cells cannot be identified (16); in such cases, the smears can be classified as false negatives. Hindering factors included technically unsatisfactory smears, low frequencies of abnormal cells in the smears, difficulty in distinguishing immature squamous metaplasias and cohesive fragments of HSIL, difficulty in distinguishing small HSIL cells, small cell carcinomas and some AIS of endometrial cells. Additionally, false positive cytology results are possible: for example, cells of the lower uterine segment or endometrial tissue, because of its high N / C ratio, hyperchromasia and mitotic activity, can be replaced with squamous or glandular lesions. Lymphocytes and plasma cells in the cervical smears of postmenopausal women (follicular cervicitis) may be replaced with HSIL, lymphoma, or endometrial cells, among others (16).

Table 7 H^2 test—Testing hypotheses about the relation between the cytological and histological tests

			DGPHX2 (Histological test)			Total
			NEGATIVE	LSIL	HSIL+CA INSITU+CA INVASIVA	
DGSUX2 (Cito-Test)	Neg/unsat	Count	20	15	4	39
		% within DGSUPX2	51,3%	38,5%	10,3%	100,0%
		% within DGPHX2	24,4%	14,2%	17,4%	18,5%
		% of Total	9,5%	7,1%	1,9%	18,5%
	Atypical cells	Count	28	34	5	67
		% within DGSUPX2	41,8%	50,7%	7,5%	100,0%
		% within DGPHX2	34,1%	32,1%	21,7%	31,8%
		% of Total	13,3%	16,1%	2,4%	31,8%
	Low G	Count	23	41	5	69
		% within DGSUPX2	33,3%	59,4%	7,2%	100,0%
		% within DGPHX2	28,0%	38,7%	21,7%	32,7%
		% of Total	10,9%	19,4%	2,4%	32,7%
	High G	Count	11	16	9	36
		% within DGSUPX2	30,6%	44,4%	25,0%	100,0%
		% within DGPHX2	13,4%	15,1%	39,1%	17,1%
		% of Total	5,2%	7,6%	4,3%	17,1%
Total		Count	82	106	23	211
		% within DGSUPX2	38,9%	50,2%	10,9%	100,0%
		% within DGPHX2	100,0%	100,0%	100,0%	100,0%
		% of Total	38,9%	50,2%	10,9%	100,0%

The database in this study included patients after secondary cytological examination. Compared with the pilot project in the Branicevo region, which included patients with primary and secondary cytological screenings, we received expected data on the percentages for the presence of cytological findings: slightly lower presence of normal findings and slightly higher presence of abnormal and unsatisfactory cytology findings (17).

In our study population, patients varied widely in age, which reflects the real situation in the general population. The number of patients who were outside the range of years envisaged by a screening program (i.e., younger than 25 and older than 64 years) minimally contributed (4.16%) to the total number of patients.

It is noted that there was a slight asymmetry in the distribution of patients who participated in the screening, weighted towards a younger age. The distribution of mean values for different age groups of cytological findings in our study was related to the fifth decade of life, except for squamous cell carcinoma and adenocarcinoma in situ, which were detected cytologically in the sixth decade, in agreement with the field's knowledge of the pathophysiology of cervical cancer.

Results of a large meta-analysis, reported by Melnikova et al., have been drawn from data from 1966 to 1996; these data linked women with cervical atypia per cytology (ASC-US) and those with low-grade lesions to a low level of invasion of cervical cancer over a follow-up duration of 24 months, and they also showed that the high level of the lesion (HSIL) had a higher likelihood of progression to cancer than the probability of finding a regression to normal, which is consistent with the biological theory of the origin process for cervical dysplasia (13).

According to this study, the regression of ASC-US and LSIL without treatment within 24 months occurred approximately 53% of the time, whereas the progression for these results in the same period was less than 1%. The regression rate within 24 months was 68.19% for ASC-US and 47.39% for LSIL, whereas the rate of regression for HSIL over the same period was 35.03%. The rate of progression to invasive cancer within 24 months of follow-up was 0.25% for ASC-US, 0.15% for LSIL and 1.44% for HSIL. From these results, it can be observed that HSIL has the highest rate of progression to invasive carcinoma, whereas LSIL had the lowest rate of progression within 24 months (Table 8)(13). This finding is consistent with the pathogenesis of low-grade dysplasia, which is normally considered to be the consequence of transient infection of HPV. The cumulative rate of progression over 24 months resulting

Table 8 Rates of progression and regression of pre-invasive squamous lesions within 24 months (13,16)

	Regression (%)	Progression to HSIL (%)	Progression to invasive cancer (%)
ASC-US	68	7	0,25
LSIL	47	21	0,15
HSIL	35	/	1,4

from the ASC-US/LSIL Triage Study of ASC-US in CIN was 8-9%, whereas the cumulative rate of progression from LSIL to HSIL was 6.6% over 6 months and 20.8% over 24 months (11, 18).

Several studies have shown that the diagnosis of ASC-US cannot be ignored. The percentage of the participation of ASC-US cytology results in abnormal cytology is the largest, accounting for 90-95% of all results (11). The percentage of squamous intraepithelial lesions obtained after biopsies of these findings ranges from 36% to 63%. According to a study by Massad et al. (2001), on the basis of histological results of cytological findings, 30% of ASC-US findings really are histologically negative, 47% as associated with lumps, and 18% reflected CIN 1, 3% CIN 2 and 3% CIN 3 (19). Thus, the introduction of this category of Bethesda classification seems justified because eliminating this category would result in a portion of high-grade lesions being unidentified.

In a clinical study, Barcelos et al. found that after repeated cytology, 42.8% of the female patients with ASC-USA results are sent to biopsy, 16.6% of which have an HPV infection, and 30% have CIN (10% CIN1, 10% CIN2 and 10% CIN 3), whereas 53.3% have a normal biopsy. It is believed that 9-17% of women with ASC-US results have definitive CIN (11).

The frequency of atypical squamous cells of undetermined significance in abnormal cytological findings in our study is somewhat lower (41.8%) than expected based on the results of other studies (11). In our study, we used strict criteria for the interpretation of ASCUS cytology according to standardized instructions provided for the interpretation of cervical cytology smears (12). It should be noted that, in this study, the population consisted of women without any disease symptoms included in a screening program, where the expected presence of HPV infection was lower than in a population of women with gynaecological complaints and cervical changes.

In our study, after a colposcopy and/or an HPV typing or a repeated cytology, a biopsy was carried out on a certain percentage of cytological ASC-US results. The results of our study showed that a definite CIN was found for 8.5%, which is slightly lower compared to previous published data (19). In biopsies with a cytological ASC-US result, the LSIL category dominates in relation to the HSIL, which is in accordance with the results of other authors (19). For ASC-US, CIN1 and CIN2 alone have been confirmed following biopsy.

The ASC-H result comprises 5-10% of all ASC results. In our study, 34.6% of CIN results were confirmed within the ASC-H cytology results, 15.79% of which were CIN2 and CIN3. The results of our study are consistent with the results of other studies, which in 30-40% of the ASC-H cases, CIN is diagnosed, 25-50% of which is CIN2 or CIN3 (8, 11, 20).

It is noted that the colposcopic results after the ASC cytology were significantly more abnormal in the ASC-H group than in ASC-US group (21). This is explained by the fact that the ASC-H category possesses a higher predictive value for HSIL compared to the ASC-US category and a lower one than the predictive value of the HSIL category (8).

In women with a LSIL result, 17% had CIN2 and 12% had CIN3 (11). In our study, female patients with LSIL cytology results had a dominant histological CIN1 (LSIL) result, whereas the HSIL result was confirmed in only 3.4%. The results of our study have shown that the percentage of benign HP findings in the LSIL category corresponded to approximately one-third of the sample, which is in accordance with the data from previous studies, which show that approximately 30% of LSIL findings are over-diagnosed (15).

Studies show that, with a cytological HSIL result, more than 50% of the female patients will have CIN2 or a more severe finding, and 2% will have invasive carcinomas (11). Data also show that between 20% and 30% of HSIL results are not diagnosed by cervical cytology (15).

Our study showed that, for 68.57% of the biopsies performed, there was a CIN result and that the prevalence of invasive cancer within the HSIL cytology results in this study fits with previous published data (11).

In 44% of women with atypical glandular cells, squamous lesions are found after further histological procedures (8). In our study, the cytological result of AIS with clear cytological elements of this cancer in the biopsy was confirmed as HSIL, which confirms the results of previous research (8)—i.e., squamous and glandular lesions coexist at high rates in the pathology of the cervix.

In this study, there were no cases of a negative cytology after which the biopsy showed an invasive carcinoma. This coincides with the conclusions made by other studies—that is, the Pap test detects all malignant lesions, but there are very frequent false-positive results (15). Of the total number of Pap tests performed in this study, 2.65% were false positives, i.e., of the total number of biopsies performed, 38.9% had benign results.

We see from the study results that, in the cytological categories of ASC-H and HSIL, there is a percentage shift towards a category of dysplasia, lower than would be expected based on other studies. This can be explained by the possible coexistence of large LSIL and small focal HSIL lesions. It is possible that, in some cases, these small lesions are not revealed by a targeted biopsy. If one takes into consideration the quantity of cells that can be found in the Pap test with an ASC-H result (a small number of cells with given characteristics), we can hypothesize that the number of cells in the cytological smear corresponds to the size of the lesions in the conizate.

In this study, HSIL was confirmed for small percentages in the ASC-US and LSIL results. Based on data from the ASC-US LSIL Triage study, Sherman et al. concluded that CIN3 lesions found after LEEP excision were smaller (<10 mm) than those expected for micro invasive carcinoma (63.5 mm). The researchers in this study drew the conclusion that the CIN3 lesions detected after a less severe cytological result than HSIL tend to be small. The small CIN3 result also has a slightly increased risk of being related to a false negative HPV test (22).

The bias that is present in this study exists as an inevitability, given that histological diagnoses were not performed for all participants in the study, because in cases of normal cytological and colposcopic results, it would represent over-treatment.

A certain percentage of patients with cytological HSIL results, where a histological examination would be expected, were lost in this study, and we had no follow-up on further diagnosis. According to this study, approximately 1% of the total number of patients were women diagnosed with squamous lesions of high-grade cytology, for which data on further histological examination was missing. Because the screening was carried out on a voluntary basis, which for now is a reality in our society, it is necessary to educate patients and physicians about the need to comply with all clinical pathways, protocols and guidelines.

When interpreting the results of this study, it should be noted that the works of M. H. Stoler and Schiffman show that, between different clinicians, there is moderate consistency in the identification of monolayer cytology smears, as well as a moderate consistency in the identification of histopathological biopsy interpretation. The major sources of disagreement in monolayer cytology are the ASC-US results. Another significant source of disagreement between cytologists is the interpretation of the HSIL results that are sometimes referred to as LSIL or even as ASC-US. The explanation for the down-grading of HSIL results to LSIL is noted in literature as the often-difficult distinction between mild and moderate dysplasia, whereas in the case of down-grading to ASC-US, controversy may arise in connection with small atypical squamous cells of the immature metaplasia type, when it cannot be determined with certainty whether the entity is distinct from HSIL. With the biopsy results, histological variability results in the highest percentage of the disagreement over stage CIN1 (23). There are also cases that record errors in the assessment of HP and when CIN that is present is not properly diagnosed (15). The data indicate the possibility of "false-negative" histological results in which very small CIN2 or CIN3 lesions are not diagnosed, despite cytology and virology records. According to a study by Castle et al., cases of "false-positive HSIL cytology" in fact are not false positives but instead are CIN3 lesions that were not detected upon initial colposcopy but were then discovered during intensive monitoring of the female patient. It is important to emphasize that a colposcopy diagnosis has limitations in the detection of very small CIN3 lesions, which on the other hand, can be detected as minor cytological changes in a Pap test (24).

A positive characteristic of this study compared to similar performed studies is the involvement of a large number of female patients in both the reproductive and the postmenopausal period, comprising a representative sample that reflects the true state of the prevalence of cervical lesions and the actual representation of HPV infection in the general population. Different degrees of interpretation of cytological smears in organized screening have been reduced by the two-way analysis of smears, with the secondary smear interpretation given by a clinical supervisor with many years of experience in cervical cytology, according to the standards and in compliance with the uniformity of the Bethesda system for reporting cervical cytology.

This study demonstrated that cervical cytology and cervical biopsy are dependent features, between which there is a connection of medium strength, as established by other researchers using a similar methodology (15).

As in similar studies, this study has confirmed the high sensitivity, specificity, positive and negative predictive value of the procedure . However, this study also shows that cervical cytology does not represent an absolutely valid method. The overall recorded accuracy of this method in our study was greater than 90%. In relation to cytology, the HPV test has a higher sensitivity in the detection of cervical intraepithelial lesions of a high grade. The data obtained from organized tests, the subject of which was a comparison of primary screening organized by HPV testing and a screening organized on a basis of cervical cytology, have shown that the HPV-based screening test has a high sensitivity but a low specificity in comparison with screening-based cytology (25). Based on these results, the cytological cervical smear (Pap smear) is legally provided as a primary screening test in this country (26). The HPV test, due to its high specificity, has proved to be useful in determining the level of oncogenic potential of the cervical lesions present, which is directly related to the presence/absence of HPV with high oncogenic potential. The data show that 32.6-44.7% of women with ASC-US results and the majority of women with LSIL results are positive for HPV of high oncogenic potential (27,28).

INSTEAD OF A CONCLUSION— CLINICAL RECOMMENDATIONS

In this study, it was shown that none of the clinical methods used in the diagnosis of precancerous and cancerous changes in the cervix is absolutely accurate, whether it be cytology, colposcopy or histopathological analysis. Accordingly, a decision on further procedure and treatment of patients with present changes in the cervix should be made in a multi-disciplinary way, with respect to clinical recommendations from the 2011 National clinical guidelines for the diagnosis and treatment of cervical cancer in the Republic of Serbia and on the basis of European and American guidelines for the management of women with abnormal cytology (11, 29, 30). An individualize approach to treatment of the disease based on the patient's medical history is also necessary.

In this study, we have shown that cervical cytology is not only a consultative result but also a diagnostic tool that plays an active role in the decision about further treatment and the treatment of cervical disease.

In the case of positive cytology accompanied by negative colposcopy and/or histopathology, it is necessary to

actively monitor and determine the cytological HPV status of the female patient.

After obtaining a histopathological result, it is also necessary to respect the recommendations made in the guidelines of good clinical practice for the treatment of this disease.

In order to determine the type of treatment, it is of special importance to determine oncogenic potential through HPV testing. Our clinical recommendation is that, in the case of an LSIL result in combination with an HPV virus of high oncogenic potential, further active treatment of these lesions should be applied, regardless of age.

ACKNOWLEDGEMENTS

Funding—The authors would like to express their gratitude to the Ministry of Science and Technological Development of the Republic of Serbia for Grant N°175014, out of which the clinical trial that served as the basis for this original scientific paper was partially financed.

REFERENCES

1. Broćić M, Dodić V, Perišić Ž. Ginekološka citodijagnostika i kolposkopija. Beograd: Eksportpress. 1991.
2. -Department for Prevention and Control of Noncommunicable Diseases. Cancer incidence and mortality in central Serbia 2011 Cancer registry of central Serbia. Beograd: Institute of Public Health of Serbia "Dr Milan Jovanović Batut". 2013. report No. 13.
3. Mitrovic-Jovanovic A, Stanimirovic B, Nikolic B, Zamurovic M, Perisic Z, Pantic-Aksentijevic S. Cervical, vaginal and vulvar intraepithelial neoplasms. Vojnosanitetski pregled 2011;68(12):1051-1056. doi: 10.2298/VSP1112051M
4. Matovina M, Sabol I, Grubisic G, Gasperov NM, Grce M. Identification of human papillomavirus type 16 integration sites in high-grade precancerous cervical lesions. Gynecol Oncol 2009;113(1):120-7. PubMed PMID: 19157528. doi: 10.1016/j.ygyno.2008.12.004
5. Djordjevic M. Citoloski atlas malignih tumora. Beograd: Elit- Medica.
6. Vrdoljak-Mozetic D. Papa test danas - nova, "Bethesda" klasifikacija. Medix 2005;11(58):85-89.
7. Pavlovic S. Citopatologija zenskih genitalija. Nis: Prosveta. 1980.
8. Apgar BS, Zoschnick L, Wright TC. The 2001 Bethesda System terminology. Am Fam Physician 2003;68(10):1992-8. PubMed PMID: 14655809
9. Mathilde EB, Albert JHS. The Pap Smear, 3rd ed. Amsterdam: Harwood academic publishers. 1996.
10. Solomon D, Nayar R. The Bethesda System for Reporting Cervical Cytology. Definitions, Criteria, and Explanatory Notes, 2nd ed. New York: Springer Science; New York: Business Media, LLC.. 2004.
11. -Ministarstvo zdravlja Republike Srbije, -Republička stručna komisija za izradu i implementaciju vodiča dobre kliničke prakse . Nacionalni vodič dobre kliničke prakse za dijagnostifikovanje i lečenje raka grlića materice. Klinički vodič 12/14. Beograd: Agencija za akreditaciju zdravstvenih ustanova Srbije. 2013.
12. Pogacnik A, et al. . Uputstva za tumačenje briseva cervikalne citologije-klasifikacija prema Bethesdi. Beograd: Institut za javno zdravlje Srbije "Dr Milan Jovanović-Batut". 2012. (prevod slovenačke skraćene verzije).
13. Melnikow J, Nuovo J, Willan AR, Chan BK, Howell LP. Natural history of cervical squamous intraepithelial lesions: a meta-analysis. Obstet Gynecol 1998;92(4 Pt 2):727-735.
14. Koss LG, Melamed MR. Koss' Diagnostic Cytology and Its Histopathologic Bases, 5th ed. USA: Lippincott Williams & Wilkins. 2006.
15. Milenkovic V, Sparic R, Dotlic J, Tulic L, Mirkovic L, Milenkovic S, Atanackovic J. Reliability and relationship of colposcopical, cytological and histopathological findings in the diagnostic process. Vojnosanitetski Pregled 2012;69(10):869-873.
16. Cibas ES, Ducatman BS. Cytology: Diagnostic principles and clinical correlates, 3rd ed. Philadelphia: Saunders. 2009.
17. Kesic V, Jovicevic-Bekic A, Vujnović M. Cervical cancer screening in Serbia. Coll Antropol 2007;31(2):31-6. PubMed PMID: 17598502
18. Schiffman M, Solomon D. Findings to date from the ASCUS-LSIL Triage Study (ALTS). Arch Pathol Lab Med 2003;127(8):946-9. PubMed PMID: 12873166
19. Massad LS, Collins YC, Meyer PM. Biopsy correlates of abnormal cervical cytology classified using the Bethesda system. Gynecol Oncol 2001;82(3):516-22. PubMed PMID: 11520149. doi: 10.1006/gyno.2001.6323
20. Chivukula M, Shidham VB. ASC-H in Pap test - definitive categorization of cytomorphological spectrum. Cytojournal 2006;3(1):14. doi: 10.1186/1742-6413-3-14
21. Barcelos ACM, Antoniazi Michelin M, Adad SJ, Candido Murta EF. Atypical Squamous Cells of Undetermined Significance: Bethesda Classification and Association with Human Papillomavirus. Infectious Diseases in Obstetrics and Gynecology 2011;904674:9. doi: 10.1155/2011/904674
22. Stoler MH, Schiffman M. Interobserver reproducibility of cervical cytologic and histologic interpretations: realistic estimates from the ASCUS-LSIL Triage Study. JAMA 2001;285(11):1500-5. PubMed PMID: 11255427
23. Sherman ME, Wang SS, Tarone R, Rich L, Schiffman M. Histopathologic extent of cervical intraepithelial neoplasia 3 lesions in the atypical squamous cells of undetermined significance low-grade squamous intraepithelial lesion triage study: implications for subject safety and lead-time bias. Cancer Epidemiol Biomarkers Prev 2003;12(4):372-9. PubMed PMID: 12692113

24. Castle PE, Cox JT, Schiffman M, Wheeler CM, Solomon D. Factors Influencing Histologic Confirmation of High-Grade Squamous Intraepithelial Lesion Cytology. Obstet Gynecol 2008;112(3):637-645.
25. Perisi Z, Plesinac-Karapandzic V, Dzinic M, Zamurovic M, Perisic N. Cervical cancer screening in Serbia. Vojnosanitetski pregled 2013;70(1):86-89. doi: 10.2298/VSP1301086P
26. -Vlada Republike Srbije. Uredba o nacionalnom programu ranog otkrivanja karcinoma grlića materice. Službeni glasnik RS 2013 73/2013 :83/2013.
27. Plummer M, Schiffman M, Castle PE, Maucort-Boulch D, Wheeler CM. A 2-year prospective study of human papillomavirus persistence among women with a cytological diagnosis of atypical squamous cells of undetermined significance or low-grade squamous intraepithelial lesion. J Infect Dis 2007;195(11):1582-9. PubMed PMID: 17471427
28. Stoler MH, Wright TC, Sharma A, Apple R, Gutekunst K, Wright TL. High-risk human papillomavirus testing in women with ASC-US cytology: results from the ATHENA HPV study. Am J Clin Pathol 2011;135(3):468-75. PubMed PMID: 21350104. doi: 10.1309/AJCPZ5JY6FCVNMOT
29. Wright TC, Massad L, Dunton CJ, Spitzer M, Wilkinson EJ, Solomon D. 2006 consensus guidelines for the management of women with abnormal cervical cancer screening tests. Am J Obstet Gynecol 2007;197(4):346-55. PubMed PMID: 17904957
30. Jordan J, Arbyn M, Martin-Hirsch P, Schenck U, Baldauf J, Da SD, Prendiville W. European guidelines for quality assurance in cervical cancer screening: recommendations for clinical management of abnormal cervical cytology, part 1. Cytopathology 2008;19(6):342-54. PubMed PMID: 19040546. doi: 10.1111/j.1365-2303.2008.00623.

ASSESSMENT OF SERVICE QUALITY AT COMMUNITY PHARMACIES IN THE CITY OF BELGRADE, REPUBLIC OF SERBIA

Andjelka Prokic[1], Mirjana Davidovic[2], Nenad Gunjic[3]
[1]Faculty of Medical Sciences, University of Kragujevac, Kragujevac
[2]Faculty of Pharmacy, University of Belgrade, Belgrade
[3]University of Kragujevac, Kragujevac

PROCENA KVALITETA USLUGE U APOTEKAMA U GRADU BEOGRADU, REPUBLIKA SRBIJA

Anđelka Prokić[1], Mirjana Davidović[2], Nenad Gunjić[3]
[1]Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac
[2]Farmaceutski fakultet, Univerzitet u Beogradu, Beograd
[3]Univerzitet u Kragujevcu, Kragujevac

Received / Primljen: 18.06.2014. Accepted / Prihvaćen: 15.09.2014.

ABSTRACT

One of the principles of rational drug use is informing patients about their prescribed drug and its effects. A pharmacist has to contribute to safe and appropriate drug use and give patients adequate drug information. Service quality in pharmacies is examined by measuring drug use indicators provided by the World Health Organisation. Indicators relevant for pharmacies include patient care indicators and health facility indicators.

The goal of this paper is to measure indicators of drug use in both private and state-owned pharmacies.

Drug use indicators were measured prospectively in private and state-owned-owned pharmacies in Belgrade, Serbia. The study is designed as a cross-sectional study. The research was conducted withon 100 patients at each of 14 pharmacies, 7 of which were state-owned and 7 of which were private. Pharmacies were selected randomly.

Drug use indicators were not significantly different between private and state-owned pharmacies, except for their essential drugs lists. To improve pharmaceutical health care and achieve rational pharmacotherapy, all pharmacists should dedicate more time to patients.

Key words: *community pharmacies, drug use indicators, WHO/INRUD, essential drugs list*

SAŽETAK

Jedan od principa racionalne upotrebe lekova je informisanje pacijenta o leku i njegovim efektima. Farmaceut je dužan da obezbedi sigurno i primereno korišćenje lekova, pružajući pacijentu adekvatne informacije o leku. Način da se ispita kvalitet usluge u apotekama je merenje pokazatelja upotrebe lekova koje je dala Svetska zdravstvena organizacija. Pokazatelji, relevantni za apoteke su: pokazatelji zaštite pacijenta i pokazatelji zdravstvenih ustanova.

Cilj ovog rada je da izmeri pokazatelje upotrebe lekova u privatnim i državnim apotekama.

Pokazatelji korišćenja lekova mereni su prospektivno u privatnim i državnim apotekama u Beogradu. Studija je dizajnirana kao studija preseka. Istraživanje je sprovedeno u 14 apoteka, 7 državnih, 7 privatnih i u svakoj je ispitano 100 pacijenata. Apoteke su izabrane po principu slučanosti.

Pokazatelji korišćenja lekova nisu se značajno razlikovali u privatnim i državnim apotekama, izuzev liste esencijalnih lekova. U cilju poboljšanja farmaceutske zdravstvene zaštite i postizanja racionalne farmakoterapije svi farmaceuti bi trebali više vremena da posvete pacijentu.

Ključne reči: *apoteke, pokazatelji korišćenja lekova, INRUD*

ABBREVIATIONS

SPSS - Service Provisioning System Software **WHO** - World Health Organisation

DE GRUYTER OPEN

UDK: 615.03(497.11) / Ser J Exp Clin Res 2014; 15 (4): 217-221
DOI: 10.2478/SJECR-2014-0027

Correspondence: Andjelka Prokic; 064 492 9499; andjelkap88@open.telekom.rs

INTRODUCTION

Rational drug use refers to patients receiving drugs that correspond to their clinical needs in appropriate doses, for adequate durations and at the lowest prices for them and the society they live in (1). One of the principles of rational drug use is informing patients about each drug and its effects (2).

A pharmacist has to provide safe and appropriate drug use by giving patients adequate information about their prescribed drugs (3, 4). The quality of pharmaceutical services depends on pharmacists' training, knowledge, communication skills, and time spent consulting with patients (4).

The optimal drug dispensing time is 60 seconds (5). In the eastern part of Saudi Arabia, the average drug dispensing time is 100 (58-180) seconds (6) and in Egypt (Alexandria) it is less than 60 (47.4) seconds (7). In Kragujevac, the average drug dispensing time was between 20.4 and 48.2 seconds, and times were longer in private pharmacies (8). In contrast, in Jagodina, the drug dispensing times were similar in state-owned (54.4 seconds) and private (53.3 seconds) pharmacies (9).

Since January 2013, private pharmacies in Serbia have been able to accept prescriptions as well, which can influence the quality of pharmacy services.

The goal of this paper is to measure the values of drug use indicators in private and state-owned pharmacies using the methodology recommended by the WHO. Service quality data from pharmacies can be useful for the planning and implementation o f corrective measures for improving health outcomes and patient welfare.

MATERIALS AND METHODS

The best way to examine service quality in pharmacies is by measuring the values of drug use indicators provided by the World Health Organisation/International Network for the Rational Use of Drugs (INRUD). The indicators relevant for pharmacies are patient care indicators (of which there are four) and health facility indicators (of which there are two)[5].

The study was designed as a cross-sectional study. Drug use indicators were measured prospectively in private and state-owned community pharmacies in Belgrade, Serbia, from December 28, 2013 to February 19, 2014. Belgrade has 89 state-owned and 95 private pharmacies. This study included 7 (7.86%) state-owned and 7 (7.36%) private pharmacies. Pharmacies were selected consecutively. Because the present study dealt with the current practice of drug supplies in community and private pharmacies, the WHO recommended that at least 30 patients from each of 20 health institutions be enrolled. If a smaller number of health institutions are considered, more patients (at least 600) should be included in the study. This study was conducted in a total of 14 pharmacies, and each pharmacy enrolled 100 patients[5].

All private pharmacies included in the study have a contract with the NHIF (National Health Insurance Fund) and dispense prescription drugs. Private pharmacies that do not dispense prescription drugs were not considered in the study. The study was conducted by three researchers; in each pharmacy, one researcher measured the drug dispensing time, another researcher controlled the number of drugs actually dispensed and the dosage regimen labelling on the prescription forms. The third researcher questioned the patients in front of the pharmacy about their drugs. The patients were asked whether they could report the dosage regimen. Once the data were collected for 100 patients at each pharmacy, the pharmacists were questioned about the availability of key and A-list drugs. Because Serbia has no essential drugs list, the A-list was used as a substitution. This is a list of drugs that are prescribed and supplied on prescription forms.

Observed variables

Patient care indicators: a) average drug dispensing time describes how much time a pharmacist devotes, on average, to a patient while dispensing drugs (5). This is calculated as: the average drug dispensing time = total time spent dispensing drugs to a series of patients divided by the number of patient encounters. *b)* Percentage of drugs actually dispensed describes the ability of the health facility to provide drugs that patients need (5). This parameter is calculated as the: percentage of drugs actually dispensed = (the number of drugs actually dispensed / total number of drugs prescribed) × 100. *c)* To ensure that patients have the correct understanding of the dosage regimen, labelling must be legible and understandable. According to WHO recommendations, labels should contain the drug name, patient name and dosage regimen (5). This parameter is calculated in the following way: percentage of adequately labelled drugs = (percentage of drug adequately labelled / total number of drugs dispensed) × 100. *d)* Measurement th e effectiveness of information given to patients was performed in the following way: percentage of patients familiar with the way their drug is dosed = (number of patients who responded correctly to a question about the dosage regimen (schedule) of each drug correctly / total number of patients interviewed) × 100.

Health facility indicators: *a)* key drugs availability is a parameter for measuring available drugs used for the treatment of the most common diseases in the region under examination. The Pharmacology Department at the Faculty of Medical Sciences in Kragujevac established a list of essential drugs in Serbia based on their clinical significance and the frequency of their use. This list includes the following medicines: epinephrine, hydrocortisone, aspirin, morphine, penicillin, diazepam, aminophylline, furosemide, insulin, diclofenac, captopril, aminoglycosides, digoxin, glyceryl trinitrate and intravenous solutions (NaCl 0.9% or 5% glucose). (5,8) The essential drugs list, which was used in previous studies conducted in Serbia, was also used in this study. This parameter was calculated as the: key drugs availability = (the number of key drugs available in stock / total number of key drugs on the essential drugs list) × 100.

b) According to World Health Organisation regulations, all pharmacies should own a copy of the list of essential drugs. This paper used a positive list of drugs (drugs supplied based on health insurance requirements). Availability of a copy of the list of essential drugs was set as a dichotomous variable: yes or no for each health unit.(5,8)

Statistics

Continuous variables are summed as arithmetic means, medians and standard deviations, and categorical variables as proportions (percentages of categories). Student's t-test for independent samples was used to determine statistical significance in continuous variables values between the compared groups with normal distributions. If the data were not normally distributed, a nonparametric alternative (the Mann-Whitney U test) was used. The Kolmogorov-Smirnov test was used to determine if whether the data were normally distributed. A two-factorial analysis of variance allowed for the assessment of the individual influence of two categorical independent variables on the observed outcome, in this case the influence of each state-owned and private pharmacy on drug dispensing times and the interactions between them. Values of $p<0.05$ were considered statistically significant.

All statistical analyses were performed using SPSS software, version 21.

RESULTS

This study included 1395 patients. Of those, 697 were from state-owned pharmacies and 698 were from private pharmacies. The average drug dispensing time for patients in state-owned pharmacies was 15.58 seconds, whileand the average dispensing time in private pharmacies was 18.5 seconds. On average, state-owned pharmacies dispensed 92.7% of the drugs that patients needed, and private pharmacies dispensed 91.9% of the needed medicines. On average, 58.71% of the patients could correctly repeat their dosage regimen after picking up their medicines in state-owned pharmacies, whereas in private pharmacies, this percentage was 56.2%. The availability of key drugs was 77.14% on average in state-owned pharmacies and 80.95% in private pharmacies (Table 1).

Using a two-factor analysis of the variance of different groups, the influences on drug dispensing times of state-owned pharmacies, private pharmacies and each pharmacy separately were examined. It was determined that there is a statistically significant difference in drug dispensing times when each pharmacy is observed separately (F=52.595; p<0.001). However, the partial eta-square is 0.314, which means that the influence is of moderate strength.

Additional comparisons performed using Tukey's HSD test showed that the mean value of drug dispensing was statistically significantly different for the fourth state-owned pharmacy (M=33.64; SD=36.557; p=0,000; HSD= -18,20) and the seventh state-owned pharmacy (M=3.44; SD=1.166; p<0.001; HSD=12). For private pharmacies, statistical significance was noted in the cases of the fourth (M=43.81; SD=24.972; p<0.001; HSD=-28,37), sixth (M=25.33; SD=14.607; p=0,002; HSD=-9,89) and seventh (M=4.89; SD=4.452; p<.001; HSD =10,57) pharmacies.

Neither private nor state-owned pharmacies did labelled drugs adequately. None of the pharmacies marked the patients' names on the drug packaging. Dosage regimens were written illegibly and indistinctly on the original drug package.

Table 1. Drug use indicators in private and state-owned pharmacies in Belgrade

Pharmacy	Average drug dispensing time in seconds per patient (SD)	Actually dispensed drugs (%)	Patients familiar with drug use (%)	Key drugs availability (%)
State-owned				
1.	15.44 (15.574)	193 (95.5)	81	12 (80)
2.	7.67 (7.171)	162 (92.5)	67	12 (80)
3.	9.06 (9.630)	232 (95.0)	54	11 (73.3)
4.	33.64 (36.557)	200 (96.1)	51	13 (86.6)
5.	23.18 (14.739)	146 (91.8)	57	10 (66.6)
6.	16.58 (10.518)	189 (91.7)	47	12 (80)
7.	3.44 (1.166)	158 (84.9)	54	11 (73.3)
Total	**15.58 (19.565)**	**1280 (92.7)**	**58.71**	**81 (77.14)**
Private				
1.	13.79 (12.117)	162 (94.1)	51	12 (80)
2.	8.43 (10.783)	137 (90.7)	52	10 (66.6)
3.	11.16 (16.951)	154 (97.4)	51	14 (93.3)
4.	43.81 (24.972)	163 (92.0)	68	11 (73.3)
5.	19.47 (16.450)	159 (92.4)	53	12 (80)
6.	25.33 (14.607)	164 (86.3)	63	13 (86.6)
7.	4,89(4.452)	159 (91.3)	56	13 (86.6)
Total	**18.15 (19.701)**	**1098 (91.9)**	**56.2**	**85 (80.95)**
Compared state-owned and private pharmacies	U=231686.500; p=0.122	U=13.500; p=0.159	U=22.00; p=0.748.	t (14)= -0.910; p=0.381

All state-owned pharmacies possessed an essential drugs list, which was not observed at all of the private pharmacies (42.8% of private pharmacies possessed an essential drugs list).

DISCUSSION

This paper showed that there is no statistically significant difference in drug dispensing times between state-owned and private pharmacies in Belgrade, Serbia. These results are in accordance with previous observations in Jagodina (6). In the city of Kragujevac, it was noted that private pharmacies took significantly longer to dispense drugs (8), but, this time is not long enough to explain all of the necessary information about the drugs to patients (5). The average drug dispensing time in pharmacies in this study is 16.87 seconds. The optimal drug dispensing time is 60 seconds (5), so pharmacists in both private and state-owned pharmacies should extend drug dispensing times and provide patients with at least basic information about their drugs. Serbia is a country undergoing a socio-economic transition. Thus, material and other health system resources are limited, the drug supply system is not efficient enough, and there is a lack of an list essential drugs for treating diseases that are generally a burden to the health system and society in general. Good pharmacy practice standards still have not been implemented despite the fact that they were established and adopted by The Pharmaceutical Chamber. The personal incomes of pharmacists employed in state sectors are usually insufficient and there is lack of adequate communication and cooperation between doctors and pharmacists in everyday practice, all of which can influence both pharmacists' motivation and the quality of pharmaceutical services.

Written information about drugs in Australia is given in the form of *Medical Information for the Patient* and printed for the patient or given as flyers (12). The same study showed that patients want to read and discuss this information, especially if they received it from a doctor or pharmacist (12). This paper showed that even basic drug labelling was not in line with the recommendations established by the WHO (5), in that it was illegible and indistinct and in that none of the pharmacists wrote patient names on the packaging, which is consistent with the behaviour of pharmacists observed in previous studies in Serbia (8, 9).

Forty to eighty percent of patients can correctly repeat their dosage regimen, with results varying from pharmacy to pharmacy, but there is no statistically significant difference in this result between private and state-owned pharmacies. In developing countries, the results vary from 18% to 82% (6, 10, 11, 13, 15). All patients who pick up drugs in a pharmacy should know how to use them. Extending drug dispensing times order toto providegive necessary information to the patient about using their drugs, as well as improving the quality of drug labelling, would most likely improve the status of this important issue.

A statistically significant difference in the number of drugs actually dispensed between private and state-owned pharmacies was not observed.

This paper demonstrates that pharmacies in Serbia are currently better supplied with medicines than in the past (8, 9).

The 79% average availability of key drugs from the essential drugs list did not differ significantly between state-owned and private pharmacies. In the previous study conducted in Kragujevac, the availability of drugs from the key list was 77.5%[8] on average.

In Brazil (Brasilia) (15) and Egypt (Alexandria) (7), 83.2% and 78.3% of key list drugs, respectively, were available, respectivelywhich are both lower than the results found in our study. Drugs used for treating the most common health problems are not available in appropriate quantities (the value of this parameter should be 100%[5]).

The essential drugs list was present in all of the state-owned pharmacies, and the pharmacists were familiar with this WHO concept. Only two private pharmacies possessed essential drugs lists, but the pharmacists were familiar with the conceptit. In the future, it will be necessary to provide equal educational conditions for all pharmacists. Serbia has no essential drugs list. Its role is performed by the drugs list created by the NHIF (a so-called 'positive list') , which may be the reason for the differences noted between the private and state-owned pharmacies.

Drug use indicators were not significantly different between private and state-owned pharmacies, except for the essential drugs list. To improve the rational use of medicines and pharmaceutical health care in general, pharmacists should dedicate more time to patients. Continuing education of students and pharmacists and the implementation of standards of good apothecary practice adopted by the Ministry of Health to harmonise pharmacists' work in everyday practice are measures that would improve the current conditions.

ACKNOWLEDGEMENTS

We would like to thank Pharmacy Belgrade and Filly Farm for allowing us access to their pharmacies and thus enabling us to perform our study.

REFERENCES:

1. World Health Organisation. How to develop and implement a national drug policy (Guidelines for Developing National Drug Policies). Geneva: WHO; 2001.
2. Akici A, Kalaça S, Uğurlu MU, Toklu HZ, Iskender E, Oktay S. Patient knowledge about drugs prescribed at primary healthcare facilities. Pharmacoepidemiol Drug Saf. 2004; 13(12): 871-6.
3. International Pharmaceutical Federation. Standard for Quality of Pharmacy Services (Good Pharmacy Practice). Hague: FIP; 1997.

4. Toklu HZ, Akici A, Oktay S, Cali S, Sezen SF, Keyer-Uysal M. The pharmacy practice of community pharmacists in Turkey. Marmara Pharm J 2010; 14(1): 53-60.
5. World Health Organisation. How to investigate drug use in health facilities (Selected drug use indicators). Action program on essential drugs. Geneva: WHO; 1995.
6. El Mahalli AA, Akl OA, Al-Dawood SF, et al. WHO/INRUD patient care and facility-specific drug use indicators at primary health care centres in Eastern province, Saudi Arabia. East Mediterr Health J. 2012; 18(11): 1086-90.
7. Akl OA, El Mahalli AA, Elkahky AA, Salem AM. WHO/INRUD drug use indicators at primary healthcare centres in Alexandria, Egypt. J Taibah Univ Med Sci 2013; 9(11): 54-64.
8. Jankovic SM, Maksimovic MR, Vusanovic A et al. Quality of work in state-owned and private pharmacies in city of Kragujevac. CMJ 2001; 42(1): 88-91.
9. Stojanovic BB, Jankovic SM.An analysis of drug use indicators within primary care health facilities in Jagodina. Medicus. 2002; 3(2): 30-6.
10. Otoom S, Batieha A, Hadidi H, Hasan M, Al-Saudi K. Evaluation of drug use in Jordan using WHO patient care and health facility indicators. East Mediterr Health J. 2002; 8(4-5): 544-9.
11. Dahal P, Bhattarai B, Adhikari D, Shrestha R, Baral SR, Shrestha N. Drug use pattern in Primary Health Care facilities of Kaski District, Western Nepal. Sunsari Technical College Journal. 2012; 1(1): 1-8.
12. Hamrosi KK, Raynor DK, Aslani P. Pharmacist, general practitioner and consumer use of written medicine information in Australia: Are they on the same page? Res Social Adm Pharm. 2013; pii:S1551-7411(13): 00186-10doi:1016.
13. Chedi BAZ, Abdu-Aguye I, Kwanashie HO. Analysis of Patient Care and Facility Indicators in Public Health Institutions in Kano State-owned, Nigeria. NJPS 2009; 8(2): 72-8.
14. Hogerzeil HV, Ross-Degnan D, Laing RO, et al. Field tests for rational drug use in twelve developing countries. Lancet. 1993; 342(8884): 1408-10.
15. Naves Jde O, Silver LD. Evaluation of pharmaceutical assistance in public primary care in Brasilia, Brazil. Rev Saude Publica. 2005; 39(2): 223-30.

THE ROLE OF AUTOPHAGY IN IMMUNITY AND AUTOIMMUNE DISEASES

Bojana Simovic Markovic[1], Ljubica Vucicevic[2], Sanja Bojic[3], Vladislav Volarevic[1]
[1] Centre for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
[2] Institute for Microbiology and Immunology, Faculty of Medicine, Univesity of Belgrade, Serbia
[3] Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia

ULOGA AUTOFAGIJE U IMUNSKOM ODGOVORU I AUTOIMUNSKIM BOLESTIMA

Bojana Simović Marković[1], Ljubica Vučicević[2], Sanja Bojić, Vladislav Volarević[1]
[1] Centar za molekulsku medicinu istraživanje matičnih ćelija, Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija
[2] Institut za mikrobiologiju i imunologiju, Medicinski fakultet, Univerzitet u Beogradu, Srbija
[3] Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija

Received / Primljen: 19.08.2014. Accepted / Prihvaćen: 23.09.2014.

ABSTRACT

Autophagy is a catabolic mechanism in the cell that involves the degradation of unnecessary or dysfunctional cellular components by the lysosomal machinery. Recent studies have indicated that autophagy is a source of autoantigens, thus highlighting its potential role in the pathogenesis of autoimmunity. There are at least three different forms of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). The physiological role of autophagy is to maintain cellular homeostasis by removing long-lived, damaged proteins and dysfunctional organelles and by providing energy. Aberrant autophagy may contribute to chronic inflammatory diseases and autoimmune diseases.

An understanding of the complex relationships between autophagy and autophagy-related genes in each autoimmune disease creates the possibility of developing more specific and effective therapeutic strategies. Given the importance of autophagy in immune functions, this review article summarises current knowledge about the role of autophagy in the pathogenesis of autoimmune diseases.

Keywords: *Autophagy, Autoimmune diseases, mTOR, ATG16L1*

SAŽETAK

Autofagija je lizozomalni katabolički proces razgradnje proteinskih agregata i oštećenih organela. Nedavne studije ukazuju na autofagiju kao izvor autoantigena i na taj način potvrđuju potencijalnu ulogu autofagije u patogenezi autoimunskih oboljenja. Postoje tri različite forme autofagije: makroautofagija, mikroautofagija i autofagija posredovana šaperonima. Fiziološka uloga autofagije se ogleda u održavanju ćelijske homeostaze, uklanjanjem dugoživećih oštećenih proteina, kao i disfunkcionalnih organela i održavanjem energije. Poremećaji procesa autofagije mogu da posreduju u hroničnim inflamatornim i autoimunskim bolestima.

Razumevanjem složenih odnosa između samog procesa autofagije i gena koji taj proces kodiraju u određenim autoimunskim bolestima otvaraju nove mogućnosti za razvoj specifičnijih i efektivnijih terapeutskih agensa. U ovom revijskom radu smo sumirali već postojeće činjenice u vezi sa ulogom procesa autofagije u patogenezi autoimunskih bolesti.

Ključne reči: *Autofagija, autoimunske bolesti, mTOR, ATG16L1*

INTRODUCTION

Autophagy is a catabolic mechanism in the cell that involves the degradation of unnecessary or dysfunctional cellular components by the lysosomal machinery. The word "autophagy" is derived from the Greek words "auto," meaning "self," and "phagy," meaning "to eat." Initially, autophagy was recognised as a survival mechanism during starvation, but now, an ever-growing body of evidence shows that autophagy has a homeostatic function in many physiological processes, such as cell growth; cell development; and cell survival during hypoxia, oxidative stress and DNA damage. Moreover, over the past few years, research in the field has demonstrated the involvement of autophagy in immunity and infection. This mechanism has important roles in the detection and clearance of pathogens, as well as in antigen presentation, in lymphocyte survival and homeostasis, and in the mediation of cytokine production (1-7). Recent studies indicated that autophagy is a source of autoantigens, thus highlighting its potential role in the pathogenesis of autoimmunity (8). Given the importance of autophagy in immune functions,

DE GRUYTER OPEN
UDK: 616-097:612-015.3 / Ser J Exp Clin Res 2014; 15 (4): 223-229
DOI: 10.2478/SJECR-2014-0028

Correspondence: Bojana Simovic Markovic, medical doctor, Centre for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Serbia, e mail: bojana.simovic@gmail.com

this review article summarises current knowledge about the role of autophagy in the pathogenesis of autoimmune diseases.

The autophagy process

Autophagy is an evolutionarily conserved pathway in which long-lived, denatured proteins and damaged organelles are delivered to lysosomes for degradation. There are at least three different forms of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). CMA describes the chaperone-mediated, direct translocation of unfolded proteins across the lysosomal membrane, whereas during microautophagy, lysosomal uptake of cytosolic substrates is performed by invagination of the lysosomal membrane. In contrast, macroautophagy has several stages, starting with membrane isolation, or phagophore formation. The phagophore then expands across the cytosolic material and structures, resulting in a double-membrane cytosolic vesicle known as an autophagosome or autophagic vacuole. The completed autophagosome subsequently fuses with a lysosome to form a single-membrane autolysosome, allowing lysosomal hydrolases access to the inner autophagosomal membrane and its cargo, which is degraded and recycled. Among the three main types of autophagy, macroautophagy is the best-characterised process and is generally referred to as autophagy (9-15).

Autophagy is a tightly regulated process, and to date, at least 35 autophagy-related genes (*ATG*) involved in autophagosome formation have been defined (16,17). These genes are conserved from yeast to human. The main autophagy regulator is mammalian target of rapamycin (mTOR), which negatively regulates autophagy induction. Inhibiting mTOR kinase activity allows ULK1 activation, translocation of the mTOR substrate complex (ULK1/2, ATG13, FIP200 (also known as RB1CC1) and ATG101) and initiation of autophagy (18,19). This leads to recruitment of the class III phosphatidylinositol-3-OH kinase (PI(3)K) complex (PIK3C3/VPS34-BECN1) and *de novo* autophagosomal membrane formation (20). The source of membrane could be different organelles, including the ER, the Golgi complex, the mitochondria and the plasma membrane (21-23). The subsequent elongation and closure of autophagosomes involves the activation of two ubiquitin conjugation complexes, the ATG12 (ATG12-ATG5-ATG16L1) and LC3 (Atg 8) conjugation systems (24). The status of the autophagy repressor mTOR is controlled by two principal upstream regulatory pathways, or phosphatidylinositol 3-kinase (PI3K)-Akt and an AMP-activated protein kinase (AMPK)-dependent signalling network (25, 26).

Autophagy was originally regarded as a non-selective, bulk degradation process, but now, it has been demonstrated that autophagy degrades substrates in a selective manner and that proteins, such as sequestosome 1 (SQSTM1)/p62, NBR1, Nix and others, act as autophagic adaptors or cargo receptors. All autophagic adaptors contain an LC3-interacting domain through which they directly bind to the phagophore (27).

The physiological role of autophagy is to maintain cellular homeostasis by removing long-lived, damaged proteins and dysfunctional organelles and by providing energy. Under stress conditions, autophagy also represents a survival mechanism by providing energy and/or interfering with apoptosis. In contrast, during *Drosophila melanogaster* development or when there is excessive or abnormal cell proliferation in mammals, autophagy acts as a cell death pathway (programmed cell death type II) (28, 29). Recent data indicate that depending on the conditions, autophagy can either impair or promote cell survival (30, 31, 32).

Autophagy and innate immunity

In addition to clearing endogenous substrates, autophagy plays an important role in the clearance of intracellular pathogens, and this form of selective autophagy is termed xenophagy. In xenophagy, pathogens are captured within autophagosomes and subsequently destroyed, which leads to the activation of pattern recognition receptors

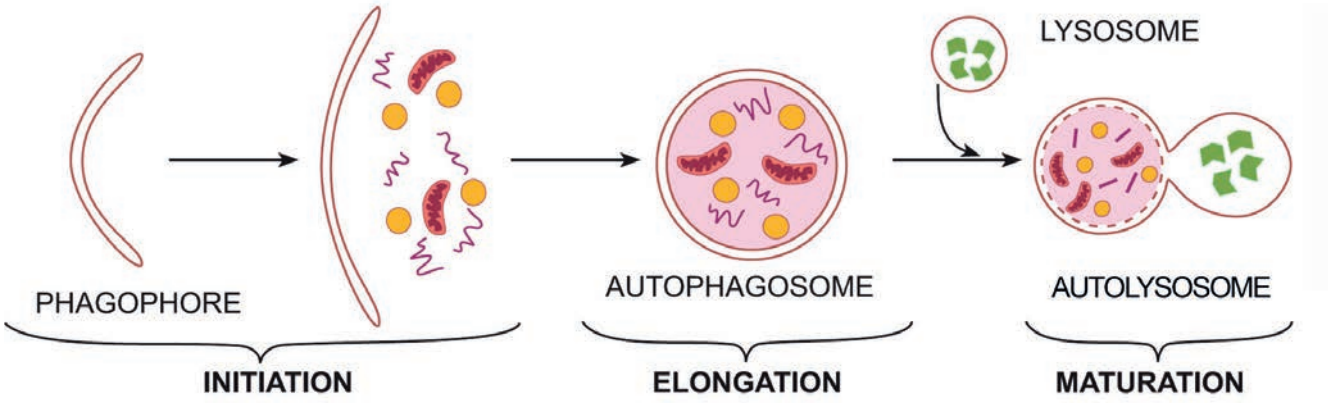


Figure 1. The process of autophagy.

and enhanced MHC presentation of antigens (33-36). The precise membrane dynamics and specificity of xenophagy are not fully understood. Possible mechanisms of capturing autophagy-dependent viruses, bacteria and parasites involve wrapping of pathogen-containing phagosomes or endosomes with autophagosomal membranes, the fusion of pathogen-containing phagosomes or endosomes with autophagosomes, and the direct autophagic capture of a pathogen that has escaped into the cytoplasm (6). Different cellular adaptors are required for capturing intracellular bacteria and viruses than for endogenous substrates, such as the cellular factor nuclear dot protein 52 (NDP52), which plays a role in autophagosomal targeting of *Salmonella typhimurium* (37).

Signalling pathways that regulate the inflammatory response may also have a role in the regulation of autophagy, which may indicate the importance of autophagy in innate immunity. It has been demonstrated that autophagy is induced by different families of pathogen recognition receptors, including Toll-like receptors, Nod-like receptors, damage-associated molecular patterns (DAMPs), and pathogen receptors such as CD46; by interferon (IFN)-γ; and by downstream immunity-related guanosine triphosphatases (GTPases) (36, 38, 39). Additionally, autophagy-related proteins function in both stimulating and suppressing immune and inflammatory responses. Recent studies revealed a negative influence of autophagic proteins on inflammasome-associated proinflammatory cytokine maturation in macrophages. Induced transcription of pro-interleukin (IL)-1β and IL-18 is followed by inflammasome complex appearance and caspase 1 activation as a response to cellular infection. Inflammasomes are cytosolic complexes that contain NOD-like receptor proteins that serve as adaptors. It has been suggested that autophagy represents a feedback mechanism in macrophages to sustain the inflammatory process (40). For example, increased IL-1β and IL-18 secretion from Atg16l1- or Atg7-deficient mouse macrophages was observed after TLR4 stimulation. The same increased cytokine activation was also detected in LC3B- or beclin-deficient macrophages (41). The relationship between autophagy and immunity/inflammation is complex and bidirectional.

Autophagy and adaptive immunity

Autophagy also participates in adaptive immunity, including the development/homeostasis of haematopoietic stem cells and lymphocytes and antigen presentation. Autophagy is involved in antigen processing for MHC class II presentation on professional antigen-presenting cells (APCs), or macrophages, B cells and dendritic cells. Dysfunction of Atg5 and Atg12, which are autophagy-related genes, during Epstein-Barr and herpes simplex 2 virus infections down-regulates antigen processing and MHC class II presentation on APCs (42- 44). In contrast, inhibition of autophagy by pharmacological or gene manipulations does not affect the expression of MHC class I molecules (45). Another crucial function of autophagy in adaptive immunity is the elimination of autoreactive T cells from the thymus. High levels of autophagy are present in thymic epithelial cells and participate in delivering endogenous proteins to MHC class II molecules. Transplantation of embryonic thymi from $Atg5^{-/-}$ mice into athymic nude mice was shown to induce systemic autoimmunity (46). It was also shown that autophagy specifically marks apoptotic cells for phagocytic clearance during embryonic development (47). Faulty removal of apoptotic cells in the embryonic lung and retina has been associated with an increased number of inflammatory cells, followed by endothelial damage, increased vascular permeability and oedema formation (48).

Autophagy and cytokines

Many cytokines are strong inducers of autophagy (49). A classic example of the involvement of cytokines in the regulation of autophagy is macrophages' response to *Mycobacterium tuberculosis*, demonstrating the stimulatory effect of tumour necrosis factor (TNF)-α and Th1 cytokines (IFN-γ and IFN-α) on autophagy in human and murine macrophages, human leukemic T lymphocytes, human vascular cells and rat epithelial cells (50, 51). In contrast, Th2 cytokines such as IL-4 and IL-13 antagonise autophagy through signal transducer and activator of transcription (STAT)-6 dependent stimulation of type I PI3-K, leading to the activation of mTOR, a serine/threonine protein kinase (50). Autophagy is regulated by cytokines, and this is not a unidirectional activity; the self-digestion process affects the transcription, selection and secretion of certain cytokines (51). Macrophages and dendritic cells with disrupted autophagy secrete IL-1β in response to TLR agonists (52). Rapamycin-induced autophagy inhibits release of IL-1β in murine dendritic cells activated by LPS, which establishes positive feedback for a self-sustaining inflammatory process.

Autophagy and susceptibility to autoimmune diseases

Aberrant autophagy may contribute to chronic inflammatory diseases and autoimmune diseases. There is a well-established connection between mutations in autophagy-related genes and Crohn's disease. Crohn's disease is a chronic inflammatory bowel disease associated with ulceration and neutrophil influx in the intestinal epithelia (53). Mutations in the autophagy-related genes autophagy-related 16-like 1 (ATG16L1) and immunity-related p47 GTPase family M (IRGM) are associated with an increased risk of Crohn's disease in humans (54-57). These two autophagy-related genes are believed to be important for both autophagy and antigen presentation (6). A single-nucleotide polymorphism (SNP) of ATG16L1 (threonine 300 is replaced with alanine, or T300A) has been associated with Crohn's disease, but the exact role of the human ATG16L1 protein and the consequences of this mutation have not been determined (58). One study suggest-

ed that dendritic cells expressing T300A from patients with Crohn's disease are defective in presenting bacterial antigen to $CD4^+$ T cells (49). Knocking out ATG16L1 in mice impairs autophagosome formation and enhances the generation of IL-1β by macrophages stimulated with endotoxin (59). ATG16L1-deficient mice die within the first day after delivery, but mice that express a low level of Atg16L1 exhibit Paneth cells with abnormal granule exocytosis and are more susceptible to experimentally induced colitis (60). Mice deficient in IRGM have a decreased capability to fight intracellular bacteria and therefore are more susceptible to infection (61). Two polymorphisms of IRGM have been correlated with Crohn's disease in humans (62). Another link between autophagy and pathogenesis in Crohn's disease has been demonstrated with the expression of NOD2 genetic variants (63). The induction of autophagy by muramyl dipeptide, a component of the bacterial peptidoglycan cell wall, requires NOD2-ATG16L1 interaction. NOD2 polymorphism associated with Crohn's disease results in impaired autophagy-dependent bacterial clearance and processing (64, 65).

Systemic lupus erythematosus (SLE) is a multifactorial disease with an unknown pathogenesis. The autophagy process has not yet been fully defined in SLE. Several SNPs in ATG5 (a key autophagy gene required for the formation of autophagosomes) seem to be predisposing factors for SLE. Loss of ATG5-dependent processes, including negative thymic selection, regulation of IFN and proinflammatory cytokine secretion, clearance of dying cells and antigen presentation, may contribute to autoimmunity and inflammation in SLE patients (66, 67). The impaired clearance of apoptotic cells may explain the accumulation of nuclear autoantigens in various tissues of SLE patients. It seems that dead cells are an abundant source of autoantigens in the setting of a clearance deficiency (68). Phagocytosis by macrophages (69) and phagocytosis by neutrophils are clearly depressed in SLE patients, and this deficient clearance of cellular debris may be a reason for the accumulation of apoptotic material in the tissues of certain SLE patients (68). Macroautophagy to remove cell debris may be insufficient. Autophagy was shown to be required for the activation of T cells and their survival after stimulation and differentiation (70, 71). A recent study clearly demonstrated that disrupted autophagy promoted the survival of autoreactive T cells in a lupus-prone mouse model and in lupus patients. One of the crucial roles of autophagy is in the regulation of peripheral T cell homeostasis in SLE patients (72). Other studies have suggested that an autophagy-independent role of ATG5 may contribute to the pathogenesis of SLE (73, 74).

Autophagy has a role in the pathophysiology of both type 1 and type 2 diabetes mellitus. It is assumed that the regulatory pathways of autophagy in insulin-generating β-cells in type 1 diabetes are different from those following the onset of insulin resistance in the tissues in type 2 diabetes. The role of autophagy in diabetes and metabolic disorders is critical because insulin and glucagon are well-known modulators of autophagy and are important to the function and survival of the pancreatic β-cells. Insulin and intracellular molecules such as mTOR inhibit autophagy, whereas glucagon stimulates autophagy. Recent studies have strongly suggested that basal autophagy is important in the maintenance of β-cell volume and function. Deficiency of autophagy can lead to islet degeneration and reduced insulin secretion, which proves the crucial role of autophagy in islet function and survival (75). Atg7-deficient mice showed a reduction in β-cell mass and pancreatic insulin content as well as hypoinsulinaemia and hyperglycaemia (25). In addition, the role of an important autophagy-related protein, SQSTM1/p62, has been shown. SQSTM1/p62 knockout in mice causes metabolic disorders and insulin resistance, leading to type 2 diabetes (76). Suppression of autophagy can lead to the accumulation of reactive oxygen species in the mitochondria, and this may cause initiation of early diabetic nephropathy. Because $CD4^+$ and $CD8^+$ T cells are effector cells in the autoimmune destruction of β-cells (77), autophagy may initiate the pathogenesis of type 1 diabetes by affecting the function of T cells.

The association between autophagy and autoimmunity has also been detected in multiple sclerosis (MS). Increased ATG5 expression was detected in blood samples from mice with experimental autoimmune encephalomyelitis and in T cells from active relapsing-remitting MS patients (78).

Concanavalin A (Con A) can induce T cell-dependent and T cell-independent acute hepatitis in both immunocompetent and immunodeficient mice (79). Hepatocyte injury in NKT/CD4+ T cell-mediated acute hepatitis is caused by apoptosis, whereas in Con A-induced T cell-independent acute hepatitis in SCID/NOD mice, hepatocyte death is mediated by autophagy (80). After binding to the mannose moiety of the cell membrane glycoprotein, Con A is internalised through endocytosis and accumulates preferentially on the mitochondria. Consequently, the mitochondrial membrane permeability is altered, and autophagy is activated, leading to lysosomal degradation of the affected mitochondria and caspase-independent cell death (80). Chang et al. reported that IFN-γ, a potent Th1 cytokine, enhances autophagic flux and causes necrotic-type cell death in Con A-treated hepatocytes (81). The same group showed that the liver's blood vessels are the first target in both T cell-independent and T cell-dependent hepatitis. The infused Con A bonds to the hepatic vascular endothelial cells and causes damage to the liver's blood vessels before the induction of T cell-independent hepatitis via autophagy (82). Con A induces autophagy of endothelial cells, and haemorrhage is also enhanced by IFN-γ (82). Necrostatin-1 (Nec-1), an inhibitor of programmed cell necrosis, inhibits Con A-induced acute liver injury and cell death. It seems that autophagy is one of the key mediators of this hepatoprotective effect. It was shown that autophagosome formation was significantly reduced following Nec-1 treatment, as was the expression of the autophagy-related proteins beclin-1 and LC3. The fact that Nec-1 treatment can reduce the level of autophagy in hepatocytes after Con A-induced injury provides new ideas and targets for the treatment of acute hepatitis (83). A recent study showed that metformin exacerbates Con A-induced hepatitis by promoting autophagy (84).

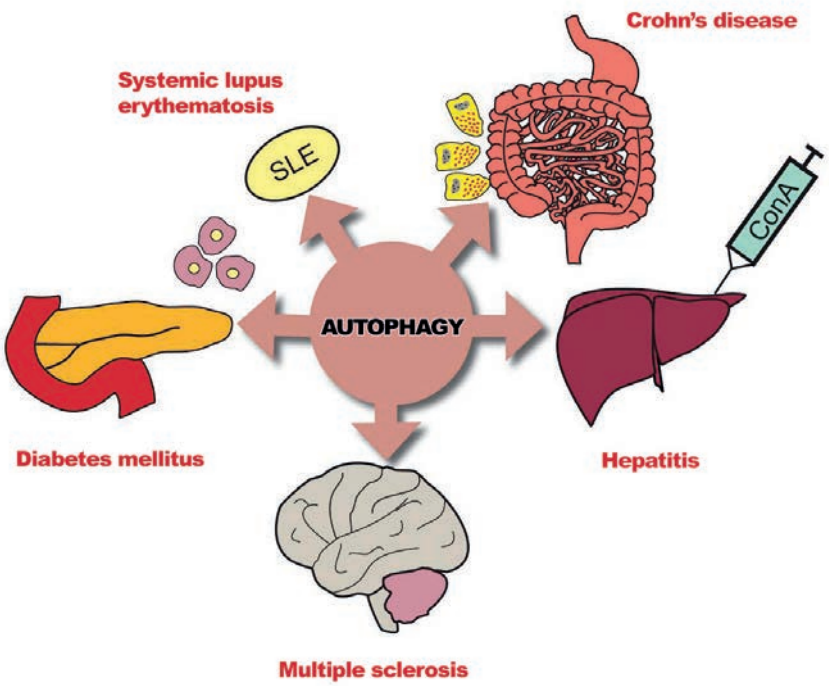


Figure 2. Pathological functions of autophagy.

CONCLUSION

Over the past few years, there have been numerous reports on the functional correlation between autophagy and autoimmune diseases. This is not surprising, given the role of autophagy in T cell homeostasis and function. The potential implications of autophagy in autoimmune diseases could also explain the beneficial therapeutic effect of chloroquine, an autophagy inhibitor, in SLE and rheumatoid arthritis. There is a need for more studies in this field because autophagy has different roles in autoimmune diseases, depending on the pathophysiology. Understanding the complex relationships between autophagy and autophagy-related genes in each autoimmune disease creates the possibility of developing more specific and effective therapeutic strategies.

CONFLICT OF INTEREST

The authors who have taken part in this study declare that they do not have anything to disclose regarding funding or a conflict of interest with respect to this manuscript.

ACKNOWLEDGEMENT

We thank Bojan Koturanovic for excellent technical assistance.

REFERENCES

1. Deretic, V., Autophagy in innate and adaptive immunity. Trends Immunol, 2005. 26(10): p. 523-8.
2. Deretic, V., Autophagy as an immune defense mechanism. Curr Opin Immunol, 2006. 18(4): p. 375-82.
3. Deretic, V., Multiple regulatory and effector roles of autophagy in immunity. Curr Opin Immunol, 2009. 21(1): p. 53-62.
4. Deretic, V., Autophagy as an innate immunity paradigm: expanding the scope and repertoire of pattern recognition receptors. Curr Opin Immunol, 2012. 24(1): p. 21-31.
5. Levine, B. and V. Deretic, Unveiling the roles of autophagy in innate and adaptive immunity. Nat Rev Immunol, 2007. 7(10): p. 767-77.
6. Levine, B., N. Mizushima, and H.W. Virgin, Autophagy in immunity and inflammation. Nature, 2011. 469(7330): p. 323-35.
7. Saitoh, T. and S. Akira, Regulation of innate immune responses by autophagy-related proteins. J Cell Biol, 2010. 189(6): p. 925-35.
8. Lleo, A., et al., Autophagy: highlighting a novel player in the autoimmunity scenario. J Autoimmun, 2007. 29(2-3): p. 61-8.
9. Chen, Y. and D.J. Klionsky, The regulation of autophagy - unanswered questions. J Cell Sci, 2011. 124(Pt 2): p. 161-70.
10. He, C. and D.J. Klionsky, Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet, 2009. 43: p. 67-93.

11. Levine, B. and G. Kroemer, Autophagy in the pathogenesis of disease. Cell, 2008. 132(1): p. 27-42.
12. Meijer, A.J. and P. Codogno, Autophagy: regulation by energy sensing. Curr Biol, 2011. 21(6): p. 007.
13. Mizushima, N., Autophagy: process and function. Genes Dev, 2007. 21(22): p. 2861-73.
14. Mizushima, N. and M. Komatsu, Autophagy: renovation of cells and tissues. Cell, 2011. 147(4): p. 728-41.
15. Rubinsztein, D.C., T. Shpilka, and Z. Elazar, Mechanisms of autophagosome biogenesis. Curr Biol, 2012. 22(1): p. 034.
16. Wirawan, E., et al., Autophagy: for better or for worse. Cell Res, 2012. 22(1): p. 43-61.
17. Mizushima, N., T. Yoshimori, and Y. Ohsumi, The role of Atg proteins in autophagosome formation. Annu Rev Cell Dev Biol, 2011. 27: p. 107-32.
18. Jung, C.H., et al., ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. Mol Biol Cell, 2009. 20(7): p. 1992-2003.
19. Mizushima, N., The role of the Atg1/ULK1 complex in autophagy regulation. Curr Opin Cell Biol, 2010. 22(2): p. 132-9.
20. Itakura, E. and N. Mizushima, Characterization of autophagosome formation site by a hierarchical analysis of mammalian Atg proteins. Autophagy, 2010. 6(6): p. 764-76.
21. Geng, J. and D.J. Klionsky, The Golgi as a potential membrane source for autophagy. Autophagy, 2010. 6(7): p. 950-1.
22. Hailey, D.W., et al., Mitochondria supply membranes for autophagosome biogenesis during starvation. Cell, 2010. 141(4): p. 656-67.
23. Tooze, S.A. and T. Yoshimori, The origin of the autophagosomal membrane. Nat Cell Biol, 2010. 12(9): p. 831-5.
24. Tanida, I., T. Ueno, and E. Kominami, LC3 conjugation system in mammalian autophagy. Int J Biochem Cell Biol, 2004. 36(12): p. 2503-18.
25. Jung, C.H., et al., mTOR regulation of autophagy. FEBS Lett, 2010. 584(7): p. 1287-95.
26. Ravikumar, B., et al., Regulation of mammalian autophagy in physiology and pathophysiology. Physiol Rev, 2010. 90(4): p. 1383-435.
27. Johansen, T. and T. Lamark, Selective autophagy mediated by autophagic adapter proteins. Autophagy, 2011. 7(3): p. 279-96.
28. Yang, Z.J., et al., The role of autophagy in cancer: therapeutic implications. Mol Cancer Ther, 2011. 10(9): p. 1533-41.
29. Berry, D.L. and E.H. Baehrecke, Autophagy functions in programmed cell death. Autophagy, 2008. 4(3): p. 359-60.
30. Tovilovic G, Ristic B, Siljic M et al. mTOR-independent autophagy counteracts apoptosis in herpes simplex virus type 1-infected U251 glioma cells. Microbes Infect. 2013 Jul-Aug;15(8-9):615-24.
31. Krmpot AJ, Janjetovic KD, Misirkic MS et al. Protective effect of autophagy in laser-induced glioma cell death in vitro. Lasers Surg Med. 2010 Apr;42(4):338-47.
32. Harhaji-Trajkovic L, Vilimanovich U, Kravic-Stevovic T, Bumbasirevic V, Trajkovic V. AMPK-mediated autophagy inhibits apoptosis in cisplatin-treated tumour cells. J Cell Mol Med. 2009 Sep;13(9B):3644-54.
33. Levine, B., Eating oneself and uninvited guests: autophagy-related pathways in cellular defense. Cell, 2005. 120(2): p. 159-62.
34. Delgado, M., et al., Autophagy and pattern recognition receptors in innate immunity. Immunol Rev, 2009. 227(1): p. 189-202.
35. Dupont, N., N. Temime-Smaali, and F. Lafont, How ubiquitination and autophagy participate in the regulation of the cell response to bacterial infection. Biol Cell, 2010. 102(12): p. 621-34.
36. Sumpter, R., Jr. and B. Levine, Autophagy and innate immunity: triggering, targeting and tuning. Semin Cell Dev Biol, 2010. 21(7): p. 699-711.
37. Cemma, M., P.K. Kim, and J.H. Brumell, The ubiquitin-binding adaptor proteins p62/SQSTM1 and NDP52 are recruited independently to bacteria-associated microdomains to target Salmonella to the autophagy pathway. Autophagy, 2011. 7(3): p. 341-5.
38. Bertin, S. and V. Pierrefite-Carle, Autophagy and toll-like receptors: a new link in cancer cells. Autophagy, 2008. 4(8): p. 1086-9.
39. Virgin, H.W. and B. Levine, Autophagy genes in immunity. Nat Immunol, 2009. 10(5): p. 461-70.
40. Shi, C.S., et al., Activation of autophagy by inflammatory signals limits IL-1beta production by targeting ubiquitinated inflammasomes for destruction. Nat Immunol, 2012. 13(3): p. 255-63.
41. Nakahira, K., et al., Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nat Immunol, 2011. 12(3): p. 222-30.
42. Paludan, C., et al., Endogenous MHC class II processing of a viral nuclear antigen after autophagy. Science, 2005. 307(5709): p. 593-6.
43. Lee, H.K., et al., In vivo requirement for Atg5 in antigen presentation by dendritic cells. Immunity, 2010. 32(2): p. 227-39.
44. Gannage, M., R.B. da Silva, and C. Munz, Antigen processing for MHC presentation via macroautophagy. Methods Mol Biol, 2013. 960: p. 473-88.
45. Crotzer, V.L. and J.S. Blum, Autophagy and adaptive immunity. Immunology, 2010. 131(1): p. 9-17.
46. Nedjic, J., M. Aichinger, and L. Klein, Autophagy and T cell education in the thymus: eat yourself to know yourself. Cell Cycle, 2008. 7(23): p. 3625-8.
47. Pattingre, S., et al., Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell, 2005. 122(6): p. 927-39.
48. Choi, A.J. and S.W. Ryter, Autophagy in inflammatory diseases. Int J Cell Biol, 2011. 732798(10): p. 30.
49. Cooney, R., et al., NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. Nat Med, 2010. 16(1): p. 90-7.

50. Harris, J., et al., T helper 2 cytokines inhibit autophagic control of intracellular Mycobacterium tuberculosis. Immunity, 2007. 27(3): p. 505-17.
51. Harris, J., Autophagy and cytokines. Cytokine, 2011. 56(2): p. 140-4.
52. Crisan, T.O., et al., Inflammasome-independent modulation of cytokine response by autophagy in human cells. PLoS One, 2011. 6(4): p. 0018666.
53. Podolsky, D.K., Inflammatory bowel disease. N Engl J Med, 2002. 347(6): p. 417-29.
54. Parkes, M., et al., Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. Nat Genet, 2007. 39(7): p. 830-2.
55. Xavier, R.J., A. Huett, and J.D. Rioux, Autophagy as an important process in gut homeostasis and Crohn's disease pathogenesis. Gut, 2008. 57(6): p. 717-20.
56. Barrett, J.C., et al., Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. Nat Genet, 2008. 40(8): p. 955-62.
57. Cadwell, K., Crohn's disease susceptibility gene interactions, a NOD to the newcomer ATG16L1: Gastroenterology. 2010 Nov;139(5):1448-50. doi: 10.1053/j.gastro.2010.09.023. Epub 2010 Sep 25.
58. Hampe, J., et al., A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. Nat Genet, 2007. 39(2): p. 207-11.
59. Saitoh, T., et al., Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. Nature, 2008. 456(7219): p. 264-8.
60. Cadwell, K., et al., A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. Nature, 2008. 456(7219): p. 259-63.
61. Feng, C.G., et al., Mice deficient in LRG-47 display increased susceptibility to mycobacterial infection associated with the induction of lymphopenia. J Immunol, 2004. 172(2): p. 1163-8.
62. McCarroll, S.A., et al., Deletion polymorphism upstream of IRGM associated with altered IRGM expression and Crohn's disease. Nat Genet, 2008. 40(9): p. 1107-12.
63. Travassos, L.H., et al., Nod proteins link bacterial sensing and autophagy. Autophagy, 2010. 6(3): p. 409-11.
64. Travassos, L.H., et al., Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. Nat Immunol, 2010. 11(1): p. 55-62.
65. Plantinga, T.S., et al., Crohn's disease-associated ATG16L1 polymorphism modulates pro-inflammatory cytokine responses selectively upon activation of NOD2. Gut, 2011. 60(9): p. 1229-35.
66. Harley, I.T., et al., Genetic susceptibility to SLE: new insights from fine mapping and genome-wide association studies. Nat Rev Genet, 2009. 10(5): p. 285-90.
67. Zhou, X.J., et al., Genetic association of PRDM1-ATG5 intergenic region and autophagy with systemic lupus erythematosus in a Chinese population. Ann Rheum Dis, 2011. 70(7): p. 1330-7.
68. Munoz, L.E., et al., SLE--a disease of clearance deficiency? Rheumatology, 2005. 44(9): p. 1101-7.
69. Herrmann, M., et al., Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus. Arthritis Rheum, 1998. 41(7): p. 1241-50.
70. Arsov, I., et al., A role for autophagic protein beclin 1 early in lymphocyte development. J Immunol, 2011. 186(4): p. 2201-9.
71. Pua, H.H., et al., A critical role for the autophagy gene Atg5 in T cell survival and proliferation. J Exp Med, 2007. 204(1): p. 25-31.
72. Gros, F., et al., Macroautophagy is deregulated in murine and human lupus T lymphocytes. Autophagy, 2012. 8(7): p. 1113-23.
73. Yousefi, S., et al., Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. Nat Cell Biol, 2006. 8(10): p. 1124-32.
74. Zhao, Z., et al., Autophagosome-independent essential function for the autophagy protein Atg5 in cellular immunity to intracellular pathogens. Cell Host Microbe, 2008. 4(5): p. 458-69.
75. Jung, H.S., et al., Loss of autophagy diminishes pancreatic beta cell mass and function with resultant hyperglycemia. Cell Metab, 2008. 8(4): p. 318-24.
76. Okada, K., et al., The alpha-glucosidase inhibitor acarbose prevents obesity and simple steatosis in sequestosome 1/A170/p62 deficient mice. Hepatol Res, 2009. 39(5): p. 490-500.
77. Wang, Y., et al., The role of CD4+ and CD8+ T cells in the destruction of islet grafts by spontaneously diabetic mice. Proc Natl Acad Sci U S A, 1991. 88(2): p. 527-31.
78. Alirezaei, M., et al., Elevated ATG5 expression in autoimmune demyelination and multiple sclerosis. Autophagy, 2009. 5(2): p. 152-8.
79. Chang, C.P. and H.Y. Lei, Autophagy induction in T cell-independent acute hepatitis induced by concanavalin A in SCID/NOD mice. Int J Immunopathol Pharmacol, 2008. 21(4): p. 817-26.
80. Lei, H.Y. and C.P. Chang, Induction of autophagy by concanavalin A and its application in anti-tumor therapy. Autophagy, 2007. 3(4): p. 402-4.
81. Chang, C.P., M.C. Yang, and H.Y. Lei, Concanavalin A/IFN-gamma triggers autophagy-related necrotic hepatocyte death through IRGM1-mediated lysosomal membrane disruption. PLoS One, 2011. 6(12): p. 5.
82. Yang, M.C., C.P. Chang, and H.Y. Lei, Endothelial cells are damaged by autophagic induction before hepatocytes in Con A-induced acute hepatitis. Int Immunol, 2010. 22(8): p. 661-70.
83. Zhou, Y., et al., Protective effects of necrostatin-1 against concanavalin A-induced acute hepatic injury in mice. Mediators Inflamm, 2013. 706156(10): p. 1.
84. Volarevic V, Misirkic M, Vucicevic L et al. Metformin aggravates immune-mediated liver injury in mice. Arch Toxicol. 2014 Apr 26.

INSTRUCTION TO AUTHORS FOR MANUSCRIPT PREPARATION

Serbian Journal of Experimental and Clinical Research is a peer-reviewed, general biomedical journal. It publishes original basic and clinical research, clinical practice articles, critical reviews, case reports, evaluations of scientific methods, works dealing with ethical and social aspects of biomedicine as well as letters to the editor, reports of association activities, book reviews, news in biomedicine, and any other article and information concerned with practice and research in biomedicine, written in the English.

Original manuscripts will be accepted with the understanding that they are solely contributed to the Journal. The papers will be not accepted if they contain the material that has already been published or has been submitted or accepted for publication elsewhere, except of preliminary reports, such as an abstract, poster or press report presented at a professional or scientific meetings and not exceeding 400 words. Any previous publication in such form must be disclosed in a footnote. In rare exceptions a secondary publication will acceptable, but authors are required to contact Editor-in-chief before submission of such manuscript. the Journal is devoted to the Guidelines on Good Publication Practice as established by Committee on Publication Ethics-COPE (posted at www.publicationethics.org.uk).

Manuscripts are prepared in accordance with „Uniform Requirements for Manuscripts submitted to Biomedical Journals“ developed by the International Committee of Medical Journal Editors. Consult a current version of the instructions, which has been published in several journals (for example: Ann Intern Med 1997;126:36-47) and posted at www.icmje.org, and a recent issue of the Journal in preparing your manuscript. For articles of randomized controlled trials authors should refer to the „Consort statement“ (www.consort-statement.org). Manuscripts must be accompanied by a cover letter, signed by all authors, with a statement that the manuscript has been read and approved by them, and not published, submitted or accepted elsewhere. Manuscripts, which are accepted for publication in the Journal, become the property of the Journal, and may not be published anywhere else without written permission from the publisher.

Serbian Journal of Experimental and Clinical Research is owned and published by Medical Faculty University of Kragujevac. However, Editors have full academic freedom and authority for determining the content of the journal, according to their scientific, professional and ethical judgment. Editorial policy and decision making follow procedures which are endeavoring to ensure scientific credibility of published content, confidentiality and integrity of auth ors, reviewers, and review process, protection of patients' rights to privacy and disclosing of conflict of interests. For difficulties which might appear in the Journal content such as errors in published articles or scientific concerns about research findings, appropriate handling is provided. The requirements for the content, which appears on the Journal internet site or Supplements, are, in general, the same as for the master version. Advertising which appears in the Journal or its internet site is not allowed to influence editorial decisions.

MANUSCRIPT

Manuscripts for Serbian Journal of Experimental and Clinical Research are available for submission through the Editorial Manager System http://www.editorialmanager.com/sjecr/.

For papers that are accepted, Serbian Journal of Experimental and Clinical Research obligatory requires authors to provide an identical, electronic copy in appropriate textual and graphic format.

The manuscript of original, scinetific articles should be arranged as following: Title page, Abstract, Introduction, Patients and methods/Material and methods, Results, Discussion, Acknowledgements, References, Tables, Figure legends and Figures. The sections of other papers should be arranged according to the type of the article.

Each manuscript component (The Title page, etc.) should begins on a separate page. All pages should be numbered consecutively beginning with the title page.

All measurements, except blood pressure, should be reported in the System International (SI) units and, if necessary, in conventional units, too (in parentheses). Generic names should be used for drugs. Brand names may be inserted in parentheses.

Authors are advised to retain extra copies of the manuscript. Serbian Journal of Experimental and Clinical Research is not responsible for the loss of manuscripts in the mail.

TITLE PAGE

The Title page contains the title, full names of all the authors, names and full location of the department and institution where work was performed, abbreviations used, and the name of corresponding author.

The title of the article should be concise but informative, and include animal species if appropriate. A subtitle could be added if necessary.

A list of abbreviations used in the paper, if any, should be included. The abbreviations should be listed alphabetically, and followed by an explanation of what they stand for. In general, the use of abbreviations is discouraged unless they are essential for improving the readability of the text.

The name, telephone number, fax number, and exact postal address of the author to whom communications and reprints should be sent are typed et the end of the title page.

ABSTRACT

An abstract of less than 250 words should concisely state the objective, findings, and conclusions of the studies described in the manuscript. The abstract does not contain abbreviations, footnotes or references.

Below the abstract, 3 to 8 keywords or short phrases are provided for indexing purposes. The use of words from Medline thesaurus is recommended.

INTRODUCTION

The introduction is concise, and states the reason and specific purpose of the study.

PATIENTS AND METHODS/MATERIAL AND METHODS

The selection of patients or experimental animals, including controls, should be described. Patients' names and hospital numbers are not used.

Methods should be described in sufficient detail to permit evaluation and duplication of the work by other investigators.

When reporting experiments on human subjects, it should be indicated whether the procedures followed were in accordance with ethical standards of the Committee on human experimentation (or Ethics Committee) of the institution in which they were done and in accordance with the Helsinki Declaration. Hazardous procedures or chemicals, if used, should be described in details, including the safety precautions observed. When appropriate, a statement should be included verifying that the care of laboratory animals followed accepted standards.

Statistical methods used should be outlined.

RESULTS

Results should be clear and concise, and include a minimum number of tables and figures necessary for proper presentation.

DISCUSSION

An exhaustive review of literature is not necessary. The major findings should be discussed in relation to other published work. Attempts should be made to explain differences between the results of the present study and those of the others. The hypothesis and speculative statements should be clearly identified. The Discussion section should not be a restatement of results, and new results should not be introduced in the discussion.

ACKNOWLEDGMENTS

This section gives possibility to list all persons who contributed to the work or prepared the manuscript, but did not meet the criteria for authorship. Financial and material support, if existed, could be also emphasized in this section.

REFERENCES

References should be identified in the text by Arabic numerals in parentheses. They should be numbered consecutively, as they appeared in the text. Personal communications and unpublished observations should not be cited in the reference list, but may be mentioned in the text in parentheses. Abbreviations of journals should conform to those in Index Serbian Journal of Experimental and Clinical Research. The style and punctuation should conform to the Serbian Journal of Experimental and Clinical Research style requirements. The following are examples:

1. Introduction

This document describes standards for preparing the references in the APA style. The following sections give detailed instructions on citing books, journal articles, newspaper articles, conference papers, theses, webpages and others.

Please provide all the required elements in the references to your paper. Please pay particular attention to spelling, capitalization and punctuation. Accuracy and completeness of references are the responsibilities of the author. Before submitting your article, please ensure you have checked your paper for any relevant references you may have missed.

A complete reference should give the reader enough information to find the relevant article. And most importantly, complete and correct references may allow automatic creation of active links by the MetaPress technology that we use for making the electronic version of our journal. Active reference linking is regarded as the greatest benefit of electronic publishing and it adds a lot of value to your publication.

2.Book

a. Book (one author)

Format:

Author. (Year of publication). *Book title*. Place of publication: Publisher.

Example:

Baxter, R. (1982). *Exactly Solvable Models in Statistical Mechanics.* New York: Academic Press.

b. Book (two or more authors)

Format:

Author1, Author2 & Author3. (Year of publication). *Book title*. Place of publication: Publisher.

Example:

Kleiner, F.S., Mamiya C.J. & Tansey R.G. (2001). *Gardner's art through the ages* (11th ed.). Fort Worth, USA: Harcourt College Publishers.

c. Book chapter or article in an edited book

Format:

Author(s) of chapter. (Year of publication). Chapter title. In Editors of the book (Eds.), *Book title* (Chapter page range). Place of publication: Publisher.

Example:

Roll, W.P. (1976). ESP and memory. In J.M.O. Wheatley & H.L. Edge (Eds.), *Philosophical dimensions of parapsychology* (pp. 154-184). Springfield, IL: American Psychiatric Press.

d. Proceedings from a conference

Format:

Author(s). (Year of publication). Title. In Conference name, Date (Page range). Place of publication: Publisher.

Example:

Field, G. (2001). Rethinking reference rethought. In Revelling in Reference: Reference and Information Services Section Symposium, 12-14 October 2001 (pp. 59-64). Melbourne, Victoria, Australia: Australian Library and Information Association.

e. ebook

Format:

Author(s). (Year of publication). *Title*. Publisher. Retrieving date, http address. DOI.

Example:

Johnson, A. (2000). *Abstract Computing Machines.* Springer Berlin Heidelberg. Retrieved March 30, 2006, from SpringerLink http://springerlink.com/content/w25154. DOI: 10.1007/b138965.

f. Thesis

Format:

Author(s). (Year of publication). *Title*. Information, Place of publication.

Example:

Begg, M. M. (2001). *Dairy farm women in the Waikato 1946-1996: Fifty years of social and structural change*. Unpublished doctoral dissertation, University of Waikato, Hamilton, New Zealand.

g. Report

Format:

Author(s). (Year of publication). *Title*. Place of publication: Publisher. (Report number)

Example:

Osgood, D. W., & Wilson, J. K. (1990). *Covariation of adolescent health problems*. Lincoln: University of Nebraska. (NTIS No. PB 91-154 377/AS)

h. Government publication

Format:

Institution name. (Year of publication). *Title.* Place of publication: Publisher.

Example:

Ministerial Council on Drug Strategy. (1997). *The national drug strategy: Mapping the future*. Canberra: Australian Government Publishing Service.

3. Article

a. Journal Article (one author)

Format:

Author. (Year of publication). Article title. *Journal Title. Volume* (issue), range of pages. DOI.

Example:

Nikora, V. (2006). Hydrodynamics of aquatic ecosystems: spatial-averaging perspective. *Acta Geophysica*, 55(1), 3-10. DOI: 10.2478/s11600-006-0043-6.

b. Journal Article (two or more authors)

Format:

Author1, Author2 & Author3. (Year of publication). Article title. *Journal Title. Volume* (issue), range of pages. DOI.

Example:

Cudak, M. & Karcz J. (2006). Momentum transfer in an agitated vessel with off-centred impellers. *Chem. Pap.* 60(5), 375-380. DOI: 10.2478/s11696-006-0068-y.

c. Journal article from an online database

Format:

Author(s). (Year of publication). Article title [Electronic version]. *Journal Title. Volume* (issue), range of pages. Retrieved date of access, from name of database. DOI.

Example:

Czajgucki Z., Zimecki M. & Andruszkiewicz R. (2006, December). The immunoregulatory effects of edeine analogues in mice [Abstract]. *Cell. Mol. Biol. Lett.* 12(3), 149-161. Retrieved December 6, 2006, from PubMed database on the World Wide Web: http://www.pubmed.gov. DOI: 10.2478/s11658-006-0061-z.

d. Newspaper article (no author)

Format:

Article title. (Publication date). Journal Title. page.

Example:

Amazing Amazon region. (1989, January 12). New York Times, p. D11.

e. Encyclopedia article

Format:

Author. (Year of publication). Article title. In Encyclopedia title (volume number, pages). Place of publication: Encyclopedia name.

Example:

Bergmann, P. G. (1993). Relativity. In The new encyclopedia britannica (Vol. 26, pp. 501-508). Chicago: Encyclopedia Britannica.

4. Other formats

a. Web page

Format:

Author/Sponsor. (last update or copyright date). *Title*. Retrieved date of access, from URL.

Example:

Walker, J. (1996, August). *APA-style citations of electronic resources*. Retrieved November 21, 2001, from http://www.cas.usf.edu/english/walker/apa.html

b. Lecture note

Format:

Author(s). (Date of presentation). *Lecture title*. Lecture notes distributed in the unit, at the name of the teaching organisation, the location.

Example:

Liffers, M. (2006, August 30). *Finding information in the library*. Lecture notes distributed in the unit Functional Anatomy and Sports Performance 1102, University of Western Australia, Crawley, Western Australia.

c. Patent

Format:

Author. (Year). Patent number. The location. Issue body.

Example:

Smith, I. M. (1988). U.S. Patent No. 123,445. Washington, D.C.: U.S. Patent and Trademark Office.

d. Standard

Format:

Issue body. (Year). Standard name. Standard number. The location.

Example:

Standards Association of Australia. (1997). Australian standard: Pressure equipment manufacture. AS4458-1997. North Sydney.

e. Video

Format:

Producer, P. P. (Producer), & Director, D.D. (Director). (Date of publication). Title of motion picture [Motion picture]. Country of origin: Studio or distributor.

Example:

Zhang, Y. (Producer/Director). (2000). Not one less [Motion Picture].China: Columbia Pictures Industries, Inc.

f. Audio recording

Format:

Songwriter, W. W. (Date of copyright). Title of song [Recorded by artist if different from song writer]. On Title of album [Medium of recording]. Location: Label. (Recording date if different from copyright date).

Example:

Taupin, B. (1975). Someone saved my life tonight [Recorded by Elton John]. On Captain fantastic and the brown dirt cowboy [CD]. London: Big Pig Music Limited.

g. Mailing list

Format:

Author. (Exact date of posting). Subject line of message. Message posted to followed by name of mailing list, archived at followed by address for the archived version of the message

Example:

Hammond, T. (2000, November 20). YAHC: Handle Parameters, DOI Genres, etc. Message posted to Ref-Links electronic mailing list, archived at http://www.doi.org/mail-archive/ref-link/msg00088.html

h. Computer software

Format:

Author(s). (Year). Title [computer software]. The location: Company.

Example:

Ludwig, T. (2002). PsychInquiry [computer software]. New York: Worth.

MOST COMMON REFERENCE STYLES

MetaPress can capture data from every style of references, but using one of the listed will increase the number of active links in the references. Once you have chosen one of the styles, please do not change it.

APA style[1]

Article in a journal:

Lippke, S., & Ziegelmann, J. (2006). Understanding and modelling health behaviour change: The multi-stage model of health behaviour change. *Journal of Health Psychology*,*11*(1), 37-50,
DOI:10.2478/s11533-007-0023-3.

Book:

Jones, E., Farina, A., Hastorf, A., Markus, H., Miller, D., & Scott, R. (1984). *Social stigma: The psychology of marked relationships*. New York: W. H. Freeman.

Chicago style[2]

Article in a journal:

Spitzer, Steven. Review of *The Limits of Law Enforcement*, by Hans Zeisel. *American Journal of Sociology* 91 (1985): 726-29; DOI:10.2478/s11533-007-0023-3.

Book:

Lloyd, Donald A., and Harry R. Warfel. *American English and Its Cultural Setting*. New York: Alfred A. Knopf, 1956.

Harvard style[3]

Article in a journal:

Conley, TG & Galenson, DW 1998, 'Nativity and wealth in mid-nineteenth century cities', *Journal of Economic History*, vol. 58, no. 2, pp. 468-493, DOI:10.2478/s11533-007-0023-3.

Book:

Hodgson, A 1998, *Accounting theory*, John Wiley & Sons, Brisbane.

Oxford style[4]

Article in a journal:

KHOO, G.K. Accounting for leases. *The Chartered Accountant in Australia*, 46(5): Nov. 1975: 19-23: DOI:10.2478/s11533-007-0023-3.

Book:

GIBBS, Graham. *Teaching students to learn: a student-centred approach*. Milton Keynes, Open University Press, 1981.

MLA style[5]

Article in a journal:

Joyce, Michael. "On the Birthday of the Stranger (in Memory of John Hawkes)." Evergreen Review 5 Mar. 1999. 12 May 1999 http://www.evergreenreview.com/102/evexcite/joyce/nojoyce.html. DOI:10.2478/s11533-007-0023-3.

Book:

Bird, Isabella L. A Lady's Life in the Rocky Mountains. New York, 1881. Victorian Women Writers Project. Ed. Perry Willett. 27 May 1999. Indiana U. 4 Oct. 1999

IEE style[6]

Article in a journal:

I.E. Sutherland, R.F. Sproull, and R.A. Schumaker, "A Characterization of 10 Hidden-Surface Algorithms," *ACM Computing Surveys*, Mar. 1974, pp. 1-55, DOI:10.2478/s11533-007-0023-3.

Book:

W.M. Newman and R.F. Sproull, *Principles of Interactive Computer Graphics*, McGraw-Hill, 1979, p. 402.

Vancouver style[7]

Article in a journal:

You CH, Lee KY, Chey WY, Menguy R. Electrogastrographic study of patients with unexplained nausea, bloating and vomiting. Gastroenterology 1980;79:311-4; DOI:10.2478/s11533-007-0023-3.

Book:

Eisen HN. Immunology: an introduction to molecular and cellular principles of the immune response. 5th ed. New York: Harper and Row; 1974.

TABLES

Tables should be typed on separate sheets with table numbers (Arabic) and title above the table and explanatory notes, if any, below the table.

[1] Read more: http://www.library.uwa.edu.au/education_training___and___support/guides/how_to_cite_your_sources/apa_style

[2] Read more: http://www.wisc.edu/writing/Handbook/DocChiWorksCited.html

[3] Read more: http://www.library.uwa.edu.au/education_training___and___support/guides/how_to_cite_your_sources/citing_your_sources_-_harvard_style#Reference

[4] Read more: http://www.usq.edu.au/library/help/ehelp/ref_guides/oxford.htm

[5] Read more: http://www.bedfordstmartins.com/online/cite5.html

[6] Read more: http://www.computer.org/portal/site/ieeecs/menuitem.c5efb9b8ade9096b8a9ca0108bcd45f3/index.jsp?&pName=ieeecs_level1&path=ieeecs/publications/author/style&file=refer.xml&xsl=generic.xsl&

[7] Read more: http://www.library.uwa.edu.au/education_training___and___support/guides/how_to_cite_your_sources/citing_your_sources_-_vancouver_style

FIGURES AND FIGURE LEGENDS

All illustrations (photographs, graphs, diagrams) will be considered as figures, and numbered consecutively in Arabic numerals. The number of figures included should be the least required to convey the message of the paper, and no figure should duplicate the data presented in the tables or text. Figures should not have titles. Letters, numerals and symbols must be clear, in proportion to each other, and large enough to be readable when reduced for publication. Figures should be submitted as near to their printed size as possible. Figures are reproduced in one of the following width sizes: 8 cm, 12 cm or 17 cm, and with a maximal length of 20 cm. Legends for figures should be given on separate pages.

If magnification is significant (photomicrographs) it should be indicated by a calibration bar on the print, not by a magnification factor in the figure legend. The length of the bar should be indicated on the figure or in the figure legend.

Two complete sets of high quality unmounted glossy prints should be submitted in two separate envelopes, and shielded by an appropriate cardboard. The backs of single or grouped illustrations (plates) should bear the first authors last name, figure number, and an arrow indicating the top. This information should be penciled in lightly or placed on a typed self-adhesive label in order to prevent marking the front surface of the illustration.

Photographs of identifiable patients must be accompanied by written permission from the patient.

For figures published previously the original source should be acknowledged, and written permission from the copyright holder to reproduce it submitted.

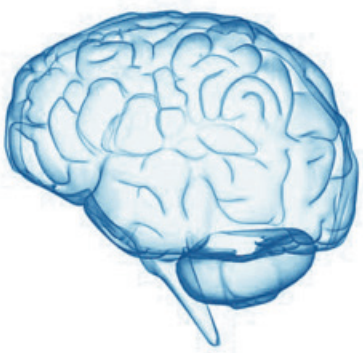
Color prints are available by request at the authors expense.

LETTERS TO THE EDITOR

Both letters concerning and those not concerning the articles that have been published in Serbian Journal of Experimental and Clinical Research will be considered for publication. They may contain one table or figure and up to five references.

PROOFS

All manuscripts will be carefully revised by the publisher desk editor. Only in case of extensive corrections will the manuscript be returned to the authors for final approval. In order to speed up publication no proof will be sent to the authors, but will be read by the editor and the desk editor.



CIP - Каталогизација у публикацији
Народна библиотека Србије, Београд

61

SERBIAN Journal of Experimental and Clinical Research
editor-in-chief Vladimir Jakovljević.
- Vol. 9, N° 1 (April 2008) -
- Kragujevac (Svetozara Markovića 69) :
Medical Faculty, 2008 - (Kragujevac : Medical Faculty). - 29 cm

Je nastavak: Medicus (Kragujevac) = ISSN 1450-7994
ISSN 1820-8665 = Serbian Journal of
Experimental and Clinical Research
COBISS.SR-ID 149695244

Serbian Journal of Experimental and Clinical Research

2000

FACULTY OF MEDICAL SCIENCES

Svetozara Markovica 69, 34000 Kragujevac, SERBIA

P.O. Box 124

Tel. +381 (0)34 30 68 00 • Tfx. +381 (0)34 30 68 00 ext. 112

e-mail: sjecr@medf.kg.ac.rs

www.medf.kg.ac.rs