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Building a fairer, healthier world for everyone!

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ORIGINAL ARTICLES / ORIGINALNI RADOVI

Building a fairer, healthier world for everyone!

Every year, on the day when the World Health Organization was founded, the World Health Day is celebrated with the aim of promoting the basic vision of this organization that every individual has the right to achieve the highest possible level of health care.
In recent months, the COVID-19 pandemic has jeopardized the progress made so far in preserving and improving the health of people around the world, pushing many into poverty and exacerbating gender, social, and health inequalities. Therefore, the main motto of this year's World Health Day is - Building a fairer and healthier world for everyone!

Svake godine, 7. aprila, na dan osnivanja Svetske zdravstvene organizacije, obeležava se Svetski dan zdravlja sa ciljem promovisanja osnovne vizije ove organizacije da svaki pojedinac ima pravo na ostvarenje najvišeg mogućeg nivoa zdravstvene zaštite.

Poslednjih meseci, pandemija COVID-19 ugrozila je ostvareni napredak u očuvanju i unapređenju zdravlja ljudi širom sveta, gurnula mnoge u siromaštvo i pojačala rodne, društvene i zdravstvene nejednakosti. Stoga je glavni moto ovogodišnjeg Svetskog dana zdravlja - Izgradnja pravednijeg i zdravijeg sveta za svakog!

ORIGINAL ARTICLES

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Differences in autonomic heart rate modulation during rest and after a supramaximal anaerobic test in relation to gender and the menstrual cycle in women

Razlike u autonomnoj modulaciji srčanog ritma u stanju mirovanja i nakon supramaksimalnog anaerobnog testa u odnosu na pol i menstrualni ciklus žena

Lana Andrić*, Vedrana Karan†, Nikola Radulović‡, Miodrag Drapšin†‡, Dea Karaba Jakovljević†‡, Aleksandar Takači§, Aleksandar Klašnja†‡

Health Center "Bečej", *Department of Women and Children's Health Care, Bečej, Serbia; University of Novi Sad, Faculty of Medicine, †Department of Physiology, ‡Faculty of Sport and Physical Education, §Faculty of Technology, Novi Sad, Serbia

Abstract

Background/Aim. Heart rate variability (HRV) and heart rate recovery (HRR) show differences between genders, and dissimilarities were also reported in women in various menstrual cycle (MC) phases. The aim of this research was to analyze cardiac autonomic indices during rest and in recovery after the Wingate test between genders in the young, sedentary population and to investigate whether a MC phase in women can influence these indices. **Methods.** Twenty-five females (20.5 ± 0.7 years) and sixteen males (20.4 ± 0.7 years) performed the Wingate anaerobic test on a cycle ergometer while their HRR and resting and recovery HRV indices were obtained. In females, data were collected during three distinctive MC phases. **Results**. The natural logarithm of low-frequency (lnLF) HRV marker and the natural logarithm of high-frequency (lnHF) HRV marker were higher in males during rest compared to women in all MC phases, except in the late follicular phase, where no differences in lnHF between genders were observed. Markedly higher lnLF and lnHF were recorded in males after the Wingate test. There were no differences in HRV between women in various MC phases during rest. Surprisingly, parasympathetic time-domain marker (the square root of the mean squared differences of successive NN intervals, RMSSD) and lnLF were both higher in the early follicular phase in comparison to the luteal phase of MC during recovery. HRR was faster in men in comparison to women in all MC phases. **Conclusion.** Males show greater HRR and total variability during rest and recovery, but it appears that resting parasympathetic activity is similar when females are in the late follicular phase of MC. Intra-female resting autonomic variability is not affected by the sex hormonal cycle. Post-exercise HRV in the early follicular phase reflects a significantly favourable autonomic profile in comparison to the luteal phase of MC.

Key words:
heart rate; autonomic nervous system; exercise test.

Apstrakt

Uvod/Cilj. Razlike u varijabilnosti srčane frekvencije (VSF) i oporavku srčanog ritma (OSR) postoje kako između polova, tako i među ženama u različitim fazama menstrualnog ciklusa (MC). Cilj istraživanja bio je da se ispitaju autonomni indeksi u stanju mirovanja i tokom oporavka nakon Vingejtovog testa između polova u mladoj, sedentarnoj populaciji i da li faza MC kod žena može imati uticaj na ove indekse. **Metode.** Dvadeset i pet ispitanica (20,5 ± 0,7 godina) i šesnaest ispitanika (20,4 ± 0,7 godina) izvodili su Vingejtov anaerobni test na bicikl ergometru pri čemu su im registrovani OSR i VSF u stanju mirovanja i tokom oporavka. Kod ispitanica, podaci su prikupljani tokom tri faze MC. **Rezultati.** brži OSR i veću ukupnu varijabilnost u stanju mirovanja i Prirodni logaritam markera niskih frekvencija (lnLF) VSF i prirodni logaritam markera visokih frekvencija (lnHF) VSF bili su veći kod muškog pola u stanju mirovanja u odnosu na žene u različitim fazama MC, osim u slučaju kasne folikularne faze gde nije bilo razlike u lnHF među polovima. Značajno veći lnLF i lnHF uočeni su kod muškaraca tokom oporavka od Vingejtovog testa. Nije bilo razlike u parametrima VSF u stanju mirovanja među ženama u različitim fazama MC. Iznenađujuće, parasimpatički marker vremenskog domena – kvadratni koren srednje vrednosti sume kvadrata razlika između sukcesivnih NN intervala (RMSSD) i lnLF bili su veći u ranoj folikularnoj fazi u odnosu na lutealnu fazu MC tokom oporavka. OSR je bio brži kod muškaraca u odnosu na žene u svim fazama MC. **Zaključak.** Muškarci pokazuju tokom oporavka, ali čini se da je parasimpatička ak-

Correspondence to: Lana Andrić, Health Center "Bečej", Department of Women and Children's Health Care, Braće Tan 3, 21 220 Bečej, Serbia. E-mail: lanaandric@yahoo.com

tivnost u stanju mirovanja slična između polova kada su žene u kasnoj folikularnoj fazi. Hormonski ciklus kod žena nema uticaj na autonomnu varijabilnost u stanju mirovanja. Rana folikularna faza pokazuje poželjniji autonomni profil tokom oporavka u poređenju sa lutealnom fazom MC.

Ključne reči:
srce, frekvencija; nervni sistem, autonomni; vežbanje, testovi.

Introduction

Besides autonomic regulation, the rhythmicity of cardiac beats is finely directed by humoral factors, hence the influence of hormonal fluctuations on heart rate variability (HRV) throughout the menstrual cycle (MC) [1, 2]. The female monthly sexual cycle is dominantly regulated through the influences of the hypothalamic releasing hormone – gonadotropin-releasing hormone (GnRH), anterior pituitary sex hormones – follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and the ovarian hormones – estrogen (mainly in the form of ß-estradiol) and progestogens (almost exceptionally progesterone). At the very beginning of the proliferative phase, there is a rise in FSH and LH levels, where FSH increases estrogen production in primary follicles leading to a peak secretion just before the ovulation. Two days prior to ovulation, the LH surge happens, rising by 6–10 fold, with the about 2–3-fold increase in FSH production at the same time. After ovulation, concentrations of progesterone and estrogen start to increase until the late luteal phase when involution of corpus luteum and cessation of progesterone and estrogen secretion removes the feedback inhibition, and levels of FSH and LH start to rise again [3]. The influence of the MC phases on HRV is not yet clear. Higher sympathetic activity in the luteal phase has been reported proposing the effect of progesterone increase on parasympathetic withdrawal [4–7]. At the same time, others have reported the opposite or did not find any significant phase differences [8, 9]. On the other side, estrogen, the leading hormone of the follicular phase, has a positive relationship with vagal activity [9]. It acts on presynaptic alpha-2 adrenoceptors leading to a decrease in norepinephrine secretion and is also associated with an increase in acetylcholine production [10], thus, it may be that the rise in FSH, LH, and progesterone levels accounts for the inhibition of estrogen-related vagal control [9]. Some studies show marked sympathetic tone in male athletes, while the parasympathetic nervous system dominates in female athletes [11, 12]. Research conducted on non-athletes showed diminished parasympathetic influence in younger and middle-aged women [13]. In another study, parasympathetic influence prevailed among adolescent female non-athletes, as opposed to their age-matched male counterparts [14]. Women have a faster vagal post-exercise recovery after a maximal aerobic capacity test [15], but the supra-maximal anaerobic test has a greater impact on autonomic reactivation in women [16, 17]. Women are, in general, underrepresented in exercise studies, and the majority of those that include them do not hold MC phases into account.

The aim of this paper was to investigate the influence of different MC phases (especially the early and late follicular phase) on resting and post-exercise autonomic modulation between genders, as well as in females solely. It was postulated that females would have higher parasympathetic indices when in the early and/or late follicular phase in comparison to males and intra-subject relations. Secondly, we wanted to examine how MC phases possibly influence the results of a supra-maximal (Wingate) test in females.

Methods

Participants

Forty-one participants (16 males and 25 females), aged 18 to 24 years (age 20.4 ± 0.7 years and 20.5 ± 0.7; height 184 ± 5 cm and 168 ± 5 cm; body weight 79.38 ± 9.42 kg and 60.96 ± 6.93 kg; body mass index 23.53 ± 2.83 and 21.57 ± 2.23, for males and females, respectively) entered the study voluntarily. All participants were regularly enrolled in the studies of medicine at the Faculty of Medicine, University of Novi Sad. Subjects were in self-reported good health, without the use of medications, and with no medical history of cardiovascular and neuromuscular diseases, including neuro-vegetative dystonia. The inclusion criterion for female participants was a regular MC. Additional criteria implied that leisure-time physical activity in the past six months did not exceed an hour of sports activity per day for no more than three days a week.

The research was approved by the Ethics Committee of the University of Novi Sad, Faculty of Medicine, and it was conducted according to the Declaration of Helsinki. Participants were thoroughly introduced to the study procedure and its goal, and they all gave written informed consent.

All measurements were conducted at the Laboratory for Functional Diagnostics of the Department of Physiology, between 10 and 12 am, at room temperature around 22–24ºC. Participants were strongly advised to restrain from intensive training and from consuming caffeinated and alcoholic beverages, including stimulant substances, 24 h before the test. Female subjects were required to come at three phases of their MC. MC phase calculation was performed via recommendations provided by Stricker et al. [18], where the 14th day of the cycle was marked as day zero. The measurements were taken during the phase of menstrual bleeding (from day -15 until -6) – the early follicular phase, when levels of both estrogen and progesterone are low; in the middle of late follicular phase (from day -5 until -1), when estrogen reaches its peak; in mid-luteal phase (from day +5 until +9), when progesterone peak is expected.

Study protocol and data acquisition

The protocol consisted of two modes of heart rate acquisition – at rest and during recovery, using a telemetric pulsometer (Polar RS800CX, Finland). Firstly, participants were required to sit quietly and breathe spontaneously for 5 minutes on a cycle ergometer (Wattbike, Wattbike Ltd, Nottingham, UK), with their feet placed on a platform in front of the pedals, knees flexed at a 90-degree angle, and arms resting on thighs while heart rate recordings were obtained. The Wingate anaerobic test was preceded by a 3-minute warm-up where resistance was set at 50 W. Throughout this period, they performed 2–3 bouts of sprint in order to get adjusted to the level of speed and exertion they had to engage for the test. After the warm-up period, subjects were instructed to pedal at full speed in a standing position against the constant breaking force (7.5% of body weight). Upon cessation of the exercise test, heart rate recording was started again for 5 minutes. During the first minute of the recovery period, participants continued pedalling without any resistance, and afterwards, they were required to stay in the same body position as before the exercise for additional 4 minutes.

Data analysis

Ergometric parameters

Peak power (PP) was a value of the highest power achieved at any 5-second stage. Mean power (MP) was defined as an average of all obtained power values.

Heart rate variability

A sampling rate of 1000 Hz was chosen, and data from the pulsometer were transferred to a laptop computer via a USB interface, where they were analyzed in Polar ProTrainer 5 TM (Polar, Finland) software. Ectopic beats and artefacts were identified with visual inspection and removed. They were deleted with the post extra systolic beat and replaced automatically with interpolated adjacent R-R interval values. HRV indices (the square root of the mean squared differences of successive NN intervals (RMSSD), low-frequency (LF) spectral power (0.04–0.15 Hz), and high-frequency (HF) spectral power (0.15–0.40 Hz) were calculated for all 5 minutes of resting period and for the 3-minute recovery period (minutes 3–5). In order to ensure the stability of the data and reduce bias arising from non-uniformity of error, natural log-transformations (ln) of spectral HRV indices were performed.

Heart rate recovery (HRR)

HRR was assessed via indices which were extracted from the 5-minute recovery recordings. HRR60 represents the absolute difference between heart rate values at 60 seconds after exercise termination (HR60) and peak heart rate values registered immediately after termination of the test (HRmax). Resting heart rate (HRrest) was presented as a mean heart rate value acquired from the pre-exercise 5-minute recordings. Heart rate readings at the end of the post-exercise period (HRend) were also obtained. T30 was a time constant of the rapid heart rate decay during the first 30 seconds of recovery and it represented the negative reciprocity of regression line slope. T was the time constant decay obtained by fitting the 5-minute post-exercise HRR into the first-order exponential curve [16, 19, 20], where heart rates were modelled with an iterative technique using MatLab software (The Math Works Inc, Natick, MA, USA) to fit the following equation:

$$HR = HR_o + HR\Delta e(-t+T)$$

Where: HR = heart rate, HR_o = stabilized heart rate following exercise, HRΔ = maximal heart rate – HR_o, t = time (s), T = time constant of exponential heart rate decay.

Statistical analysis

The normality of the distribution was assessed with the Lilliefors normality test. Microsoft Excel data analysis tool was used for statistical inspection. The F-test was performed to assess the equality of variances between groups, after which we did the two-sample *t*-test. The data are presented as means ± standard deviation (SD) with respect to 95% confidence interval (95% CI). Statistical significance was indicated at $p < 0.05$.

Results

There were significant differences in mean values of PP comparing the results in men with the results in women in early follicular ($p = 0.0000117$), late follicular ($p = 0.000016$), and luteal phase ($p = 0.0000157$). There were also significant differences in mean values for MP comparing the results in men with the ones in women in early follicular ($p = 0.000000213$), late follicular ($p = 0.00000871$), and luteal phase ($p = 0.000000209$) (Figure 1). There was no statistical significance in these parameters among menstrual cycle phases in women.

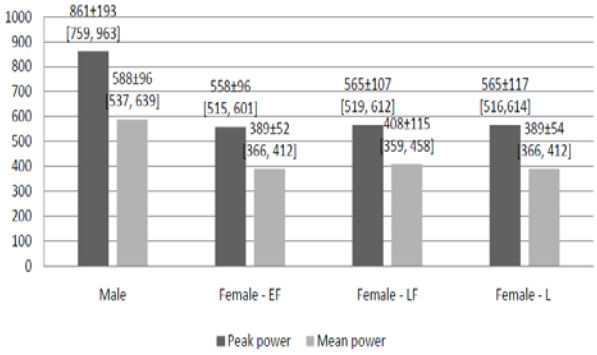

Fig. 1 – Wingate anaerobic test peak and mean power in men and women in different menstrual cycle phase [mean ± standard deviation (95% confidence interval)] EF – early follicular phase; LF – late follicular phase; L – luteal phase

Table 1

Resting heart rate variability indices in men and women in the early follicular menstrual cycle phase

Indices	Men	Women in the early follicular phase	*p*-value
RMSSD	27.25 ± 8.27 (22.84–31.66)	29.25 ± 15.82 (22.24–36.27)	> 0.05
lnLF	7.49 ± 0.51 (7.21–7.76)	6.62 ± 0.85 (6.24–6.99)	0.000376
lnHF	6.32 ± 0.58 (2.82–3.81)	5.73 ± 0.83 (5.36–6.09)	0.019372

***Note*: Results are given as mean ± standard deviation (95% confidence interval).**
RMSSD – root mean square of the successive differences; lnLF – natural log-transformations of low-frequency (LF) spectral power; lnHF – natural log-transformations of high-frequency (HF) spectral power.

Table 2

Resting heart rate variability indices in men and women in the late follicular menstrual cycle phase

Indices	Men	Women in the late follicular phase	*p*-value
RMSSD	27.25 ± 8.27 (22.84–31.66)	31.87 ± 14.92 (25.41–38.32)	> 0.05
lnLF	7.49 ± 0.51 (7.21–7.76)	6.61 ± 0.75 (6.29–6.93)	0.000248
lnHF	6.32 ± 0.58 (2.82–3.81)	5.93 ± 1.10 (5.45–6.40)	> 0.05

***Note*: Results are given as mean ± standard deviation (95% confidence interval).**
RMSSD – root mean square of the successive differences; lnLF – natural log-transformations of low-frequency (LF) spectral power; lnHF – natural log-transformations of high-frequency (HF) spectral power.

Table 3

Resting heart rate variability indices in men and women in the luteal menstrual cycle phase

Indices	Men	Women in the luteal phase	*p*-value
RMSSD	27.25 ± 8.27 (22.84–31.66)	28.66 ± 12.92 (23.20–34.11)	> 0.05
lnLF	7.49 ± 0.51 (7.21–7.76)	6.47 ± 0.82 (6.12–6.81)	0.0000232
lnHF	6.32 ± 0.58 (2.82–3.81)	5.67 ± 0.89 (5.29–6.05)	8.79E-10

***Note*: Results are given as mean ± standard deviation (95% confidence interval).**
RMSSD – root mean square of the successive differences; lnLF – natural log-transformations of low-frequency (LF) spectral power; lnHF – natural log-transformations of high-frequency (HF) spectral power.

By analyzing HRV indices during rest in relation to gender, RMSSD did not show valuable differences, lnLF was significantly higher in men in comparison to women throughout all three MC phases, and lnHF was significantly higher in men in contrast to women in the early follicular and luteal phase of MC (Tables 1–3).

After analyzing the HRV recovery parameters, it was noticed that RMSSD did not show statistical significance when compared to any of the female cycle phases with males. On the other side, lnLF and lnHF values markedly differed between men and women in all MC phases (Figure 2).

When comparing females during rest in various MC phases, no differences were observed in HRV. However, during the recovery from the Wingate anaerobic test, RMSSD was noticeably higher while females were in the early follicular phase vs. luteal phase of MC (6.29 ± 1.06, 5.20 ± 0.83; $p = 0.011415$). Moreover, in the early follicular phase, female participants had greater values of lnLF in

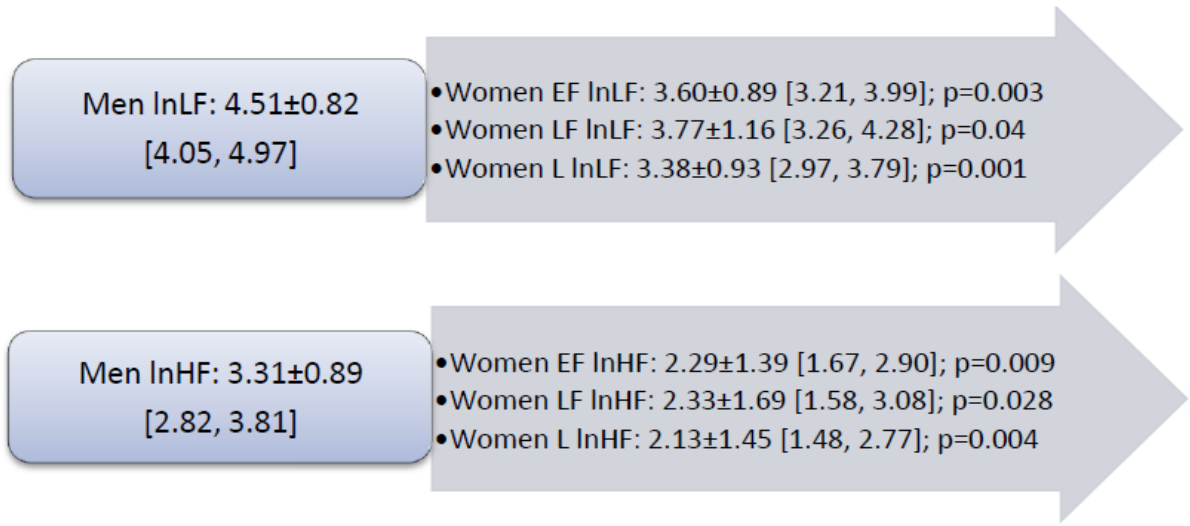

Fig. 2 – Recovery lnLF and ln HF values in men and women in different menstrual cycle phases [mean ± standard deviation (95% confidence interval)].
lnLF - natural log-transformations of low-frequency (LF) spectral power; lnHF - natural log-transformations of high-frequency (HF) spectral power; EF – early follicular phase; LF – late follicular phase; L – luteal phase.

comparison to the luteal phase of MC (3.62 ± 0.20, 3.39 ± 0.21; $p = 0.008511$).

Mean values of HRrest, HRmax, HR60, HRR60, HRend, and T did not significantly differ between men and women in the examined MC phases ($p > 0.05$), but heart rate recovery perceived through T30 was faster in men in comparison to women in all MC phases (Table 4). Not one parameter showed differences in various phases among the females ($p > 0.05$).

Discussion

As opposed to what we have expected, males had a more favourable autonomic profile than females. Our study showed that males had greater resting and post-exercise overall HRV, as well as faster HRR no matter in which phase of the menstrual cycle the women were in. Contrary to the findings of some authors [21, 22], we did not find intra-subject HRV differences regarding the cycle phase during rest. Sur-

Table 4

Resting heart rate and heart rate recovery indices after a Wingate anaerobic test in men and women in different menstrual cycle phases

Indices	Men	Women – early follicular phase	Women – late follicular phase	Women – luteal phase
HRrest	89 ± 11 (84–95)	89 ± 12 (70–95)	87 ± 14 (81–93)	90 ± 13 (84–95)
HRmax	188 ± 9 (183–193)	186 ± 7 (182–189)	185 ± 9 (181–189)	186 ± 10 (182–191)
HR60	158 ± 13 (151–166)	155 ± 8 (151–159)	156 ± 13 (150–161)	154 ± 13 (148–160)
HRR60	30 ± 9 (25–34)	30 ± 7 (27–33)	29 ± 9 (25–33)	32 ± 11 (27–37)
HRend	116 ± 6 (112–119)	113 ± 13 (107–119)	110 ± 15 (104–117)	115 ± 16 (108–122)
T30	262.1 ± 91.8 (213–311)	621.4 ± 161.1* (548–695)	607.04 ± 150.6† (538–675)	661.34 ± 206.9‡ (570–753)
T	135.7 ± 53.1 (107–164)	134.2 ± 47.1 (112–156)	123.7 ± 44.2 (102–145)	117.8 ± 43.4 (98–138)

***Note*: Results are given as mean ± SD (95% confidence interval).**
***p* = 6.88E-10; †*p* = 4.72E-10; ‡*p* = 4.56E-09.**
HRrest – resting heart rate; HRmax – peak heart rate values registered immediately after termination of the test; HR60 – the absolute difference between heart rate values at 60 seconds after exercise termination; HRR60 – the absolutwe difference between heart rate values at 60 seconds after exercise termination (HR60) and peak heart rate values registered immediately after termination of the test (HRmax); HRend – heart rate values at the end of the post-exercise period; T30 – time constant of the rapid heart rate decay during the first 30 seconds of recovery; T – time constant decay obtained by fitting the 5-minute post-exercise heart rate recovery into a first-order exponential curve.

prisingly, both RMSSD and lnLF were augmented in the early follicular in comparison to the luteal phase of MC.

The anaerobic capacity results (PP, MP) after the Wingate test were in accordance with the existing literature [16, 23, 24]. Muscle hypertrophy and variations in muscle fibre type are, allegedly, the main causes for higher values of PP and MP in men. There is a prevalence of slow twitching fibres in skeletal muscle sections in women [25, 26]. Furthermore, some authors suspect that differences in sarcomeral metabolism might influence the divergence in muscle power between sexes [27]. Similar to the recently published results [28, 29], the difference in anaerobic power parameters in women concerning MC phases was not observed in our study.

In the general population, sympathetic nervous system activity is higher in men and parasympathetic one in premenopausal women [12, 14, 30–32]. Time and frequency domain differences between genders gradually fade out with age. In fact, they fade more progressively after the third decade [33]. Numerous papers point out that the differences disappear after 50 years of age [12, 34], which is addressed to postmenopause and a lowered protective effect of endogenous sex hormones in women. In our study, resting values of lnLF were higher in men, which is in agreement with the above mentioned if we consider LF as a marker of solely sympathetic activity. However, LF portrays joined actions of both autonomic branches with a slight predominance in sympathetic activity, especially after a workout [35]. LF also represents oscillations in the baroreceptors system [36, 37], and baroreflex sensitivity (BRS) is said to be higher in men while at rest [38]. Our participants had their HRV registered in a sitting position, which provokes the sympathetic response, but we saw no change in lnLF while comparing females in different phases, although the baroreflex response of a sympathetic component in women is found to be more pronounced in the menstrual and/or luteal phase [21]. In fact, a significant number of papers imply that the sympathetic nervous system is more active during the luteal phase [5, 7, 10]. However, there are also papers where no differences between phases were observed [6, 9]. On the other hand, our results also showed that parasympathetic influence (lnHF) during rest was more prominent in the male sex in comparison to women in the early follicular and luteal MC phase. The lack of differences between genders when females were in the late follicular phase might express the evolving vagal tone while approaching peak levels of estrogen. Despite a much greater number of opposing results [39–41] that did not take MC into account, it appears that working in shifts can indeed influence female HRV depending on the MC phase [8]. In this case, the follicular phase shows a fall in vagal and an increase in sympathetic activity. The results we got might have an explanation for stress and lack of sleep that medical students deal with, which may have heightened sympathetic tone in the male and lessened parasympathetic tone in female participants. On the other side, our study lacks information on physical activity levels. Greater participation in recreational sport could explain prevailed vagal indices in men.

Markedly higher lnLF and lnHF values were obtained in males after the Wingate test in comparison to women in all menstrual cycle phases. These findings contrast the ones found by authors who reported higher values of HF in women during recovery from the test for maximal oxygen consumption and concluded that women have faster vagal post-exercise reactivation [15]. In general, HRR is faster after the Wingate test, and recovery after an incremental VO2max test sometimes takes several days [42, 43], but supramaximal exercise has a greater impact on autonomic modulation in women. Significantly decreased HF power after the Wingate test in females in contrast to males was reported in one study with the upright sitting position where only vagal indices were analyzed [16], and a significant increase in LF power was reported in another study where recovery took place in a supine position [17]. Despite that, men have accentuated resting baroreflex sensitivity (BRS), and women might possess a higher diapason of its effect during post-exercise recovery. This was supported by a persistent reduction in heart rate in women while seated, but not in men [38]. Contrary to this, another study found that seated position provokes less favourable recovery than supine [19]. In our study, women had a slower HRR and a lesser lnLF after exercise. Although stress can be addressed for suppressed BRS [44], our participants were subjected to the same levels. Maybe poor engagement in sport in our female participants can be held responsible for such results, but we do not have evidence to support that.

Intra-subject differences in HRV during recovery were observed in females. A marker of vagal activity, RMSSD, was higher in the early follicular phase in comparison to the luteal one. Among eumenorrheic women, even in those who report premenstrual symptoms, resting RMSSD is mostly higher in the follicular in comparison to the luteal phase [10, 45]. However, some authors consider the follicular phase as the one that follows the menstrual phase, starting on day 8 or 9 of MC, which is by our classification addressed as the late follicular phase. We also found that post-exercise lnLF was higher in the early follicular phase in relation to the luteal phase. Whether dysmenorrhoea can be the cause of disrupted autonomic modulation was a matter of subject in various studies [46, 47], which stated that a slight increase in LF/HF can exist during menstruation pointing out to a fall in parasympathetic activity. However, in our female population vagal index – RMSSD, was marked in the early follicular phase. It is possible that BRS during post-exercise recovery is more pronounced in the early follicular phase in comparison to other phases, or that the parasympathetic component of the low-frequency domain is augmented.

Resting heart rate did not differ between the sexes in our study. Literature shows favourably lower resting heart rates in men [16, 48, 49]. The lack of this difference in our results might be because of the small sample size but also because of the specificity of the medical student population. There is proof that stress and working in shifts can significantly lower the HRV indices (SDNN, TP, HF) among the male health workers without greater interfering with these indices in females [39–41]. The similar stressful life milieu of our participants could have influenced diminished differences in genders. Maximally achieved heart frequencies did not stand in

contrast between men and women in our study, which is consistent with previous results [15, 17, 23].

In accordance with the previous findings [16], our results showed faster vagal reactivation in males, perceived by T30, which is an immediate post-exercise index of vagally mediated cardiac rate decay [50, 51]. HRR was found to be in a strong correlation with the level of physical activity [52]. It was also found to negatively correlate with resting supine parasympathetic markers of HRV when in a standing position during the first minute of recovery, but the higher the indices of combined autonomic modulation (LF, lnLF), the greater the HRR in the third and fifth-minute post-exercise [53–55]. The existing data report no correlation of estradiol levels with the initial HRR dynamics [38]. Similar to a study by Pestana et al. [29], we did not find statistical differences in HRR among the MC phases.

Conclusion

Men have greater total variability and a more favourable autonomic profile during rest and in a seated recovery after the Wingate test. Our study supports the notion that supra-maximal exercises present a heavier load to the female autonomic nervous system. Products of anaerobic metabolism and muscle metaboreflexes, in a way, may be responsible for this. We would also like to instigate more research towards understanding HRV dynamics concerning the early and late follicular phases. We guess that in a resting state, vagal influence could be expected in the late follicular phase coinciding with the peak levels of estrogen. On the other hand, in a recovery state, vagal reactivation might preferably be recorded in the early follicular phase before the preovulatory FSH and LH surge happen.

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Association of fracture configuration and callus formation with a concentration of proinflammatory cytokines in children with long bone fractures

Povezanost tipa preloma i formiranja kalusa sa koncentracijom proinflamatornih citokina kod dece sa prelomima dugih kostiju

Zoran Paunović*, Sanja Milutinović†, Nikola Stanković*, Džihan Abazović‡, Ivan Stanojević†§, Mia Rakić‖¶, Mirjana Djukić, Gordana Šupić†§, Danilo Vojvodić†§, Dušan Marić††, Wasim S Khan‡‡, Srdjan Starčević†§§**

***Institute for Health Protection of Mother and Child of Serbia "Dr. Vukan Čupić", Belgrade, Serbia; †University of Defence, Faculty of Medicine of the Military Medical Academy, Belgrade, Serbia; ‡Emergency Medical Center of Montenegro, Podgorica, Montenegro; Military Medical Academy, §Institute for Medical Research, §§Clinic for Orthopedics and Traumatology, Belgrade, Serbia; ‖University of Nantes, Faculty of Dental Surgery, Nantes, France; University of Belgrade, ¶Institute for Biological Research "Siniša Stanković", **Faculty of Pharmacy, Belgrade, Serbia; ††University of Novi Sad, Faculty of Medicine, Novi Sad, Serbia; ‡‡University of Cambridge, Addenbrooke's Hospital, Division of Trauma and Orthopedic Surgery, Cambridge, United Kingdom**

Abstract

Background/Aim. The inflammatory response is of utmost importance in bone healing, but the precise role of cells and cytokines remains unclear. In our study, we examined the association between interleukin-1β (IL-1β), tumor necrosis factors alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) concentrations, fracture configuration, and callus formation. **Methods.** Serum cytokine concentrations were determined in 78 non-obese children with long bone fractures (group 1), 10 children with finger fractures (group 2), and 10 healthy controls (group 3). Blood samples were taken immediately after fracture upon hospital admission for groups 1 and 2. Differences in cytokine concentrations were analyzed among groups and categorized according to fracture configuration and callus formation. **Results.** IL-1β and TNF-α levels were lower in patients that went on to produce incomplete callus compared with patients that formed complete callus. Surprisingly, the average IL-1β concentration was highest in the healthy control group. The only significant correlation between IL-1β and TNF-α was in the group with moderate callus formation. MCP-1 level was slightly increased in all patient groups compared to controls, with no mutual difference. An average IL-8 level showed a clear decrease tendency from the group with incompletely formed callus toward the group with completely formed callus compared to controls, without significant difference. Children with epiphysiolysis had the lowest concentrations of cytokines compared with all other fracture types including transverse, oblique, and spiral. There were significantly lower concentrations of IL-1β and MCP-1 in patients with less fragment displacement compared with patients with greater fragment displacement. **Conclusion.** The systemic inflammatory response is important in physiological bone healing. High early production of IL-1β, TNF-α, and MCP-1 is associated with greater callus formation and better healing outcome, while increased IL-8 level is associated with poor callus formation and worse healing outcome. Our results indicate that epiphysiolysis and larger fragment displacement are associated with delayed fracture healing.

Key words:
fractures, bone; fracture healing; child; adolescent; bony callus; interleukin-1beta; tumor necrosis factor-alpha; monocyte chemoattractant protein-1; interleukin-8; prognosis.

Apstrakt

Uvod/Cilj. Inflamatorni odgovor je od izuzetne važnosti u zarastanju koštanih preloma, iako je precizna uloga ćelija i citokina nejasna. U našoj studiji ispitivali smo povezanost vrednosti interleukina-1β (IL-1β), faktora nekroze tumora-alfa

Correspondence to: Srdjan Starčević, Military Medical Academy, Clinic for Traumatology and Othopaedic Surgery, Crnotravska 17, 11 000 Belgrade, Serbia. E-mail: starcevicdrsrdjan@gmail.com

(TNF-α), monocitni hemoatraktantni protein-1 (MCP-1) i interleukina-8 (IL-8) sa konfiguracijom preloma i formiranjem kalusa. **Metode.** Serumska koncentracija citokina određivana je kod 78 negojazne dece sa prelomom dugih kostiju (grupa 1), 10 dece sa prelomom prstiju (grupa 2) i 10 zdrave dece (grupa 3 – kontrolna grupa). Uzorci krvi kod dece sa prelomima kostiju uzimani su odmah po prijemu u bolnicu (grupe 1 i 2). Razlike u koncentracijama citokina su analizirane između grupa i kategorisane prema konfiguraciji preloma i formiranju kalusa. **Rezultati.** Vrednosti IL1-β i TNF-α bile su niže kod bolesnika sa nedovoljno formiranim kalusom u odnosu na one sa kompletnim kalusom. Iznenađujuće, prosečna IL1-β koncentracija bila je najveća u kontrolnoj grupi. Jedina značajna korelacija između IL1-β i TNF-α bila je u grupi sa intermedijarno formiranim kalusom. MCP-1 je imao povišene vrednosti kod svih bolesnika u odnosu na kontrolnu grupu, bez međusobnih razlika. Prosečna vrednost IL-8 pokazala je jasan pad u grupi sa nekompletno formiranim kalusom u odnosu na grupu sa kompletno formiranim kalusom i kontrolnu grupu, ali bez značajne razlike. Deca sa epifiziolizom imala su najmanje koncentracije citokina u poređenju sa svim drugim tipovima preloma. Takođe smo detetkovali značajno niže koncentracije IL-1β i MCP-1 kod bolesnika sa manjim stepenom dislokacije u odnosu na veće dislokacije fragmenata. **Zaključak.** Sistemski inflamatorni odgovor je važan u fiziološkom zarastanju kostiju. Visoka rana produkcija IL1-β, TNF-α i MCP-1 je udružena sa boljim formiranjem kalusa i boljim zarastanjem kostiju, dok je povećana IL-8 koncentracija udružena sa lošim formiranjem kalusa i lošim zarastanjem kostiju. Naši rezultati su pokazali da su epifizioliza i veći stepen dislokacije fragmenata udruženi sa odloženim zarastanjem fraktura.

Ključne reči:
prelomi; prelomi, zarastanje; deca; adolescenti; kalus; interleukin-1beta; faktor nekroze tumora-alfa; monocitni hemoatraktantni protein-1; interleukin-8; prognoza.

Introduction

The most common injuries in children are fractures. In the past few decades, treatment has been improved, but still, some fractures heal slower and come with complications. For this issue, a deeper understanding of the bone healing process is of utmost importance.

One of the most astonishing processes in the human body is the healing of a fracture because the result of this process is not in scar formation but in forming a tissue similar to the preexisting. Fracture healing is a highly regulated process that consists of the following phases: the inflammatory phase, phase of reparation, and remodeling phase [1]. Although it is highly known that chronic production of inflammatory cytokines has a negative effect on bones, brief and precisely monitored production of proinflammatory cytokines is crucial for tissue regeneration [2]. The inflammatory phase is of utmost importance for the successful healing of the fracture because the proinflammatory cytokines released during this phase initiate further signaling pathways, which culminate with the healing of the above-mentioned fracture. For the first 24 hours, some proinflammatory cytokines like tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL1-β) are produced in the area of the injury [3, 4]. Values of the TNF-α and IL-1β achieve their maximum levels at the beginning of the fracture healing [4, 5]. The influx of the inflammatory cells at the place of the injury is affected by chemoattractive chemokines, where the most important factor is monocyte chemoattractant protein-1 (MCP1) which controls the movement of monocytes from the bone marrow to the blood flow and from there towards the place of inflammation [6]. Few hours after the fracture, the phase of reparation starts, and it is affected by local and systemic production of numerous growth and differentiation factors [7]. When the fracture is finally mended, the newly formed tissue must adapt to its function, and all this is happening during remodeling phase [1].

There are a few studies about the correlation of proinflammatory cytokines and fracture types, or fragment displacement, but it is proven that highly unstable fragments do slow down angiogenesis and make the newly formed bone tissue unable to fill the gaps [8].

All this leads us to further study the association between a few proinflammatory cytokines: IL-1β, TNF-α, MCP-1, and interleukin-8 (IL-8), and callus formation, also types of bone fractures and fragment displacement. Last but not least, we need a better understanding of these interactions so that we could further improve the methods of fracture healing.

Methods

All child patients were admitted, diagnosed, and treated at the Department of Orthopedics and Joints/Bone Trauma, Institute for Health Protection of Mother and Child ”Dr. Vukan Čupić”, Belgrade, Serbia. The parents of the tested children gave informed written consent for the participation of their children in the study. This study was approved by the Ethics Committee of this institution (No 8/26, 13/10/2015). The study included both boys and girls, age span from 4 to 18 years. There was one group of children with long bone fractures (group 1, n = 78), one group with fingers or small bone fractures (group 2, n = 10), and one control group of children that were admitted to the Clinic with a diagnosis of extremity trauma, but without proof of a fracture (group 3, n = 10). None of the children were obese [body mass index (BMI) 15–24 kg/m^2].

Children with other injuries apart from long bone fracture, children with systemic diseases of connective tissue, malignant, metabolic diseases, obese children (BMI over 24.0 kg/m^2), and children with congenital anomalies of the skeletal system were not involved in the study.

The study was a cross-sectional investigation. Sample of 2 mL venous blood was taken from the cubital vein in all children 1 hour after admission. After the serum separation, the samples were frozen at -70°C until testing. The concentrations of cytokines were determined with a

commercial flow cytometric test (LEGENDplex 13-plex Human Adipokine Panel) on a flow cytometer Beckman Coulter FC500. The concentrations of IL1-β, TNF-α, MCP-1, and IL-8 in all patients were measured.

All patients had anteroposterior (AP) and lateral radiographs taken at 5-time points: immediately, preoperatively, postoperatively, 7 days postoperatively, 21 days postoperatively, and after removing the plaster cast. The type of fracture was classified as epiphysiolysis, transverse, oblique, or spiral. The fragment displacement was classified as undisplaced, displaced < 1 cm, and displaced > 1 cm. Callus formation was classified according to radiological analysis as incomplete (< 25%), partial (< 50%), and complete (> 75%). Patient files were written about their earlier fractures, infections of the upper respiratory tract, allergies, and eating habits.

We used parameters of descriptive statistics to analyze group variability and to estimate the central tendency of data. Analysis among more than two groups, groups according to fracture type (epiphysiolysis, transverse, oblique, spiral), fragment displacement (without displacement, displacement < 1 cm, displacement > 1 cm), and among groups depending on the degree of callus formation (< 25%, < 50%, > 75%) were performed using one-way analysis of variance (ANOVA), with Bonferroni post-testing. The Mann-Whitney test was used for all other comparisons between two independent groups. All statistical analyses were done using the statistical package GraphPad Prism 5.01 (GraphPad Prism Software Inc. California, USA).

Results

The results are shown in Tables 1 and 2.

Cytokines and callus formation

The average IL-1β concentration was lower in the groups 1 and 2 compared to the group 3. There was a significant difference between patients that had complete callus and those in the group 2. Although IL-1β concentration was lower in patients with insufficient callus compared with patients with completely formed callus, these differences were not statistically significant. On the other hand, the group 1 had almost twice the TNF-α concentrations of the groups 2 and 3. Patients with complete callus had the highest average TNF-α concentration, significantly greater than the patients in the groups 2 and 3.

Because of simultaneous increases in both IL-1β and TNF-a, a correlation analysis was performed. The only significant correlation was in the patients with incomplete callus formation.

MCP-1 and IL-8 levels were also higher in the group 1 than in the groups 2 and 3, but the differences were not significant. Patients with incompletely formed callus had the highest IL-8 concentrations.

Cytokines and fracture configuration

There were significant differences in IL-1β concentrations in children with oblique, transverse, and spiral long bone fractures when compared with patients in the group 2 (Table 1) and when comparing the groups 2 and 3 (Table 2). Children with epiphysiolysis had lower IL-1β values comparing with children with spiral long bone fractures.

TNF-α concentration was significantly higher in children with transverse long bone fracture compared with those in the groups 2 and 3.

Table 1

Average concentrations of IL1-β, TNF-α, MCP-1, and IL-8 in blood samples of children with bone fractures

Groups	n	Cytokines (pg/mL), mean ± standard deviation			
		IL-1β	TNF-α	MCP-1	IL-8
Group 1 (long bone fractures)					
callus formation					
incomplete	6	46 ± 10	97 ± 54	98 ± 18	2,823 ± 1,117
intermediary	53	53 ± 18	82 ± 50	98 ± 51	2,351 ± 1,027
complete	19	60 ± 19 [a]	114 ± 63 [a, b]	101 ± 27	2,141 ± 437
fracture type					
epiphysiolysis	8	36 ± 21 [b]	47 ± 21	57 ± 19 [a,b,c]	1,807 ± 446
oblique	20	54 ± 16 [c]	87 ± 70	99 ± 36 [d]	2,476 ± 970 [a]
transverse	39	52 ± 18 [e]	104 ± 63 [c, d]	94 ± 42 [e]	2,404 ± 1038
spiral	11	72 ± 25 [b,d,f]	57 ± 38	148 ± 60 [c,d,e,f,g]	2,038 ± 680
dislocation					
no	8	63 ± 18 [h,]	99 ± 20 [e]	97 ± 31	2,955 ± 1,028 [b,c,d]
< 1 cm	62	50 ± 19 [i, j]	92 ± 64 [f]	91 ± 35 [h]	2,369 ± 966 [e]
> 1 cm	8	70 ± 20 [i, k]	68 ± 72	158 ± 68 [h, i, j]	1,666 ± 666 [b]
Group 2 (finger fractures)	10	38 ± 13 [h, j, k, l]	48 ± 30	74 ± 24 [i]	1,915 ± 446 [c]
Group 3 (controls)	10	65 ± 22 [l]	44 ± 29 [e,f]	88 ± 39 [j]	1,784 ± 348 [d, e]

Superscripted letter depicts significant difference between specific groups (precise data are given in Table 2).
IL-1β – interleukin-1β; TNF-α –tumor necrosis factor-α; MCP-1 – monocyte chemoattractant protein-1; IL-8 – interleukin-8.

Table 2

Significant differences in cytokine concentrations among analyzed groups of patients according to degree of callus formation, fracture type, and fragment dislocation

Cytokine	Marker	Group	vs.	Group	*p*
IL-1β					
callus	a	complete	/	control finger fractures	0.0450
fracture	b	epyphysiolysis	/	spiral	0.0485
	c	oblique	/	control finger fractures	0.0213
	d	transverse	/	spiral	0.0191
	e	transverse	/	control finger fractures	0.0258
	f	spiral	/	control finger fractures	0.0070
	g	control	/	control finger fractures	0.0104
dislocation	h	no	/	control finger fractures	0.0208
	i	< 1 cm	/	> 1 cm	0.0343
	j	< 1 cm	/	control finger fractures	0.0346
	k	> 1 cm	/	control finger fractures	0.0109
	l	control finger fractures	/	control	0.0104
TNF-α					
callus	a	complete	/	control finger fractures	0.0131
	b	complete	/	control	0.0177
fracture	c	transverse	/	control finger fractures	0.0040
	d	transverse	/	control	0.0014
dislocation	e	no	/	control	0.0012
	f	< 1 cm	/	control	0.0095
MCP-1					
fracture	a	epyphysiolysis	/	oblique	0.0369
	b	epyphysiolysis	/	transverse	0.0433
	c	epyphysiolysis	/	spiral	0.0167
	d	oblique	/	spiral	0.0291
	e	transverse	/	spiral	0.0024
	f	spiral	/	control finger fractures	0.0022
	g	spiral	/	control	0.0140
dislocation	h	< 1 cm	/	> 1 cm	0.0066
	i	> 1 cm	/	control finger fractures	0.0016
	j	> 1 cm	/	control	0.0025
IL-8					
fracture	a	oblique	/	control	0.0312
dislocation	b	no	/	> 1 cm	0.0186
	c	no	/	control finger fractures	0.0207
	d	no	/	control	0.0045
	e	< 1 cm	/	control	0.0441
	b	no	/	> 1 cm	0.0186

Superscripted letter depicts significant difference between specific groups, as given in Table 1 (Mann-Whitney test). IL-1β – interleukin-1β; TNF-α – tumor necrosis factor-α; MCP-1 – chemoattractant protein-1.

Significantly lower values of MCP-1 were found in children with epiphysiolysis than with other fracture configurations. Children with spiral long bone fractures had significantly higher MCP-1 values than with any other fracture configuration or groups.

Significant differences in IL-8 concentrations were only seen between children with oblique long bone fractures and controls.

Cytokines and bone fragment displacement

All patients in the groups 1 and 3 had significantly higher IL-1β concentrations compared with children in the group 2. In children with displacement < 1 cm, the concentrations of IL-1β were significantly lower than in children with greater displacement.

Patients without fragment displacement and with displacement < 1 cm had significantly higher TNF-α concentrations compared with patients in the group 3. The highest concentration of IL-8 was seen in patients without fragment displacement, and it was significantly higher than in patients from the groups 2 and 3 and patients with displacement > 1 cm.

Children with displacement < 1 cm, as well as those in the groups 2 and 3 had significantly lower MCP-1 concentrations compared with children with displacement > 1 cm.

Discussion

Even with countless studies, the process of fracture healing remains mostly unexplored. It goes on in 3 different but overlapping phases: the early inflammatory phase, phase of regeneration, and remodeling phase [9]. The use of anti-inflammatory drugs during the early phase of fracture healing in many studies has shown that the healing process

was considerably worse and slower. This implies that the inflammatory phase could be crucial for the successful healing of the fracture [10–12]. Proinflammatory cytokines are known for their destructive and catabolic effect on bones, but these same proinflammatory cytokines are making the fracture healing faster [13]. Differing from the uncontrolled inflammation that we see in bone pathology, inflammation in fracture healing of a healthy subject is short and highly regulated [1]. Many studies have shown that the precise regulation of the initial inflammatory phase is one of the determining factors for fracture healing and that the proinflammatory cytokines, like TNF-α and IL-1β, have a major role in initiating the repair process [2, 14, 15].

TNF-α shows maximal values in the first 24 hours after the fracture. After that, it shows decreasing levels during the cartilage formation. Then the values grow again during the remodeling phase [14, 15]. This is in accordance with our results, where all the patient groups upon admission had almost doubled TNF-α concentration compared to the healthy controls. Studies conducted on mice show that the absence of TNF-α slows the healing of a fracture for at least a couple of weeks. Contrary to that, TNF-α deficient mice have a normal skeleton which implies that TNF-α has a specific role in postnatal fracture healing [15]. These findings are also consistent with our results where patients from the group 2 with completely formed callus had the highest average TNF-α concentration, significantly increased compared to both control groups, and values of this cytokine were rising among patient groups from the group with minimal callus formation, where they were the lowest, towards the group with completely callus formation, where they were the highest. Macrophages are the main source of TNF-α and IL-1β during the inflammatory phase [3, 4]. Whilst they use different signal pathways, effects of IL1-β on the bone mostly overlap with effects of TNF-α [16], which was also shown in our study where a similar increase in TNF-alpha and IL-1β was seen from patients with insufficient callus to the group with completely formed callus. It is of utmost importance to specify that a balanced immune response is crucial for a successful fracture healing [17, 18] because the scarcity of TNF-α delays the fracture healing, whilst abundant production of TNF-α damages the bone [19, 20].

The conclusion is that precise regulation of signaling pathways is necessary in each of the healing phases in order to maintain the balanced concentration of different cytokines during the different phases of fracture healing. We are underlying this fact because it could be the explanation for the fact that patients from the group with minimal callus formation had the highest IL-8 concentrations and that the average IL-8 level showed a clear decrease tendency toward the group with completely formed callus. It could indicate that the high and long production of IL-8, which is a neutrophil chemoattractant factor, could lead to a high and uncontrolled neutrophil infiltration. It is widely known that excessive neutrophil degranulation is common in many inflammatory diseases, and as such, it can cause tissue damage as well as the delay of the healing process [21].

The influx of inflammatory cells to the place of the injury is also affected by chemoattractive chemokines. CCR2/CCL2 (MCP-1) controls the movement of the monocytes after the injury from bone marrow to the blood flow and from there to the place of inflammation [6]. Studies conducted on mice that were missing the CCR2 gene have shown that influx of macrophages to the place of the injury, in this case, is considerably smaller in comparison with the wild mice [22, 23]. Another study on mice with deficiency of CCR2 gene was comparing the size and thickness of the callus between these mice and the wild ones in different time spans after the injury, and it has shown that the healing of CCR2 deficient mice was delayed in comparison to the wild type [24]. The results of this study prove the importance of CCR2/CCL2 interaction for the formation of callus and are in accordance with our results where the patients with the lowest callus formation had the lowest concentration of CCL2 while this value was rising upon patients with completely formed callus.

There are not many studies about the correlation between the type of bone fractures and cytokines concentration with which we could compare our results, but the most obvious thing from our results is that the lowest concentrations of all investigated cytokines were observed in children with epiphysiolysis compared with all other types of fractures. One of the recently conducted studies has shown the importance of the formation of the initial hematoma as the first influx of the inflammatory cells, which will produce cytokines and be able to initiate further influx of immune cells to the place of the injury [25]. On the other hand, countless studies conducted to this day have shown that one of the major complications of epiphysiolysis is the insufficient blood supply of the femoral head due to the damage of epiphyseal blood vessels. Damage of epiphyseal blood vessels can be the consequence of the fracture or the result of high capsule pressure due to internal intracapsular bleeding [26, 27]. This insufficient blood supply could lead to poor formation of the initial hematoma and inadequate influx of inflammatory cells, which explains the lowest cytokine concentrations in children with epiphysiolysis in our study. Moreover, according to our previously discussed results about the influence of cytokine concentrations on callus formation and bone healing, it is probable that this poor influx of inflammatory cells leads to delayed healing of the fracture in the cases of children with epiphysiolysis.

We were also analyzing the association between bone fragment displacement and proinflammatory cytokine concentrations. Our study showed that there were significantly lower concentrations of proinflammatory cytokine IL-1 beta and MCP-1 in patients with less fragment displacement (displacement less than 1 cm) compared with patients with larger fragment displacement (displacement bigger than 1 cm). A study that has been recently conducted could explain these findings. This study was conducted on sheep, where the removal of the initially formed hematoma and its effect on fracture healing was tested [28, 29]. In this study, it has been shown that the removal of the initially formed hematoma leads to a new influx of inflammatory cells. This differs from the physiological process of healing, where the anti-inflammatory signal grows stronger for 24 hours after the fracture and thus considerably reduces the inflammation process [18]. This new inflammatory

impulse could further delay the inflammatory phase. If we compare these findings with our results, we could assume that a bigger fragment displacement produces more mechanical instability, which can damage the primarily formed hematoma and set a new inflammatory impulse. Additionally, this could be the reason for higher cytokine concentrations in patients with bigger fragment displacement. We must not forget that only a highly regulated inflammatory process can further improve fracture healing and, *vice versa*, every extension of the inflammatory phase leads to slower fracture healing [18, 27, 30].

Conclusion

The systemic inflammatory response is important in physiological bone healing. High early production of IL-1β, TNF-α, and MCP-1 is associated with greater callus formation and better healing outcome, while a high level of neutrophil chemotactic cytokine (IL-8) is associated with poor callus formation and worse healing outcome. Based on our results, there is delayed healing fracture in patients with epiphysiolysis and bigger fragment displacement.

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Clinical impact of nanophotonic blue-light filtering spectacles based on fullerene C_{60} and polymethyl methacrylate (PMMA)

Klinički efekat nanofotonskih naočara s plavim filterom na bazi fulerena C_{60} i polimetil metakrilata (PMMA)

Mirko R. Jankov*, Vesna Jakšić†, Djuro Koruga‡§

***LaserFocus Centre for Eye Microsurgery, Belgrade, Serbia; University of Belgrade, Faculty of Medicine, †University Eye Clinic Zvezdara, Faculty of Mechanical Engineering, Department of Biomedical Engineering, ‡NanoLab, Belgrade, Serbia; §TFT Nano Center, Belgrade, Serbia**

Abstract

Background/Aim. Blue light might be harmful to the retina. The nano-material based on fullerene C_{60} and polymethyl methacrylate (PMMA) transforms the light into harmonized light and absorbs violet, blue, and ultraviolet (UV) wavelength. The aim of the study was to evaluate the subjective and objective impact of the spectacles on daily activities. M**ethods**. Twenty-five healthy participants were submitted to contrast sensitivity (CS) and visual field (VF) testing and a questionnaire about the influence of spectacles on daily activities: two spectacles with different concentrations of fullerene C_{60} vs. two commercially available blue-blocking spectacles. **Results.** There was no statistical difference in CS ($p = 0.83$), in VF parameters: mean deviation (MD) ($p = 0.36$), pattern standard deviation (PSD) ($p = 0.25$), number of relative scotomas ($p = 0.31$), while the number of absolute scotomas showed a statistically significant decrease ($p < 0.05$). Spectacles B (with a lower concentration of fullerene – 0.025%) had the best overall comfort mean score ($p < 0.00001$): four-fifths of participants performed better only during the day, while two-thirds performed better both during the day and night. Spectacles B were also superior in overall satisfaction regarding all combined daily activities (4.04 ± 1.1) ($p = 0.0008$). **Conclusion.** Blue-blocking filters with fullerene C_{60} increase the overall comfort of daily tasks during and after their use. These filters might be an effective mechanism that can protect us from ocular pathologies while providing better comfort in daily activities.

Key words:
spectacles; filter; colours; field of view.

Apstrakt

Uvod/Cilj. Plavo svetlo može da bude štetno za retinu. Nano-materijal od fulerena C_{60} i polimetil metakrilata (PMMA) transformiše svetlo u harmonizovano hiperpolarizovano svetlo upijajući plavu, ljubičastu i ultraljubičastu talasnu dužinu. Cilj ovog rada bio je da se oceni subjektivni i objektivni uticaj nošenja naočara na obavljanje dnevnih aktivnosti. **Metode.** Dvadeset pet zdravih dobrovoljaca podvrgnuto je kompletnom oftalmološkom pregledu, ispitivanju kontrastne senzitivnosti (KS) i perimetrije (PM). Popunjavan je upitnik o uticaju naočara na dnevne aktivosti: dvoje naočara sa različitim koncentracijama fulerena C_{60} upoređeno je sa dvoje komercijalno dostupnih naočara sa plavim filterom. **Rezultati**. Nije postojala statistička razlika u KS ($p = 0,83$), kod PM parametara: MD (*mean deviation*) ($p = 0,36$), PSD (*pattern standard deviation*) ($p = 0,25$), i kod broja relativnih skotoma ($p = 0,31$), dok je broj apsolutnih skotoma pokazao statistički značajno smanjenje ($p < 0,05$). Naočare B (sa manjom koncentracijom fulerena od 0,025%) imale su sveukupno najbolju srednju ocenu ($p < 0,00001$): oko četiri petine ispitanika je bolje funkcionisalo danju, dok je oko dve trećine njih osećalo boljitak i danju i noću. Naočare B su isto tako bile superiorne u sveukupnoj oceni zadovoljstva pri svim kombinovanim dnevnim aktivnostima (4,04 ± 1,1) ($p = 0,0008$). **Zaključak.** Naočare sa plavim filterom na bazi fulerena C_{60} povećavaju sveukupan komfor u obavljanju dnevnih aktivnosti pri njihovom nošenju i nakon nošenja. Ovi filteri mogu da budu efikasan način zaštite od očnih bolesti uzrokovanih plavim svetlom uz povećan komfor u obavljanju svakodnevnih aktivnosti.

Ključne reči:
naočare; filteri; boje; vidno polje.

Correspondence to: Mirko R. Jankov, LaserFocus Centre for Eye Microsurgery, Cara Nikolaja Drugog 25, 11 000 Belgrade, Serbia.
E-mail: visioncare@mac.com

Introduction

The pigments in retinal photoreceptor cells absorb photons, initiating a chemical cascade of events known as phototransduction, thus converting light into electrical signals, sending them along the optical nerve to the upper neural structures for further analysis [1]. It has been documented that light causes apoptotic death of photoreceptors and retinal pigment epithelium (RPE) cells because of oxidative stress [2]. While excessive blue light is theoretically harmful, adequate blue light is necessary for normal visual function, such as colour discrimination and night vision, but also for circadian rhythm, which stimulates the brain to stay awake during the day, inhibiting melatonin secretion [3].

Artificial light sources, including light-emitting diode (LED) light bulbs and fluorescent light tubes, are the primary sources of blue light. With the increasing popularity of blue-rich LED-backlight display devices, such as smartphones, tablets, computer and television (TV) screens, our eyes are exposed to more blue light than in the past. Furthermore, not much is known about the safe levels of light exposure nor of the light spectrum for the retina and other ocular structures [4].

Blue light (short wavelength 400–455 nm) has been shown to be the most harmful to the retina [3–5]. It is known that the cornea and the lens are the structures that protect the eye from light-induced damage by preventing short wavelengths from reaching the retina. The cornea absorbs wavelengths below 295 nm, while the lens absorbs ultraviolet (UV) radiation (in the range of 300–400 nm) [6].

For this reason, many different filters which reduce that part of the visible spectrum have been developed in order to reduce the effect of blue light on the retina [7]. The nano-material based on fullerene C_{60} and polymethyl methacrylate (PMMA) has been used for nanophotonic contact lenses [8] and spectacles [9]. It has been shown that it transforms diffuse light into harmonized and hyper-polarized light, and these light photons have the same symmetry order (electrical and magnetic planes of photons in space and time) as biomolecules, which interact with light [10]. Moreover, these nanophotonic spectacles absorb the high-energy part of the visible light spectrum together with the UV light [11], resulting in a spectrum that is more comparable with the light sensitivity of the eye [12].

The aim of this prospective, interventional, comparative, non-randomized trial was to evaluate whether there is any subjective or objective impact on daily activities of the subjects during and after the use of the spectacles and any possible preference between them.

Methods

Spectacles A were commercially available lenses (Blue Glide, Pol Optic, Germany) with a narrow blue filter that blocks wavelengths below 410 nm. Spectacles B were nanophotonic lenses with a lower concentration of fullerene C_{60} (0.025%) that blocks wavelengths below 490 nm. Spectacles C were nanophotonic lenses with a higher concentration of fullerene C_{60} (0.034%) that blocks wavelengths below 530 nm. Spectacles D were commercially available lenses (Blue blocker Winter Sun, Pol Optic, Germany) with a broad blue filter that blocks wavelengths below 470 nm (Figure 1).

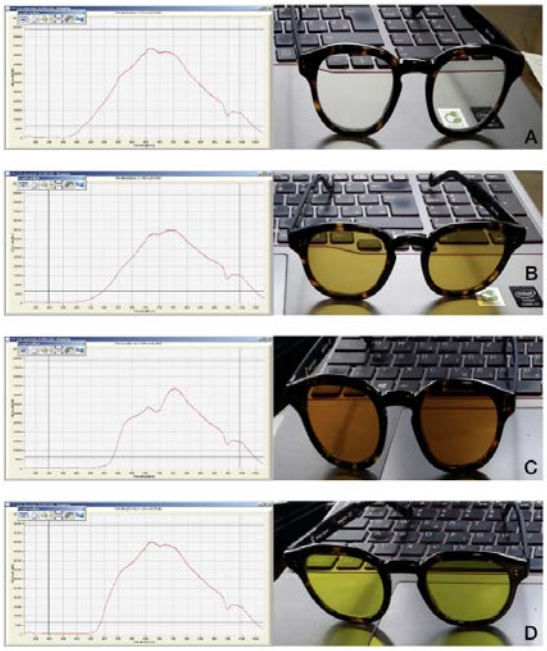

Fig. 1 – Lenses for different spectacles.
A – commercially available lenses with a narrow blue filter; B – nanophotonic lenses with concentration of fullerene (0.025); C – nanophotonic lenses with fullerene concentration of 0.034; D – commercially available lenses with a broad blue filter.

Table 1

Demographic characteristics of the study group (n = 25)

Characteristics	Values
Age (years), mean ± SD (range)	40 ± 11 (18–55)
Gender, n	
male	7
female	18
Dominant eye, n	
OD	21
OS	4
Refraction (D), mean ± SD (range)	
OD	0.23 ± 0.32 (-0.25 to 1.00)
OS	0.14 ± 0.24 (-0.25 to 0.50)
Tonometry (mmHg), mean ± SD (range)	
OD	11.84 ± 2.06 (9 to 16)
OS	11.72 ± 1.99 (9 to 16)

SD – standard deviation; OD – *oculus dexter* (right eye); OS – *oculus sinister* (left eye).

Twenty-five healthy participants (7 males and 18 females), aged 18–55 years (mean age 40 ± 11 years), were included in this pilot study. In twenty-one participants, the dominant eye was the right eye, while in four of them, the dominant eye was the left one (Table 1).

Exclusion criteria included the following: best corrected visual acuity worse than 1.0 in either eye, history of ocular diseases and surgeries, and abnormal colour vision based on the Ishihara colour vision test. All experimental procedures were approved by the Ethics Committee of the LaserFocus Centre for Eye Microsurgery (Belgrade, Serbia) and were conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from the participants, and all tests were conducted by one examiner (MJ) in the premises of the LaserFocus Centre for Eye Microsurgery.

On their first visit, the participants were subjected to a complete ophthalmological examination (distance and near visual acuity, refraction and eye dominance, slit lamp, tonometry, and fundus examination). Additionally, contrast sensitivity under standard conditions, colour discrimination, and visual field (24–2 program) were measured [13].

After four days of daily lens wear, subjective ratings of lens performance were collected by a Visual field (VF)-14 questionnaire regarding the quality of vision and comfort during different daily tasks without any spectacles. Questions about different situations, such as variable distances, object sizes, details, movement, and illumination, were combined.

All four pairs of spectacle lenses were delivered, with their identity hidden, as spectacles A, B, C, and D. The sequence of lens types was the same for each individual. All participants were asked to wear the assigned spectacles for a minimum of one day, 4 hours a day, for at least 15 minutes for each activity before assessing it in the questionnaire.

Spectacles A were commercially available lenses (Blue Glide, Pol Optic, Belgrade, Serbia) with a narrow blue filter, spectacles B were nanophotonic lenses with a lower concentration of fullerene C_{60} (0.025%), spectacles C were nanophotonic lenses with a higher concentration of fullerene C_{60} (0.034%), and spectacles D were commercially available lenses (Blue blocker Winter Sun, Pol Optic, Belgrade, Serbia) with a broad blue filter (Figure 1).

The participants were asked to use the spectacles in any daily situation, especially in the following situations: in a closed space with different types of illumination (computer, TV, tablet, mobile phone, books, newspapers and magazines); open space (walking in nature, on the streets, driving or being driven) during the day, at dusk and at night.

After the whole one-week wearing period, the visual performance and low light vision quality were assessed subjectively using a questionnaire (DA-16) and objectively - on visual acuity, contrast sensitivity, and visual field examination.

The participants would answer each of the 16 questions about how performing daily activities with a particular lens compared with the situation without the lens, and the mean score for each lens and each activity was calculated. Hence, the maximal score for all the participants for each lens would be 1.0 if all participants felt that this particular lens was better for a given activity, the minimal score would be 0 if none of the participants felt that this particular lens was better for a given activity, while the score would be 0.5 if the same number of participants stated that this particular lens was better or worse for a given activity than without using it.

The activity that was not performed by the participant was not statistically computed.

At the end of the study, the participants were asked to choose their preferred lens type among the four pairs of lenses, scoring them from 1 (very unsatisfactory) to 5 (very satisfactory).

Single-tailed paired Student's *t*-test and One-way repeated measures analysis of varience (ANOVA) were used for statistical analysis; the values of $p < 0.05$ were considered statistically significant.

Results

There was no statistical difference between the levels before and after wearing the spectacles measured by the Pelli-Robson contrast sensitivity chart with passive

illumination both under artificial light (halogen source) or natural light (sunlight) ($p = 0.83$) (Table 2).

Regarding the comfort during different activities, while using the spectacles, a universal question for each activity was whether the specific spectacles help or not in a given activity compared with the ease of performing it without them. The participant scored 1 for positive and 0 for negative, while the score was not calculated in case the subject did not perform a specific activity.

As seen in Table 3, spectacles B had the best overall comfort mean score (0 to 1), where almost two-thirds of participants had the feeling that they performed better in their overall activities than without them ($p < 0.00001$).

The results, broken down into different activities, show that the majority of participants prefer spectacles B for near activities (print reading and fine near work), intermediate activities (computer), and distance ones (signpost and face recognition, driving); two-thirds of participants perform better both during the day and night, while four-fifths of them perform better only during the day.

More details on satisfaction scores (1 to 5) from DA-16 can be found in Table 4. Spectacles B were superior regarding the following daily activities: TV, computer, reading, and open space activities with a statistically high

Table 2

Visual field results

Statistics*	MD		PSD		Absolute scotomas		Relative scotomas	
	before	after	before	after	before	after	before	after
Mean	0.04	0.03	0.58	0.64	0.88	0.48	0.92	0.80
SD	0.14	0.11	0.46	0.43	0.97	0.51	0.91	0.82
min	-0.21	-0.22	0	0	0	0	0	0
max	0.27	0.24	1.3	1.44	3	1	4	2
p		0.36		0.25		**< 0.05**		0.31

***Single-tailed paired Student's *t*-test.**
MD – mean deviation; PSD – pattern standard deviation; SD – standard deviation; min – minimum; max – maximum.

Table 3

Comfort score during different activities

Statistics*	Spectacles†			
	A	B	C	D
Mean	0.55	**0.63**	0.28	0.61
SD	0.09	0.15	0.14	0.13
min	0.30	0.38	0.00	0.42
max	0.64	0.88	0.50	0.93
p	< 0.00001			

***One-way repeated measures analysis of variance (ANOVA).**
†For explanation see Figure 1.
SD – standard deviation; min – minimum; max – maximum.

Table 4

Satisfaction score during different daily activities One-Way repeated measures ANOVA

Parameters	Spectacles†				*p**
	A	B	C	D	
Television (TV)					
mean	3.68	**4.00**	2.32	3.24	**0.0001**
SD	1.28	1.29	1.25	1.56	
Reading					
mean	3.64	**3.84**	2.44	3.28	**0.0009**
SD	1.29	1.22	1.36	1.57	
Car					
mean	**3.68**	**3.68**	2.60	3.08	**0.002**
SD	1.28	1.18	1.26	1.44	
Fine work					
mean	3.41	3.65	**3.88**	3.41	**0.00356**
SD	1.37	1.37	1.27	1.54	
Outside					
mean	3.52	**4.04**	2.60	3.60	**0.0005**
SD	1.29	1.31	1.39	1.39	

***One-way repeated measures analysis of variance (ANOVA).**
†For explanation see Figure 1.
SD – standard deviation.

difference, while they were ranked 2nd for driving and fine manual work. Spectacles C were superior for fine manual work without reaching statistical significance.

Overall satisfaction scores (1 to 5) can be found in Table 5, where spectacles B were superior in terms of overall satisfaction regarding all combined daily activities (4.04 ± 1.1), which was statistically highly significant ($p = 0.0008$).

Table 5

Overall satisfaction score One-Way repeated measures ANOVA

Statistics*	Spectacles†				*p*
	A	B	C	D	
Mean	3.12	**4.04**	2.68	3.72	0.0008
SD	1.05	1.10	1.25	1.37	

***One-way repeated measures analysis of variance (ANOVA).**
†For explanation see Figure 1.
SD – standard deviation.

Discussion

Wavelengths can activate rhodopsin range from 400 nm to almost 600 nm [12]. Not all parts of the spectrum affect retinal cells equally: the one above 500 nm wavelengths excites rhodopsin and generates toxic waste but does not cause retinal degeneration; the other part of the spectrum, below 500 nm, causes retinal degeneration in addition to toxic waste. Rhodopsin and its sub-products of excitation seem to have a major role in retinal damage [5].

After photo-stress, cones and rods die by apoptosis, thus rendering irreversible loss of retinal function. A large number of photoreceptors could survive by using the filters which allow survival, but still, there will be morphological and functional alterations in the retina [2]. On the contrary, a full recovery of functional responses after nine months of light deprivation has also been reported, even after having suffered a 50% reduction caused by photo-stress [5]. The number of absolute scotomas in our study was statistically significantly decreased after the use of the spectacles. Considering the fact that the absolute scotoma means that there is a permanent decrease in sensitivity to the light stimulus of that area of the retina, differently from the relative one where there is a relative decrease, one could postulate that we could witness the full functional recovery after using protective blue-light filtering lenses, as suggested by Vicente-Tejedor et al. [5].

Therefore, it is reasonable to think that photo-stress injury could be mitigated enough for getting a full recovery later [5]. Moreover, protecting the photoreceptors by decreasing their exposure to the high-energy blue part of the spectrum in order to reduce the risk of age-related macular degeneration (AMD) would make sense. Blue-filtering intra-ocular lenses with a UV filter that are implanted after the cataract surgery have been used with such intention without conclusive results [14, 15].

However, AMD is a multifactorial eye disease, which has risk factors including age, smoking, nutritional status, sunlight exposure, and genetic background [16, 17]. Moreover, the disease takes years to develop and progress, which makes it difficult to directly comprehend the protective efficacy of the blue-light filtering lenses in human eyes. A large population prospective study might answer that question.

Indoor lighting or screens use LED lamps that may be of concern if used for extended viewing times and at a short distance. While we can protect ourselves from the natural blue light by wearing filtering glasses, it is more difficult to do so in internal lighting. One of the suggested solutions is to restrict its use to "white warmth" lamps (2700 K). As far as organic light emmiting diode (OLED) or active matrix organic light emmiting diode (AMOLED) screens are concerned, the only effective protection is to use them occasionally and only for a short period of time [18].

On the other hand, O'Hagan et al. [19] reported that even under extreme long-term viewing conditions, none of the assessed sources (fluorescent lamps and LED, computer screens, tablet computers, laptops, and smartphones) suggested a cause for concern for public health. In terms of blue light hazard, the domestic lamps had a range from 10–20% of the exposure limit, assuming intentional long-term viewing. At the same time, knowing that the percentage of blue light transmission from the corneal surface to the retina is higher for children than for adults, such sources could be distressing for children while for adults they are uncomfortable to view [19].

Our study showed that subjectively spectacles B with a lower concentration of fullerene C_{60} were statistically significantly superior in all different daily activities: for near (reading paper print or doing fine manual work), for mid-distance (computer work), and for distance (sing posts, street signs, face recognition, driving).

It is interesting to observe that static vision (e.g., street signs or face recognition) was good both during the day and at night, while dynamic vision (driving) was more comfortable only during the day. This would be in line with the studies by Leung et al. [7] and Spalton et al. [12] that point out that adequate blue light is necessary for normal visual function, such as in colour discrimination and night vision. Under low-light conditions, the overall number of photons is generally reduced. Therefore, any additional loss that happens in the high-energy blue light with these lenses might reduce them below the threshold of comfort. Spectacle lenses with an even lower concentration of fullerene might be useful under low-light conditions while driving, as well as during foggy weather.

Although day driving has been reported as significantly more comfortable with spectacles B, some participants complained about the internal reflections of the dashboard onto the windshield. Differently from traditional polarized spectacles that prevent diffuse light from the inside of the cabin to reflect from the windshield, the hyper-polarized light does not attain the same level of comfort in this task. A combination of hyper-polarizing fullerene filter and traditionally linearly polarizing coating might make a perfect combination.

It has been shown that nanophotonic glasses transform daily sunlight, LED white light, neon light, mobile phone

and TV screen light into a light spectrum that is more comfortable to the human eye [11]. The efficiency of nanophotonic harmonized light and nanophotonic hyper-polarized light in medicine, compared to traditional light sources (linearly polarized, colour light, and laser), is 20–40% higher. The reason for this lies in the fact that nanophotonic harmonized and hyper-polarized lights affect the tissue not only from an energy point of view but because "structured light meets structured matter", as the resonance of the icosahedral (orientation-preserving) symmetry order of light (photons) and the order of structure-energy-information synergy of biomolecules.

A pilot study showed that the use of nanophotonic glasses also balances the serotonin/melatonin ratio, which also had a positive effect on behaviour, reducing anxiety and depression while meliorating sleep quality [11]. The harmonized light interacts with the biomolecules and may initiate the restoration of the disrupted symmetry. During this process, it might influence the brain waves through retinal ganglion cells rather than the photoreceptors pathway and thus influence the pineal gland function and the levels of neurotransmitters in the brain [3].

Further studies that are in progress include validation of the results presented herein in a larger sample of participants. They also include some specific modifications, such as different and longer wear regimes, diverse and more controlled ambient light sources. Moreover, these studies set with patients in whom the benefit of blue-blocking is expected, such as patients with corneal oedema and early cataract, with macular degeneration or glaucoma neural damage, in pseudophakic patientsm, or patients with low myopia or astigmatism.

Conclusion

It was shown that using blue-blocking filters with fullerene C_{60} can significantly decrease the high-energy blue part of the spectrum present in natural and artificial light sources. At the same time, they increase the overall comfort of daily tasks during and after their use. Therefore, these filters might be an effective mechanism to protect us from ocular pathologies alleviating the functional loss of retinal photosensitive cells if the expected exposure to the blue-rich light in the living ambient is high and long enough.

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Tumor budding in tumor tissue among operatively treated patients with lung adenocarcinoma

Pupljenje tumora u tumorskom tkivu kod bolesnika operisanih zbog adenokarcinoma pluća

Milena Vasilijević, Aleksandra Lovrenski, Milana Panjković

University of Novi Sad, Faculty of Medicine, Novi Sad, Serbia

Abstract

Background/Aim. The histological phenomenon of tumor budding is being recognized as an important determinant of disease progression and poor prognosis in various types of carcinoma. We aimed to evaluate the clinicopathological significance of tumor budding in adenocarcinoma of the lung. **Methods.** The study included 114 patients operatively treated for lung adenocarcinoma in a one-year period. Microscopic analysis of routine histological slides was performed to establish the presence and density of tumor buds. These results were compared in relation to gender, age, tumor size, nodal status, and pathological stage. **Results.** The budding-positive group included 34 (53.1%) men and 27 (54%) women. There were 30 (46.9%) men and 23 (46%) women in the budding-negative group. No statistical significant difference in age was found between males (64.3 ± 6.59 years) and females (63.1 ± 6.53 years) in the budding-positive group, nor in the budding-negative group (males 63.3 ± 6.02 years; females 63.2 ± 6.72 years). Statistically significant result in tumor size was found in females with the presence of tumor budding ($p < 0.05$). The budding-positive group of patients in nodal stage N1 and stage III of the disease pointed to the statistical significance ($p < 0.05$). **Conclusion.** With the statistical significance confirmed between the higher nodal status, higher pathological stage, and tumor budding found in this study, this histological phenomenon is still relatively new for the diagnostics domain of pathology. However, it increasingly receives attention as an adverse prognostic factor. These results may help tumor budding incorporate into the existing staging systems in addition to other factors known to be predictors of worse outcome.

Key words:
lung neoplasms; adenocarcinoma; neoplasm invasiveness; neoplasm staging; postoperative period; neoplasm recurrence, local.

Apstrakt

Uvod/Cilj. Fenomen tumorskog pupljenja sve više biva prepoznat kao značajna determinanta progresije i loše prognoze različitih tipova karcinoma. Cilj studije je bila klinički-patološka evaluacija ovog fenomena u adenokarcinomu pluća. **Metode.** Studija je obuhvatila 114 bolesnika operisanih od adenokarcinoma pluća u periodu od jedne godine. Prisustvo i gustina tumorskih pupoljaka analizirani su mikroskopski. Dobijeni rezultati su upoređivani u odnosu na pol i starost bolesnika, veličinu primarnog tumora, nodalni status i stadijum bolesti. **Rezultati.** U grupi bolesnika sa potvrđenim prisustvom tumorskog pupljenja bilo je 34 (55,7%) muškaraca i 27 (44,3%) žena, a u grupi bez prisustva tumorskog pupljenja 30 (56,6%) muškaraca i 23 (43,4%) žena. Nije uočena statistički značajna razlika u starosnoj dobi između muškog (64,3 ± 6,59 godina) i ženskog pola (63,1 ± 6,53 godina) kod bolesnika sa prisustvom tumorskih pupoljaka u tumorskom tkivu, kao ni kod muškog (63,3 ± 6,02 godina) i ženskog pola (63,2 ± 6,72 godina) kod bolesnika bez tog prisustva. Primarni tumor bio je značajno veći ($p < 0.05$) kod bolesnica sa fenomenom tumorskog pupljenja. U grupi bolesnika u čijem je tumorskom tkivu dokazan fenomen pupljenja dominirao je nodalni stadijum N1 ($p < 0,05$) i stadijum III bolesti ($p < 0,05$). **Zaključak.** Sa potvrđenom statističkom značajnošću između višeg nodalnog statusa, stadijuma bolesti i tumorskog pupljenja, pokazano je da ovaj fenomen, iako relativno nov u dijagnostičkom domenu patologije, privlači dodatnu pažnju kao značajan prognostički faktor. Dobijeni rezultati bi mogli pomoći u integrisanju ovog fenomena u postojeće skoring sisteme kao dodatka ostalim prediktorima koji ukazuju na lošiju prognozu bolesti.

Ključne reči:
pluća, neoplazme; adenokarcinom; neoplazme, invazivnost; neoplazme, određivanje stadijuma; postoperativni period; neoplazme, lokalni recidiv.

Correspondence to: Milena Vasilijević, University of Novi Sad, Faculty of Medicine, Hajduk Veljkova 3, 21 000 Novi Sad, Serbia.
E-mail: milena.vasilijevic@mf.uns.ac.rs; vasilijevic93@gmail.com.

Introduction

Lung cancer is confirmed to be the leading cause of cancer-related deaths worldwide, with a generally unfavorable outcome, even with a successful surgery. In recent years, the number of lung adenocarcinoma cases has been increasing. According to many studies, lung adenocarcinoma often comes with aggressive biological behavior, noting recurrence or distant metastasis soon after curative resection [1–5]. A better understanding of changes in malignant neoplasm biology that result in a more aggressive neoplastic behavior may help identify patients with a high risk of recurrent diseases and influence treatment algorithms.

In an arsenal of parameters essential in the outcome of patients, the target area in the present study was the phenomenon of tumor budding, investigated in terms of having an important role in risk stratification in lung adenocarcinoma.

The term tumor budding has been applied to the detachment and migration of single tumor cells or small clusters of cells from the neoplastic epithelium on the invasive front of tumor [6]. With an unknown molecular background, tumor budding is associated with a high incidence of local invasion and distant metastasis. Previously known as tumor dedifferentiation, this phenomenon has been likened to an epithelial-mesenchymal transition, thereby increasing cell migration and invasion [7–10]. This phenomenon is speculated to be a morphological expression of an invasive growth process that includes detachment between tumor cells, migration, and active invasion of surrounding stroma. From a morphological point of view, these groups of tumor cells tend to appear more atypical than cells in the main tumor body and may be visualized with difficulties on hematoxylin and eosin (H&E) routine slides. In addition, these cells may be obscured by a peritumoral inflammatory reaction and hardly distinguished from the reactive stromal cells [11, 12]. There are no well-established criteria used to determine how many cells should be in a cluster, therefore, it can be called a tumor bud. To date, the majority of studies used the 5-cell cutoff value. This criterion is often regarded to the presence and intensity of tumor budding in colorectal adenocarcinoma, while the number of studies referred to the budding in lung adenocarcinoma is rather small [13].

Tumor budding cannot be identified as tumor dedifferentiation [14]. The pattern composed of solid areas with numerous detached cells is often found in poorly differentiated tumors. Furthermore, high-grade tumors do not have or may have an insignificant number of tumor buds [15].

The differences in morphological features between cells in tumor buds and cells in the main tumor body are major. Budding cells have a tendency of progressively losing epithelial-cell features and resembling mesenchymal cells, therefore, they get long and spindle. In addition, these cells may degrade the extracellular matrix. Markers of motility, chemotaxis, and angiogenesis may be present on the cell surface. By performing immunohistochemistry, a positive reaction on mesenchymal cell markers may be confirmed. All the features mentioned are not found among cells in the central tumor area [9, 10, 15].

A limited number of studies, mostly in the colorectal oncology domain, recognize the presence of tumor budding as an important determinant of disease progression and poor prognosis [16–18]. Nevertheless, many disagreements are present among authors. The majority of authors consider budding as an indicator that drives aggressiveness and affects the disease-free and overall survival. Other authors, on the contrary, reported the presence of tumor budding in cases with already anticipated unfavorable prognosis due to the lymphatic and vascular invasion, as well as the infiltration of serosa.

As budding is often thought to have an independent prognostic value in patients with primary operable lung adenocarcinoma, the purpose of this study was to evaluate the clinicopathological significance of budding in adenocarcinoma of the lung.

Methods

From January 1 to December 31, 2018, a total of 114 patients with primary lung adenocarcinoma were treated by surgical resection in the regional hospital for pulmonary diseases. The cases of 64 male patients and 50 female patients who had undergone complete resection of lung adenocarcinoma were reviewed in this retrospective study. The study protocol was approved by the Research Ethics Committee of our hospital (January 26, 2018, No. 73-I/23). These cases were selected sequentially. The patients diagnosed with lung adenocarcinoma, with histological slides available for histological evaluation, and with complete follow-up data were included in this study. The exclusion criteria used in this study referred to patients who received neoadjuvant chemotherapy before the surgery and patients whose follow-up data was incomplete. The patients' characteristics that were assessed included gender, age, tumor size, stage of the disease, and histological subtypes of lung adenocarcinoma. The clinicopathological data were obtained from routine medical reports.

The histological diagnosis of primary lung adenocarcinoma was based on the 2015 World Health Organization Classification of Lung Tumors [19]. Tumor size was measured as the maximal diameter on the cut sections of the lung. The tumor subtypes, as well as the pathological stage, were determined according to the newest 2015 World Health Organization Classification of Lung Tumors and the 2014 IASLC/ATS/ERS Lung Adenocarcinoma Classification [20].

The lung tissue surgical specimens for the histological analysis were fixed by 10% neutral formalin, then routinely paraffin-embedded. The tumors were cut at approximately 5 mm intervals, sliced to 4 μm thick sections, and stained with (H&E. Full-section H&E slides were used to evaluate the presence and intensity of tumor budding, characterized by isolated tumor cells or small clusters that migrate a short distance into the neoplastic stroma at the advancing edge of neoplasms. Tumor budding was evaluated semiquantitatively, using a 20x objective lens by two pulmonary pathologists (AL and MP). In the first step, all of the slides were evaluated to determine the most representative tumor area. In a his

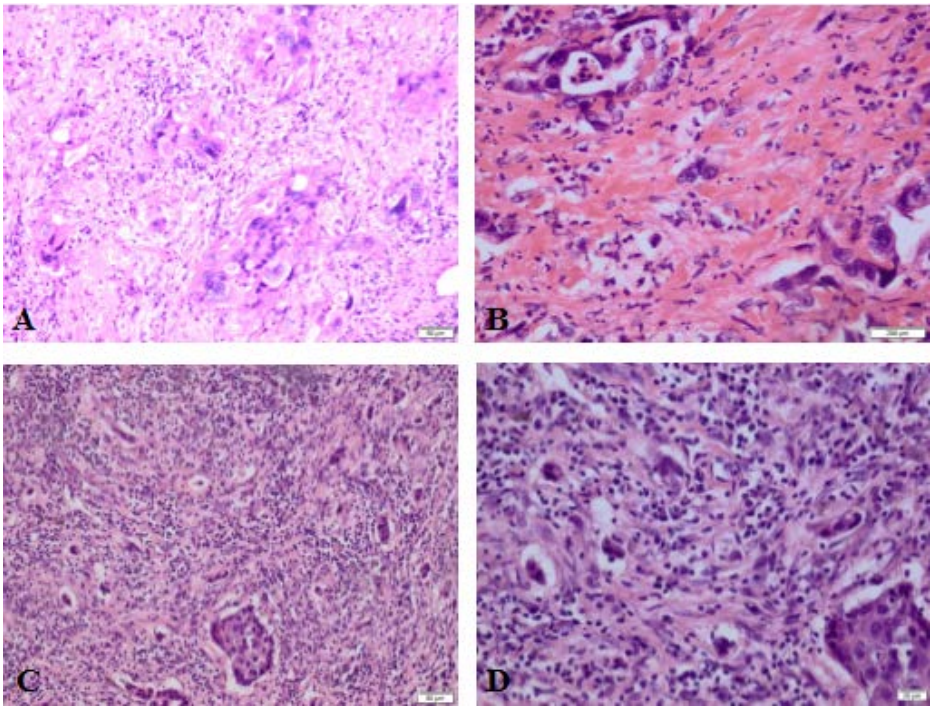

Fig. 1 – Tumor budding in lung adenocarcinoma: A, B) hematoxylin and eosin (H&E), ×20; C, D) H&E, ×40.

tological section, the maximal intensity of tumor budding was selected on the slide, and the number of tumor buds in that field was counted using a 20× objective lens. According to the presence of tumor buds per field, 2 major groups of patients were formed: 1) a budding-positive group of patients (Figure 1), and 2) a budding-negative group of patients.

In order to investigate the relationship between tumor budding and clinicopathological characteristics of the patients, we compared these results in relation to demographic parameters (gender, age), as well as with histological subtypes, tumor size, nodal status, and pathological stage of the tumor.

The data were processed in the IBM SPSS (*Statistical Package for Social Sciences*) program, version 23. Data analysis methods used descriptive and inferential statistics. Numerical variables were presented by the arithmetic mean and standard deviation, and the categorized variables through the frequencies and percentages. To determine the existence of a difference in variables between the study groups, Student's *t*-test and χ^2-test were used. Cumulative survival rates were calculated by the Kaplan-Meier method. The log-rank test was used to evaluate differences between the survival curves. All the differences were considered significant when the *p*-value was less than 0.05. The results were shown as tables and figures.

Results

Clinicopathological characteristics and histologic examination

Summarized characteristics of all 114 cases were presented through percentages. Within both groups, male patients were dominant regarding the tumor budding (Table 1). Sixty-one cases were classified as the acinar subtype, 42 as the solid subtype, 5 as the papillary subtype, 4 as the mucinous subtype, and 2 as the lepidic subtype. Tumor budding was found in 61 cases, and it was most frequently detected in the acinar subtype of lung adenocarcinoma (Table 1).

Table 2 shows the distribution of 114 cases by age and by tumor size within budding-positive and budding-negative groups. The arithmetic mean and standard deviation were calculated for these numerical variables, but, as the results

Table 1

Clinicopathological characteristics of patients with tumor budding

Characteristics of patients	All cases, n	Budding (+), n (%)	Budding (-), n (%)
Number	114	61 (53.5)	53 (46.5)
Gender			
male	64	34 (53.1)	30 (46.9)
female	50	27 (54)	23 (46)
Histological subtypes			
acinar	61	35 (57.3)	26 (42.7)
solid	42	22 (52.3)	20 (47.7)
papillary	5	3 (60)	2 (40)
mucinous	4	1 (25)	3 (75)
lepidic	2	0 (0)	2 (100)

Table 2

Distribution of patients by age and tumor size in study groups

Parameters	All cases (n)	Budding (+) mean ± SD	Budding (-) mean ± SD	Student's *t*- test	*p*-value
Age (years)					
male	64	64.3 ± 6.59	63.3 ± 6.02	0.627	0.533
female	50	63.1 ± 6.53	63.2 ± 6.72	-0.053	0.958
Tumor size (cm)					
male	64	4.12 ± 1.93	4.78 ± 2.50	-1.227	0.224
female	50	4.79 ± 2.77	3.33 ± 1.32	2.304	0.026

SD – standard deviation.

Table 3

Association between nodal and pathological stages and the presence of tumor budding

Parameters	All cases (n)	Budding (+) n (%)	Budding (-) n (%)	χ^2-test	*p*-value
Nodal stage					
N0	78	35 (44.9)	43 (55.1)	7.407	0.08
N1	20	17 (85)	3 (15)	9.669	0.02
N2	16	9 (56.2)	7 (43.8)	0.056	1
Pathological stage					
I	50	23	27	2.019	0.109
II	31	15	16	0.449	0.323
III	33	23	10	4.893	0.022

showed, the average age and tumor size were not significantly associated with the presence of tumor budding.

Table 3 shows the results of the χ^2-test used to determine the existence of a difference in nodular stage and pathological stage between the study groups. These two parameters and the presence of tumor budding were analyzed for associations, and significant associations were found between N1 status and stage III and the presence of tumor budding.

Survival analysis

From the Kaplan-Meier plots, it can be concluded that the cumulative survival proportions vary between the examined parameters. The cumulative survival proportion appeared to be much higher in the population without tumor budding compared to the population with tumor budding. It was shown that patients without tumor budding had better chances of survival (Figure 2A). Secondly, the cumulative survival proportion appeared to be equal in all nodular stages (Figure 2B). Moreover, the cumulative survival proportion appeared to be much higher in stage II compared to stage I and stage III, which did not appear to differ considerably. It was shown also that patients with the second stage of the disease had better chances of survival (Figure 2C). A log-rank test was run to determine if there were differences in the survival distribution for these three parameters. Survival distributions were not significantly different (for tumor budding: $\chi^2(2) = 1.556$, $p = 0.212$; for nodal stage: $\chi^2(2) = 1.236$, $p = 0.539$; for pathological stage: $\chi^2(2) = 5.939$, $p = 0.051$).

Discussion

The histological phenomenon of tumor budding was first described in the Japanese medical literature in 1949 [21] but revised after more than 2 decades among patients with colorectal adenocarcinoma. It is still not a part of the routine medical access and does not have a definite role in evaluating the prognosis of patients with different types of carcinoma because no consensus for the finest and most precise definition of tumor budding and the unique methodology for

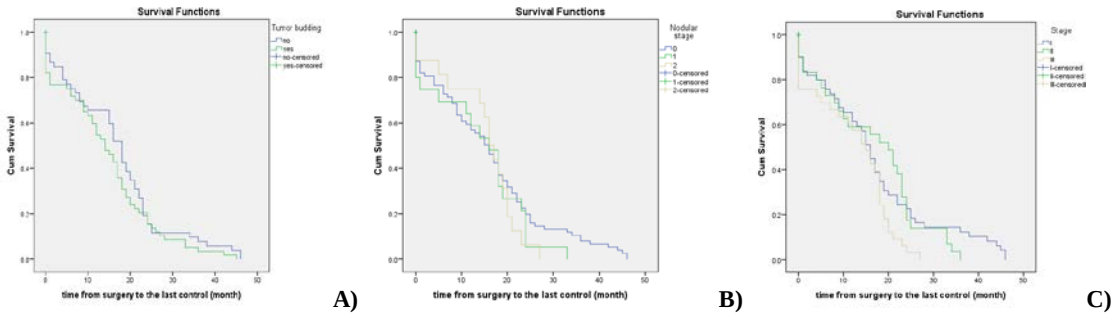

Fig. 2 – A) Cumulative overall survival curves stratified by the presence or absence of tumor budding; B) Cumulative overall survival curves stratified by the nodal stages N0, N1, and N2; C) Cumulative overall survival curves stratified by the pathological stages I, II, and III.

scoring has been formed [22]. The desire to conduct this retrospective study was based on the findings of multivariate analysis studies that show a stronger relationship between tumor budding and poor overall prognosis, unlike the singly used tumor-node-metastasis (TNM) classification [18]. In spite of these results, budding has still not been fully accepted as a factor that correlates directly with the biological behavior of the tumor.

Various ways can be used to define a histological structure as a tumor bud and to exclude bud-looking structures that are not true buds. Ueno et al. [16] defined buds as isolated malignant cells or ≤ 4 clustered malignant cells in the stroma at the invasive front of the tumor. Some authors slightly changed this definition and increased the cutoff value to foci of ≤ 5 clustered malignant cells [23, 24], thus they set the value many other authors tend to favor [25–27]. Along with the 2002 original publication of Ueno et al. [16] that was widely used in literature, there are 4 most cited methods for tumor budding assessment: Hase et al. (1993) [28], Nakamura et al. (2005) [29], and conventional method and rapid method by Wang et al. (2009) [30].

A total of 114 patients in this study were divided into two groups based on the budding-positive or budding-negative findings. The result of a dominant male distribution between the study groups may be related to the conventional fact of men being more frequently diagnosed with lung carcinoma than women. However, the study from 2015 indicated a relationship between females and low-grade budding in lung adenocarcinoma [31]. In the present study, no association between gender and the presence of tumor budding has been confirmed. The median age of the patients in our study was 63, with the range from 46 to 78 years, which is consistent with the observations of the 2016 study, where the median age was 66 (66 ± 9.9) years [32]. However, the consistency between age and tumor budding was not found.

Our attention was also dedicated to histological subtype analysis, and it was proved that the acinar subtype was dominant in male patients in both study groups, while the acinar and solid subtype were equally found in female patients, which makes these results corresponding to the reports of Kadota et al. [31] study. Tumor budding was not found in the lepidic subtype of lung adenocarcinoma [33]. These results suggest that the biological mechanism by which tumor budding is induced may vary with histological subtype.

The mean tumor size in the budding-positive group of patients was 4 cm (4.4 ± 2.34 cm), as well as in the budding-negative group (4.2 ± 2.18 cm), thus the statistical significance was not confirmed. Yamaguchi et al. [33] reported the findings of tumor budding in cases with adenocarcinoma bigger than 3 cm.

One of the most significant parameters to which tumor budding is connected is nodal status. The current result revealed that N1 status was significantly associated with the presence of tumor budding. In contrast, the absence of lymphatic invasion resulted in other studies conducted on N0 status, as opposed to our study [31]. Moreover, we analyzed the presence of tumor budding and pathological stage for associations, and significant associations were found between stage III and tumor budding. Comparing our results to other studies' results is difficult due to different stage analyses in other studies (mostly stage I) [33]. The reason why tumor budding is significantly associated with parameters that lead to a poor prognosis is not clarified. One of the satisfactory explanations is that budding cell phenotype represents a component of distant tumor invasion [22]. Taken together may explain the more aggressive behavior of the tumors that show this feature.

The overall number of studies demonstrating the presence and intensity of tumor budding in primary lung adenocarcinoma is rather small, especially because the use of corresponding immunohistochemistry methods is often required. The biggest obstacle for considering tumor budding as an integrated category in pathology reports is not having enough well-defined criteria for its evaluation. In addition, this has been pointed out in various types of carcinoma. In this manner, the budding aspect as a prognostic factor has been attracting interest [34–39]. Furthermore, it is believed that budding represents a histological basis for tumor cells to detach and invade locally and systemically [22]. According to the data reported previously, budding has been strongly linked to adverse clinicopathological features, poor overall prognosis, and disease-free survival.

Conclusion

With statistical significance confirmed between a higher nodal status, higher pathological stage, and tumor budding found in our study, this histological phenomenon is still relatively new for the diagnostics domain of pathology. However, it is receiving increasing attention as an adverse prognostic factor. It is imperative to add more clinicopathological features used to assess the risk of overall prognosis and to facilitate optimal clinical management through planning the treatment prior to surgery. These results may help tumor budding incorporate into the existing staging systems as it is associated with other factors known to portend worse outcomes, such as infiltrating tumor border, scirrhous stromal type, lymphatic, vascular, perineural and pleural invasion, nodal and distant metastases.

It is widely noted that additional studies will be needed to further define the methodology and uniform reporting of tumor budding through the most reproducible scoring method. The significance of tumor budding will need to be further evaluated in a multidisciplinary setting until further data become available.

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Association of prothrombin, FV Leiden and MTHFR gene polymorphisms in the Montenegrin patients with venous thromboembolism

Povezanost polimorfizama za protrombin, FV Leiden i MTHFR gen sa venskim tromboembolizmom kod bolesnika u Crnoj Gori

Sladjana Teofilov*, Zvonko Magić†‡, Olivera Miljanović*, Tatjana Ostojić*, Milena Bulatović*

***Clinical Center of Montenegro, Center for Medical Genetics and Immunology, Podgorica, Montenegro; †Military Medical Academy, Institute of Medical Research, Belgrade, Serbia; ‡University of Defence, Faculty of Medicine of the Military Medical Academy, Belgrade, Serbia**

Abstract

Background/Aim. Polymorphisms of the factor V Leiden (FV G1691A), prothrombin (FII G20210A), and methylenetetrahydrofolate reductase (MTHFR C677T) genes are the most commonly investigated inherited risk factors for developing venous thromboembolism (VTE). Despite this fact, there is insufficient data regarding their clinical burden and distribution in the Montenegrin population. The aim of the study was to determine the frequency of these polymorphisms in Montenegrin patients with VTE. **Methods.** This case-control study was conducted on 160 Caucasian subjects. The study group was composed of 80 patients (35 men and 45 women) with VTE. The control group consisted of 80 healthy individuals (32 men and 48 women) without previous thromboembolic episodes. Genotyping of the FV G1691A, FII G20210A, and MTHFR C677T polymorphisms was performed by allele-specific polymerase chain reaction (PCR). **Results.** The frequency of heterozygotes (HET) for FII G20210A and FV G1691A was significantly higher in the VTE group compared to the healthy control group (χ^2 = 11.7; p = 0.001 and χ^2 = 17.69; p < 0.001, respectively). The association of FII G20210A and FV G1691A polymorphisms with an increased risk of VTE [odds ratio (OR) 10.5; 95% confidence interval (CI) = 2.34 to 47.27, and OR 14.8; 95% CI = 3.34 to 65.43; p < 0.001, respectively] was confirmed. Recessive homozygotes (RH) for FII G20210A and FV G1691A were not found in any of the investigated groups. Regarding MTHFR C677T, the difference between the frequency of HET and RH in the control and VTE group was not significant. **Conclusion.** Our study showed that FII G20210A and FV G1691A polymorphisms are significantly associated with VTE. Detection of the above-mentioned polymorphisms prior to VTE development can contribute to the prevention of further VTE occurrence, especially among patients' relatives who are carriers of these polymorphisms.

Key words:
factor v; genes; mutation; polymorphism, genetic; prothrombin; thromboembolism.

Apstrakt

Uvod/Cilj. Polimorfizmi u genima koji kodiraju faktor V Leiden (FV G1691A), protrombin (FII G20210A) i metilentetrahidrofolat reduktazu (MTHFR C677T) su najčešće ispitivani nasledni faktori rizika od nastanka venskog tromboembolizma (VTE). Uprkos tome, ne postoji dovoljno podataka o kliničkom značaju i distribuciji tih polimorfizama u crnogorskoj populaciji. Cilj rada bio je da se utvrdi frekvencija tih polimorfizama kod bolesnika sa VTE u Crnoj Gori. **Metode.** Istraživanje je sprovedeno kao studija tipa slučaj-kontrola na 160 ispitanika kavkaskog porekla. Studijsku grupu sačinjavalo je 80 bolesnika (35 muškaraca i 45 žena) sa VTE, a kontrolna grupa se sastojala od 80 zdravih ispitanika (32 muškarca i 48 žena), koji nisu imali tromboembolijske epizode bolesti. Genotipizacija polimorfizama za FV G1691A, FII G20210A i MTHFR C677T izvršena je alel specifičnom, lančanom reakcijom polimeraze (PCR). **Rezultati.** Učestalost heterozigota (HET) za FII G20210A i FV G1691A bila je značajno viša u VTE grupi u poređenju sa kontrolnom grupom (χ^2 = 11,7; p = 0,001 i χ^2 = 17,69; p < 0,001). Potvrđena je povezanost polimorfizama za FII G20210A i FV G1691A sa povećanim rizikom od VTE [*odds ratio* (OR) 10,5; 95% *confidence interval* (CI) = 2,34 do 47,27; p = 0,001 i OR 14,8; 95% CI = 3,34 do 65,43; p < 0,001]. Recesivni homozigoti (RH) za FII G20210A i FV G1691A nisu pronađeni ni u jednoj

Correspondence to: Sladjana Teofilov, Ul. Zmaj Jovina, 54/57, Podgorica, Montenegro. E-mail: steofilova@yahoo.com

od ispitivanih grupa. Za polimorfizam MTHFR C677T nije pronađena značajna razlika u učestalosti HET i RH između VTE grupe i kontrolne grupe. **Zaključak.** Naša studija je pokazala da su polimorfizmi za FII G20210A i FV G1691A značajno povezani sa VTE i njihovo pravovremeno otkrivanje može doprineti prevenciji VTE, posebno kod srodnika bolesnika koji su nosioci tih polimorfizama.

Ključne reči:
faktor v; geni, mutacija; polimorfizam, genetički; protrombin; tromboembolija.

Introduction

Venous thromboembolism (VTE) is a multifactorial disease that results from the interaction between acquired and genetic risk factors with an incidence of 1–2 per 1,000 persons annually. The most common clinical manifestations of VTE are deep venous thrombosis (DVT) and pulmonary thromboembolism (PTE). Below the age of 25, thrombosis is rarely diagnosed, but after the age of 40, it shows an increasing tendency, and it is often repeated and additionally complicated by pulmonary embolism [1–3].

Thrombophilia represents a group of inherited and acquired coagulation abnormalities associated with thrombosis. Although thrombophilia itself is not a disease, it increases the risk of developing VTE in response to the provocation/perturbation by environmental factors [4, 5]. Numerous acquired and hereditary risk factors are known to be responsible for the occurrence of VTE [5, 6]. Genes encode proteins of the hemostasis system, affecting their synthesis and activity, and the acquired factors stimulate the tendency of hypercoagulability. Many studies suggested a multifactorial etiology of VTE. Therefore, the hereditary predisposition only represents an increased risk and does not determine whether the disease will necessarily manifest in the genetically affected population [7–11].

The most common single nucleotide polymorphisms (SNPs) tested in the genes associated with VTE are genes for factor II (prothrombin, FII G20210A), factor V Leiden (FV G1691A), and methylenetetrahydrofolate reductase (MTHFR C677T) [11]. Many previous studies have shown that the frequency of polymorphisms [recessive homozygotes (RH) and heterozygotes (HET)] of these genes was significantly higher in patients with VTE compared to the healthy population [11–14]. Nevertheless, there are studies with controversial results, especially on the role of MTHFR gene polymorphisms in VTE [15–16]. Besides, the prevalence of genetic polymorphisms in the healthy population can vary regarding different geographical regions and differ among ethnic groups [17, 18].

According to the literature, FV G1691A polymorphisms are the most important genetic risk factors for the manifestation of VTE. The gene for FV consists of 25 exons, and it is located on chromosome 1q23. FV G16961A mutation leads to the replacement of arginine for glutamine at position 506 in the protein resulting in reduced sensitivity of FV to the inhibitory effect of activated protein C (APC) and the balance of the hemostatic system moves to a state of hypercoagulability [19].

Coagulation factor V (FV) is an important protein (cofactor), with a double role in maintaining hemostatic balance due to the same influence in both procoagulation and the anticoagulation mechanism of blood clotting [19]. The relative risk of thrombosis in HET FV G16961A carriers is 3 to 7 times, and for the RH of FV A16961A carriers, 50 to 80 times higher, compared to noncarriers of these polymorphisms [20]. The prevalence of FV G16961A increases from West to East Europe and from North to South Europe. In a healthy European population, HET for FV G16961A is present with a frequency of 5%–7%, and 15%–50% in VTE patients [18, 21]. This mutation is rarely found in the populations of Africa, Australia, and South Asia [22, 23]. A large epidemiologic study conducted in the USA presented a 5.27% incidence of HET for FVG1691A in European individuals, 2.21% in Latinos, 1.23% in Afro-Americans, 1.25% in American Indians, and only 0.45% in Asians [22].

Another important genetic risk factor for VTE is FII G20210A (prothrombin). Prothrombin is the precursor of thrombin and has an important role in the formation of fibrin in the coagulation process. Substitution of guanine (G) for adenine (A) in the FII gene at position 20210 is associated with an increase in prothrombin plasma concentrations. Gene for FII is located on chromosome 11p11, in a 3'-untranslated region [24]. The prevalence of polymorphism of *FII* G20210A in the European population is 2%–4% [25]. The prevalence of FII G20210A in Northern Europe is 1.7%, and in the Mediterranean region is twice as high. In patients with VTE, HET for FII G20210A is present in 6%–18% of the cases [25]. This polymorphism was found to be very rare or even absent in African and Asian populations, as well as in American Indians and Australian Aborigines [25]. RH variant for FII G20210A is very rare. The risk becomes 50-fold higher among individuals with two copies of the 20210A allele [26].

MTHFR is the key enzyme in regulating the metabolism of folate and homocysteine levels, which catalyzes the reaction of 5, 10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate, which functions as a methyl donor in the conversion of homocysteine to methionine. Increased level of homocysteine in the blood has a toxic effect on the vascular structure [27]. RH and HET of MTHFR C677T result in the reduction of synthesis of 5-methylenetetrahydrofolate, leading to an increased concentration of homocysteine in the plasma, which increases the risk of arterial and venous thrombosis [27]. The gene for MTHFR is located on chromosome 1 at position 1p36.3 [28]. The HET prevalence of the MTHFR C677T gene in a healthy European population is high (30–50%), and it is not associated with an increased risk of thromboembolism. The prevalence of MTHFR C677T in Northern Europe is significantly lower than in Southern Europe, so the MTHFR C677T polymorphism in Norway accounts for about 28%, and in Italy about 44%. The prevalence of RH in the healthy European population is 5–15% [29, 30].

The SNPs in FV G16961A, FII G20210A, and MTHFR C677T genes have been broadly investigated worldwide. Although the majority of studies confirmed the importance of these polymorphisms as the most common inherited risk factors for

the development of VTE, there are still some inconsistencies, especially regarding the different ethnic populations [17, 18, 23]. To our knowledge, there is no published data on these polymorphisms in patients with VTE and the general population in Montenegro, although similar investigations of polymorphisms have been conducted on the population of pregnant women with adverse pregnancy outcomes and pregnant women with successful procreation [31, 32].

The aim of this case-control study was to determine the frequency of FV G16961A, FII G20210A, and MTHFR C677T gene polymorphisms in both patients with VTE and healthy subjects in Montenegro and to assess their association with VTE development. This is the first investigation of SNPs in genes for thrombophilia susceptibility (FII, FV, and MTHFR) in Montenegrin patients with VTE, and we believe that it could serve as knowledgeable data for comparison with different populations from the region or broader.

Methods

This case-control study consisted of 160 Caucasian subjects. The study group was composed of 80 patients (35 men and 45 women) after experiencing at least one clinically confirmed episode of VTE (DVT and/or PTE). The evidence of VTE had been documented in their medical records with appropriate diagnostic methods and specialists' expertise.

The main criterion for inclusion of patients in the study group was one or more recurrent episodes of VTE without any of well-known comorbidity (cancer, diabetes mellitus). The control group (CG) consisted of 80 healthy persons (32 men and 48 women) who had not experienced the thromboembolic episode by the time they accepted the participation in the study. For the CG, we randomly selected 80 voluntary blood donors (32 men and 48 women) with no history of the thromboembolic episode, who were as similar as possible to the VTE group regarding age and gender.

The Ethics Committee of the Clinical Center of Montenegro approved this case-control, retrospective study (Number: 03/01-5005/1). All participants provided their informed consent to take part in the research, and the study was performed in accordance with the Declaration of Helsinki. The research was conducted at the Center for Medical Genetics and Immunology, Clinical Center of Montenegro, in the period from January 2015 to July 2017.

Gene analysis

Deoxyribonucleic acid (DNA) was isolated from peripheral blood collected in 4.5 mL tubes with Na-citrate (9 N coagulation sodium citrate 3.2%) and extracted with a commercial test QIA amp DNA Blood Mini Kit (Qiagen, Germany). Extracted DNA was dissolved in 200 μl buffer AE and stored at -20°C. SNPs for FV G1691A, FII G20210A, and MTHFR C677T were detected by allele-specific polymerase chain reaction (PCR). DNA amplification was performed by Attomol Quick type, factor II 20210 G > A, factor V G1691A and MTHFR 677C > T. HotStarTaq DNA polymerase was provided by Qiagen. Heterozygous control template DNA for FII G20210A, FV G1691A, and MTHFR C677T were used as positive PCR controls. PCR was performed in a thermocycler (Mastercycler gradient Eppendorf) using the temperature regime: initial activation (15 min 95°C), 5 cycles (1 min, 94°C; 1 min, 63°C; 1min, 72°C), 30 cycles (30 s, 94°C; 30 s, 63°C, 30 s, 72°C), final elongation synthesis (2 min, 72°C). Amplified DNA samples were analyzed after electrophoresis (2.5% agarose gel, stained with ethidium bromide) and visualized on Ultra Violet (UV) transilluminator.

Statistical analyses

Statistical analyses were performed using the IBM SPSS software program (version 21.0). Descriptive statistics were used for the demographic characteristics. The significance of the differences in the distribution of HET and RH polymorphisms between the VTE group and the control group was investigated by the χ^2 test. Moreover, the odds ratio (OR) with their corresponding 95% confidence interval (CI) was used to represent the association between SNPs and VTE risk. The *p* values less than 0.05 were considered statistically significant.

Results

The baseline demographic and clinical characteristics of participants are presented in Table 1. The VTE group includes 51 (63.75%) patients with DVT, 17 (21.25%) patients with PTE, and 12 (15%) patients with both DVT and PTE. In the VTE group, the youngest patient who had DVT was 10 years old. The majority of patients from the VTE group (69%) had the first episode of DVT and/or PTE before the age of 50.

Table 1

Demographic characteristics of the patients in the venous thromboembolism (VTE) subgroups and the control group

Parameter	Groups				
	DVT	PTE	DVT + PTE	Total	Control
Patients, n (%)	51 (63.75)	17 (21.25)	12 (15)	80 (100)	80 (100)
Sex, n (%)					
male	22 (43.14)	8 (47.06)	5 (41.67)	35 (43.75)	32 (40)
female	29 (56.86)	9 (52.94)	7 (58.33)	45 (56.25)	48 (60)
Age (years)					
mean ± SD	42.88 ± 14.34	46.35 ± 14.22	47.08 ± 18.29	44.25 ± 14.91	44.61 ± 6.87
median (range)	43 (10–73)	51 (27–64)	42.5 (16–75)	43 (10–75)	43 (30–77)
Age (years) of the first episode of VTE, n (%)					
before 50				55 (69)	
after 50				25 (31)	

DVT – deep vein thrombosis; PTE – pulmonary thromboembolisms; SD – standard deviation.

The results of the allele and genotype frequencies for SNPs in FV G1691A, FII G20210A, and MTHFR C677T within the examined groups are presented in Table 2. The results obtained by comparing the VTE group with the CG showed that the frequency of HET for FII G20210A and FV G1691A were higher in the VTE group (χ^2 = 16.26; p = 0.001 and χ^2 = 17.69; p < 0.001). RH for FII G20210A and FV G1691A were not found in any investigated group. Compared to the CG, the incidence of RH alleles A for FII G20210A and FV G1691A was higher in the VTE group. Risk estimate analyses showed that the risk for VTE was significantly higher in the presence of FII G20210A (OR = 10.5; 95% CI = 2.34–47.27; p = 0.001) and FV G1691A (OR = 14.8; 95% CI = 3.34–65.43; p < 0.001). Regarding MTHFR C677T, a difference in the frequency of HET, RH, and wild type (WT) between the VTE group and the CG group was not significant (p = 0.603). Recessive allele T for the MTHFR gene was approximately equally distributed in both examined groups.

Statistically significant difference was not found when comparing the distribution of examined genotypes between genders within each group (results were not presented in the tables).

The distribution of individual and multiple polymorphisms of the investigated genes within the study groups demonstrated that only 18.75% of patients in the VTE group did not have any HET and RH for investigated polymorphisms as opposed to 36.25% in the CG group (Table 3). Furthermore, the presence of two or more investigated polymorphisms were detected in a larger percentage of subjects in the VTE group comparing with the CG (FV G1691A with MTHFR C677T: 16.25% and 1.25%, respectively; FII G20210A with MTHFR C677T: 8.75% and 2.5%, respectively).

Table 2

Allele and genotypes frequencies in patients with VTE and healthy controls

Genotype/allele, n (%)		VTE group	Control group	OR (95%CI)*	χ^2
				p value	
FV G1691A	WT (G/G)	58 (72.5%)	78 (97.5%)	14.8 (3.34–65.43)	17.69
	HET(G/A)	22 (27.5%)	2 (2.5%)	**< 0.001**	**< 0.001**
	RH(A/A)	0	0		
	allele G	138 (86.3%)	158 (98.8)	12.6 (2.90–54.52)	16.26
	allele A	22 (13.8%)	2 (1.2%)	**< 0.001**	**< 0.001**
FII G20210A	WT (G/G)	63 (78.8%)	78 (97.5%)	10.5 (2.34–47.27)	11.70
	HET(G/A)	17 (21.3%)	2 (2.5%)	**0.001**	**0.001**
	RH(A/A)	0	0		
	allele G	143 (89.4%)	158 (98.8)	9.4 (2.13–41.36)	10.96
	allele A	17 (10.6%)	2 (1.2)	**0.001**	**0.001**
MTHFR C677T	WT(C/C)	22 (27.5%)	25 (31.3%)	1.2 (0.60–2.36)	0.27
	HET(C/T)	43 (53.8%)	43 (53.8%)	0.603	0.603
	RH(T/T)	15 (18.8%)	12 (15.0%)		
	allele C	87 (54.4%)	93 (58.1%)	0.86 (0.55–1.34)	0.317
	allele T	73 (45.6%)	67 (41.9%)	0.573	0.573

***Wild – type vs. heterozygous + homozygous; WT – wild type; HET – heterozygous; RH – recessive homozygous; OR – Odds Ratio; 95% CI – 95% Confidence Interval.**

Table 3

Representation of individual and multiple polymorphisms in genes FV G1691A, FII G20210A and MTHFR C677T within the study groups

Polymorphisms	VTE group (n = 80)	Control group (n = 80)
	n (%)	n (%)
FV G1691A	4 (5)	1 (1.25)
FII G20210A	5 (6.25)	0
MTHFR 677*	31 (38.75)	47 (58.75)
FV G1691A FII G20210A	3 (3.75)	0
FV G1691A MTHFR 677 *	13 (16.25)	1 (1.25)
FII G20210A MTHFR 677*	7 (8.75)	2 (2.5)
FV G1691Aw FIIG20210A MTHFR 677*	2 (2.5)	0
None†	15 (18.75)	29 (36.25)

***MTHFR C677T and MTHFR T677T; †There is not one of heterozygous and recessive homozygous; VTE – venous thromboembolism.**

Discussion

The occurrence of the thromboembolic disease is in line with the simultaneous presence of gene polymorphisms and environmental risk factors which can partly explain the inconsistent results of similar studies conducted in different geographic regions [10, 11, 33]. The results of our study showed that the presence of polymorphisms FII G20210A and FV G1691A was significantly higher in the VTE group compared to the CG. RH for these two coagulation factors are absent both in the VTE group and the CG. A recent meta-analysis performed on a large sample, including 11,000 cases and 21,000 controls, has shown a significant association of HET for FV G1691A and FII G20210A with VTE [11], and it is in concordance to findings of our study. Literature data have shown that the combined effect of more than one genetic polymorphism for thrombophilia susceptibility can double or triple the risk for VTE [9, 29, 33]. The combination of the most significant genetic risk factor, FV G1691A and FII G20210A, with HET and RH in the MTHFR gene, has been frequently found in patients with VTE [11, 29]. We also found a higher presence of two or more polymorphisms in the VTE group compared to the CG. In two patients, polymorphisms for all three examined genes were observed simultaneously.

In our study, the frequency of FV G1691A in healthy subjects was 2.5%, which is in correlation with the published data on the white European population [11]. We found that the overall frequencies of FV G1691A polymorphisms were significantly higher in patients with VTE increasing the risk for VT in FV G1691A carriers for almost 15 times (OR = 14.8; 95% CI = 3.34–65.43; $p < 0.001$). This finding is consistent with the reported prevalence of FV G1691A for other countries in the region (Italy 15.3%, Croatia 16%, Macedonia 21.1%, Serbia 29.3%, Bulgaria 25%, Bosnia and Herzegovina 18%, and Greece 31.9%) [11, 22]. Our study showed that allele A was present in 13.8% of subjects with VTE vs. 1.2% in healthy subjects.

Polymorphism FII G20210A leads to an increased prothrombin production, which may result in 30% to 70% higher levels of prothrombin in HET and RH compared to the production in the absence of this condition [25]. In our study, 17 out of 80 patients (21.3%) with VTE were HET for FII G20210A polymorphism. In the CG, FII G20210A polymorphism was presented in 2.5% of participants. Risk for development of VTE in FII G20210A carriers was 10.5 times higher (OR = 10.5; 95% CI = 2.34–47.27; $p = 0.001$) than in wild-type carriers. The results of our research are in correlation with literature data, in which it is reported that HET of FII 20210 is present in 6–18% of patients with VTE and 2–4% in the healthy European population [24]. Our results similarly correlate with the results in Serbia (11.6% of VTE patients) [13]. Other studies from our region report different results in which HET for FII 20210 were presented in patients with VTE with lower frequency (Croatia 4% and Bosnia and Herzegovina 2.7%) [30, 33].

The role of MTHFR polymorphisms in the development of VTE is controversial: some authors have shown an association between MTHFR C677T polymorphism with VTE [32, 33], while others have proven the contrary [11, 34, 35]. Our results showed that HET and RH for MTHFR C677T were present in a large and approximately equal percentage in both examined groups (53.8% and 18.8% vs. 53.8% and 15%, respectively). We also reported previously that the frequency of HET and RH for MTHFR C677T was similar in the population of healthy pregnant women and women with adverse pregnancy outcomes [31, 32]. These results confirm previously published data of similar studies [5, 11, 13, 16]. We did not observe the difference in the frequencies of FV G1691A, FII G20210A, and MTHFR C677T polymorphisms in investigated genes between genders in the patients' group or in the CG. Determining the distribution of these polymorphisms according to gender is especially important in women because they undergo hemostatic changes during pregnancy [32].

It is estimated that 40–50% of thrombosis cases result from thrombophilia. Therefore, the genetic testing of this condition is significant in the prevention and treatment of thrombosis, with an important role in determining the duration of secondary anticoagulant prophylaxis, especially in the case of increased risk of thromboses such as surgery, pregnancy, immobilization, and trauma. Knowledge about the presence of these risk factors can support the prevention of these diseases, especially among patients' relatives who are carriers of these polymorphisms.

Our study showed a significantly higher presence of FV G16961A and FII G20210A polymorphisms in patients with VTE, compared to healthy controls with no history of VTE. In contrast, we did not detect any significant associations between the homozygous MTHFR 677TT and HET MTHFR C677T genotype and VTE. This study included a small sample of patients, and thus, conclusions should be confirmed by future research in the field.

The multifactorial etiology of VTE implies a necessity of further research of more genes related to thrombophilia and their interplay with environmental risk factors, which should lead to a more comprehensive and reliable genetic counseling and prevention of VTE.

Conclusion

Our study showed that FII G20210A and FV G1691A polymorphisms are significantly associated with VTE. Detection of the above-mentioned polymorphisms prior to VTE development can contribute to the prevention of further VTE occurrence, especially among patients' relatives who are carriers of these polymorphisms.

Conflict of interest

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

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Orthotopic ileal neobladder creation according to the original surgical method "Belgrade pouch" – A ten-years experience

Ortotopska ilealna neobešika kreirana po originalnoj hirurškoj metodi "*Belgrade pouch*"– desetogodišnje iskustvo

[1]Vladimir Bančević*[†], [1]Predrag Aleksić*[†], Dušica Stamenković[†‡]

Military Medical Academy, *Urology Clinic, [‡]Clinic for Anesthesiology and Intensive Care, Belgrade, Serbia; [†]University of Defence, Faculty of Medicine of the Military Medical Academy, Belgrade, Serbia

[1]Vladimir Bančević and Predrag Aleksić have equal authorship.

Abstract

Background/Aim. The principle of continent urinary derivations is based on the formation of a reservoir as similar as native bladder is, with satisfactory capacity, high level of continence, low pressure and small resorbent surface as possible to prevent metabolic disorders. **Methods.** This prospective and partially retreospective study included 148 patients with organ confined muscle invasive bladder cancer, operated on in a ten-year period, and followed up in a 3-year period. After a radical cystectomy, an original surgical technique of creating "U" shaped orthotopic neobladder using a short ileal segment, average length of 30 cm, called "Belgrade pouch", was performed. **Results.** The results of 126 male and 22 female patients, three years following the surgery, showed average pouch capacity of 477 (330–659) mL, insignificant residual urine of 52 (0–92) mL, and the averageurinary frequency was five times within 24 h. Day time continence was present in 90% of patients and nighttime continence in 88% of patients, 7% of patients had mild acidosis, and 3% of patients had vitamin B12 deficiency. **Conclusion.** The neobladder created according to the "Belgrade pouch" technique showed excellent results comparabile with standard techniques, with adequate capacity, high continence rate, favourable 24 h voiding frequency, insignificant residual urine, and a significantly lower rate of metabolic complications during the 3 years of the follow-up period.

Key words:
colonic pouches; postoperative complications; serbia; treatment outcome; urinary bladder neoplasms; urological surgical procedures.

Apstrakt

Uvod/Cilj. Princip kontinentnih urinarnih derivacija je zasnovan na kreiranju arteficijalnog rezervoara za urin, što sličnijeg mokraćnoj bešici, adekvatnog kapaciteta, visokog stepena kontinencije, niskog pritiska i što manje resorptivne površine, u cilju sprečavanja pojave metaboličkih komplikacija. **Metode.** Prospektivnom i delom retrospektivnom studijom obuhvaćeno je 148 bolesnika koji su u periodu od 10 godina bili operisani zbog tumora mokraćne bešike ograničenog na organ sa invazijom mišićnog sloja (pT2), i kojima je nakon radikalne cistektomije originalnom hirurškom tehnikom *Belgrade pouch* kreirana neobešika od kratkog segmenta ileuma u obliku slova „U", prosečne dužine 30 cm. Period praćenja posle operacije iznosio je tri godine. **Rezultati.** Kod 126 muškaraca i 22 žene operisanih tehnikom kreiranja neobešike *Belgrade pouch*, posle tri godine prosečna zapremina neobešike bila je 477 (330–659) mL, zapremina rezidualng urina je bila klinički beznačajna [52 (0–92) mL], a prosečna frekvencija 24-časovnog mokrenja iznosila je pet puta na dan. Dnevnu kontinenciju je imalo 90% operisanih, a noćnu 88%. Kod 7% bolesnika utvrđena je blaga acidoza, a kod 3% nedostatak vitamina B12. **Zaključak.** Neobešika kreirana po metodi *Belgrade pouch* pokazala je odlične rezultate, uporedive sa standardnim tehnikama obezbeđujući odličan kapacitet, visok stepen kontinencije, dobru 24-časovnu frekvenciju mokrenja, beznačajan rezidualni urin i značajno nižu stopu metaboličkih komplikacija u trogodišnjem periodu praćenja.

Ključne reči:
creva, rezervoari; postoperativne komplikacije; srbija; lečenje, ishod; mokraćna bešika, neoplazme; hirurgija, urološka, procedure.

Correspondence to: Vladimir Bančević, Military Medical Academy, Urology Clinic, Crnotravska 17, 11 000 Belgrade, Serbia.
E-mail: vladaban2004@yahoo.com

Introduction

Radical cystectomy (RC) represents the gold standard for managing of the muscle invasive organ-confined urinary bladder cancer [1]. The principle of continent urinary derivations is based on forming a reservoir of a satisfactory capacity, low pressure with resorbent surface as small as possible, which can ensure a high level of continence. Numerous techniques of orthotopic urinary derivations were described aimed at creating a urinary reservoir resembling a urinary bladder as much as possible. Despite that, after creating an orthotopic neobladder, numerous subsequent complications can arise, such as incontinence, urinary retention, acidosis and other metabolic disorders, infections, calculosis, vitamin B12 deficiency, stenosis in the site of ureter and neobladder anastomosis, hydronephrosis, neobladder-ureteric reflux, etc. Standard techniques most often recommend the use of the terminal ileum segment 40–65 cm long for creating a neobladder that gives satisfactory results. The length of the intestinal segment from which the neobladder is created directly affects its capacity and over time also the residual urine volume. From the time it is created to the first year of its use, the neobladder increases its volume 3.5–4 times on average, and over time this growth can be up to 8 times [2].

Methods

This prospective and partially retrospective study included 148 out of 210 patients operated on in a ten-year period (2008–2017) at the Urology Clinic, Military Medical Academy (MMA) in Belgrade. Indication for radical cystectomy was organ-confined, muscle-invasive bladder transitional cell tumor. Indication for orthotopic urinary derivation was in conformity with the European Association of Urology (EAU). The Ethics Committee of the MMA approved this study, within scientific project "Modification of surgical techniques for orthotopic ileal neobladdder creation following cystectomy – VMA/08-10/B.1." All patients gave their signed consent for approval for this surgical procedure. Patients with systemic disorders, diabetes mellitus, obstructive pulmonary disease, chemotherapy and other conditions with potential influence on the results were excluded from the study. All patients had serum creatinine levels less than 140 μmol/L. Patients were given questionnaires about continence and voiding frequency six month after the surgery, and annually in the three-year follow-up period. Pouch capacity and postvoid residual urine were measured by ultrasound.

Surgical technique "Belgrade pouch"

After cystoprostatectomy in males or cystectomy, radical hysterectomy and removal of anterior wall of vagina in females, pelvic lymphadenectomy was performed. In the absence of malignancy on the resection margins, resection of the terminal ileum with a length around 30 cm and a distance no less than 20 cm from the ileocecal valve was performed (Figure 1). Before that, mobility and tension-free postitioning

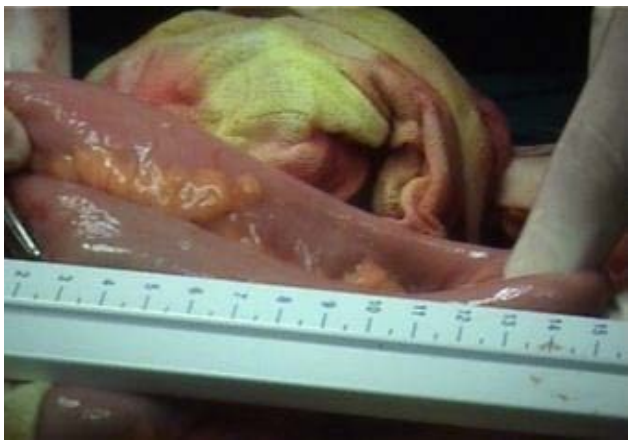

Fig. 1 – Measuring the selected segment prior to resection (in this case the pouch was made of the ileal segment about 30 cm long).

of the ileal segment on the bottom of the *pelvis minoris* was done. Transluminescence of the mesointestine may obtain maximal preservation of the intestinal segment vascularization (Figure 2). After detubularization, a "U"-shaped ileal plate was formed by using a continuous suture (Figure 3). Neobladder was created from the part of the terminal ileum 29 cm in

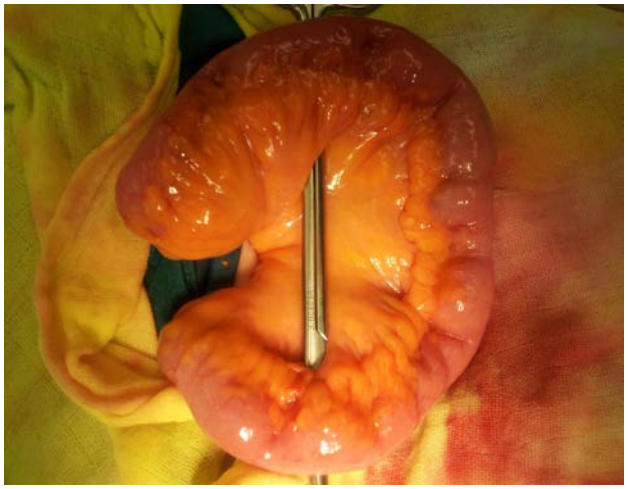

Fig. 2 – Minimal incision length on the meso allows also maximum preservation of the ileal segment vascularization.

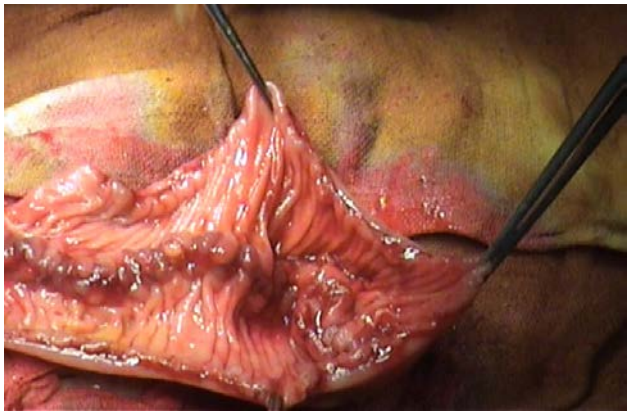

Fig. 3 – Creating an ileal plate from a 30 cm long "U" shaped intestine segment.

length (24–35 cm) on average (Figure 4). The ureters were spatulated in the length of 5 mm and implanted in the neobladder through special openings on its posterior-lateral side, bilaterally, by the direct anastomosing technique with taking out ureteral silicone tubes from the ureter through the neobladder wall to the abdominal wall skin. The neobladder roof was sutured in a letter-like manner. On the lower end of the newly formed ileal bladder, an opening of a 5–7 mm in diameter was formed, around which the perimeter suture was placed. The urethra and the neobladder anastomosis was made with 5–7 individual Vicryl 3–0 sutures (Figure 5), and the neobladder was placed in a definitive position (Figure 6).

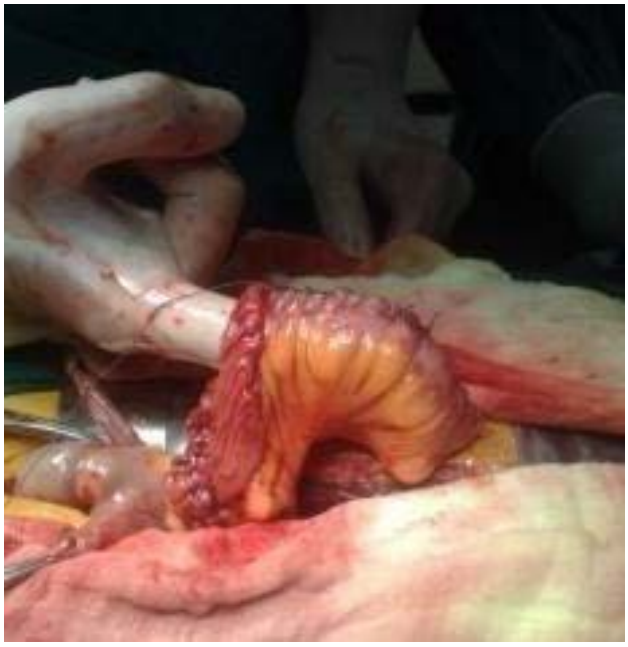

Fig. 4 – The size of the created neobladder according to the "Belgrade pouch" technique.

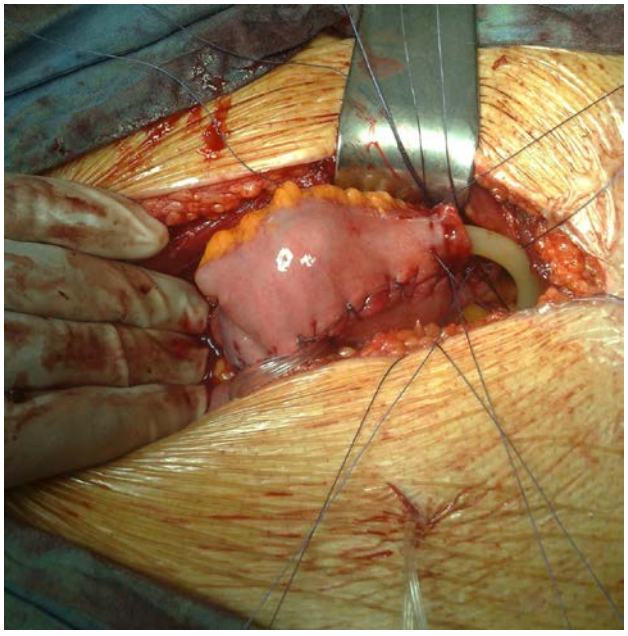

Fig. 5 – Placing of 5-7 stitches for ureteral-neobladder anastomosis.

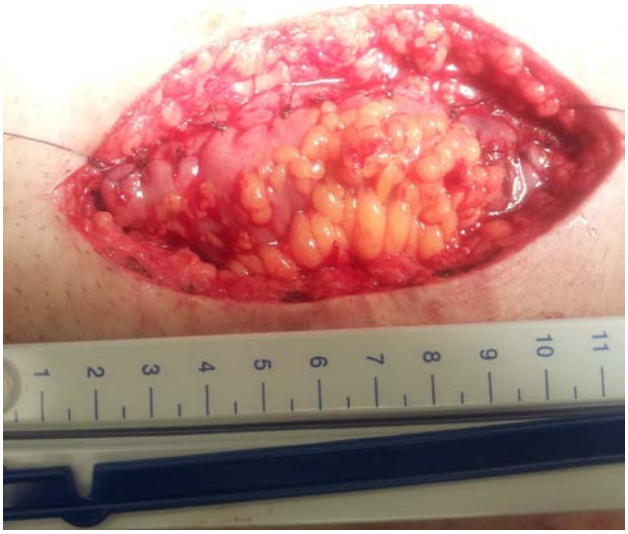

Fig. 6 – Definite position of the neobladder. Infraumbilical incision about 10 cm long, through which operation was performed.

Foley Ch18 urinary catheter was inserted and 0.5 kg traction was applied to the catheter on the first day, 0.25 kg traction on the second day and then the traction was removed from the catheter on the third day. After the removal of the urinary catheter and for quicker mastering the new method of voiding and faster achieving of continence, all patients were advised to urinate every 2 or 3 hours during the first three months even if they do not feel the need to void. It was recommended that the patients wake up twice at night to an alarm clock in order to void.

Results

The average age of the operated patients was 60 (42–73) years, 16% of them were older than 70 years. Eighty five percentage of the operated patients were males. The average operation time was 205 (150–340) min. Intraoperatively, 49% of the patients received blood transfusion with an average volume of 440 (300–940) mL of blood. Postoperatively, 8% of the patients were subjected to he blood transfusion. In 7% of the operated patients, subocclusion or paralytic ileus occurred in the immediate postopertive course where the patients were treated conservatively with the extended use of nasogastric tube and peristaltic activity stimulation with prokinetics and the correction of the electrolyte disbalance (most often potassium and calcium deficiency). The wound dehiscence occurred in one patient and it was treated by secondary suture. Extended lymphorrhea occurred in 5% of the operated patients. Urinary fistula occurred in two patients. These patients were managed conservatively, fistulas were healed spontaneously by a prolonged catheter wearing until the 27th and 29th postoperative day. Febrility that lasted for more than two days was recorded in 5% of the operated patients. In patients who had fever over 38°C, a hemoculture test was performed, but the results of all the samples were negative. Other parameters and complications are shown in Table 1.

Table 1

Characteristics of operated on patients and some postoperative complications during the three-year period after operation

Parameter	Postoperative period			
	6 months	1 year	2 years	3 years
Daytime continence (%)	72	88	91	90
Nighttime continence (%)	67	84	87	88
Neobladder capacity (mL), mean (min–max)	307 (220–378)	425 (285–514)	458 (305–582)	477 (330–659)
Residual urine volume (mL), mean (min–max)	13 (0–18)	20 (0–39)	29 (0–40)	52 (0–92)
24-h urinary frequency (n)	9	7	6	5
Acidosis (%)	0	3	4	7
Vitamin B12 deficiency (%)	0	0	0	3
Neobladder calculosis (%)	0	0	3	7
Hydronephrosis (%)				
bilateral	10 (gr. I)	3 (gr. I)	3 (gr. I)	3 (1, gr. I; 2, gr.II)
unilateral	0	1 (gr. II)	3 (1, gr. II; 2, gr. III)	6 (1, gr I; 2, gr. II; 3, gr. III–IV, twice-re-pouch-anastomosis)

gr.– gradus

Discussion

A more seldom occurrence of urinary bladder cancer in female patients and the later introduction of orthotopic derivations in women in clinical practice are the reasons for male predominance in our study. A patient's mature years may not represent a contraindication for performing this procedure, however, the emphasis is put on the so-called "biological age of a patient", comorbidity, life expectancy, etc. In patients over 75 years of age, a greater complication rate can be expected, particularly incontinence, perioperative complications, pyelonephritis and a fatal outcome in the early postoperative course. We reported 16% of patients older than 70 years in our study, and this data may influence our results.

Hautmann et al. [3] believe that by using 40–44 cm long intestinal segment for creating a neobladder, an optimum capacity of 450–500 mL can be achieved. Moreover, by using a 60 cm long segment, a pouch of double capacity can be obtained which in the early postoperative course may result in a larger continence percentage. However, a neobladder created in such a way becomes unnecessarily large over time, resulting in frequent urinary infections and a larger percentage resulting in the need for catheterization [4]. In their study, Bachor et al. [5] presented results of an average neobladder capacity in patients for whom pouches were created by a classical surgical technique shaped as a letter "W" or "M", with the capacity of 770 mL (330–2,000 mL) three months after the operation [5]. Here, the unnecessarily huge capacity of the neobladder created by Hautmann's classic technique was already emphasized in the first months following the operation, with a reminder that the pouch would continue to increase its capacity over time. Constantinides et al. [6] presented the results achieved with patients for whom a neobladder of approximately 36 cm was created, and they recorded an average pouch capacity of 672 mL one year after the operation. At the same time, according to the literature availabvle to us, there are studies where the results of creating a neobladder from the shortest intestinal segment had been presented until our technique was published. In our study, we went a step further by creating a pouch of a 30 (24–35) cm long terminal ileum on average [7].

The capacity of a neobladder and the urination biomechanics is not only influenced by the length of the intestinal segment used for creating a pouch, but by numerous other factors as well: the width of the intestinal segment from which the neobladder was created, the shape and position of the neobladder, a patient's level of training in a new voiding method, the abdominal press strength, the level of potential stenosis in the site of anastomosis of the neobladder and the urethra, body mass index etc. In our study, in the first six months the capacity of the neobladder created by using the "Belgrade pouch" technique was smaller than the average capacity in reference studies, so the percentage of continent patients in this period was significantly lower. However, the neobladder created by

using the “Belgrade pouch” technique achieved a satisfactory capacity over time, which also enabled the growth of the continence percentage in patients after the first six months, and on the other hand in a three-year follow-up period the average capacity of a neobladder created in such a way did not reach unnecessarily big capacity described in other studies and, according to this parameter, it represented a more suitable replacement for the native urinary bladder.

Stenzl et al. [8] in their studies referred to a residual urine volume larger than 100 mL as clinically important, and found that 22% of the patients over the first year of follow-up had residual urine larger than this limit value. Our study recorded a clinically insignificant increase in the residual urine volume which, on average, over a three-year follow-up period was significantly below the value of 100 mL. The latest studies show that around 12% of the patients in the first year following the operation cannot urinate spontaneously, therefore, they are inserted a urinary catheter. This percentage increases over time [9]. Urinary retention can occur quite early, in the first days of the postoperative course due to the catheter clogging by the mucus plug. The production of a larger quantity of mucus is directly proportional with the neobladder surface area. In order to prevent the clogging of the urinary catheter by the mucus plug, it is recommended that the urinary catheter is flushed out by a physiological saline solution in the early postoperative period, 2–3 times a day. Urinary retention in the pouch in the early postoperative period may result in urinary leakage between the pouch sutures, infection, pouch dehiscence and even acidosis [10]. In the later postoperative course, a larger mucus amount may indicate infection. Early and late urinary retention due to the catheter, i.e. urethra clogging by mucus plug, in reference studies occurs in 3% of the cases [10]. Unresolved urinary retention issue may result in a rare, however, life-threatening complication – pouch rupture which more often occurs in patients who receive chemotherapy, particularly cisplatin [11]. In our series of patients, there were no patients who had a catheter inserted due to increased residual urine volume and the values of residual urine in the follow-up period were better than those in the reference studies [12].

Hydronephrosis in the site of stenosis of the ureter and the neobladder anastomosis, pouch, kidney or ureteral calculi, as well as the reflux of infected urine towards the pyelon may be additional predisposing factors for the occurrence of a complicated urinary infection. In our series, there were no patients with urosepsis, the residual urine volume was small and clinically insignificant with a low percentage of neobladder calculosis which resulted in the absence of complicated urinary infections in the study group. Based on the above-stated, it can be concluded that by creating a neobladder from a shorter segment, predisposing factors for the urinary infection development can be significantly reduced.

Hydronephrosis may occur due to stenosis at the level of the ureter and the neobladder anastomosis, pouch-ureteral reflux, kidney or ureteral calculosis, recurrent urinary infections, urinary retention or novel tumor appearance etc. [11]. In the beginning, many authors favoured the antireflux techniques because it was believed that they would prevent urinary reflux towards the upper parts of the urinary tract as they represent a true imitation of a natural antireflux mechanism. Other than a major anatomic similarity with a natural antireflux mechanism, the advocates of such type of anastomosis believe that in this way they will prevent the migration of intestinal bacteria from the pouch to the ureter, i.e. the kidney and thus the occurrence of pyelonephritis [13]. Hautmann et al. [3] recommended using direct anastomosis of the ureter and the ileal pouch as this is a low pressure reservoir with a small probability of urinary reflux towards the upper parts of the urinary tract. They elaborated the results of several studies that showed the occurrence of a significant number of stenoses in the site of the ureter and the pouch anastomosis when using some of the antireflux techniques, by which kidney function is permanently weakened over time. The same authors reported that direct anastomosis of the ureter and the neobladder might pass refluxed urine towards the kidney only in case of a very large capacity and pressure within the pouch, but even then this reflux is of lower intensity. Varol and Studer [10] argue that antireflux techniques are not necessary for preventing the reflux of infected urine towards the upper parts of the urinary tract because a low pressure pouch is antirefluxive by its nature which is in agreement with the study by Thurairaja et al. [4]. Stenoses in the site of the ureter and the neobladder anastomosis most often occur in the first year after the operation and often result in asymptomatic ureterohydronephrosis due to its slow occurrence and course [14]. Stenosis in the site of anastomosis is around 3.3% in direct methods of ureteral implantation [15]. Our study showed a larger number of hydronephroses in the first six months due to reflux. However, after urinating, hydronephrosis completely spontaneously regressed in all patients except in two who were subjects to repouch-anastomosis. In time, as the neobladder capacity grew, the percentage of hydronephroses declined, so the results obtained in our series in the twenty-fourth month in this study group were in conformity with the reference studies. Thus, we showed that the application of a direct ureter and pouch anastomosis is justified even with a pouch made of the shorter ileal segment with the expected larger percentage of reversible moderate ureterohydronephrosis caused by the urinary reflux, however, without permanent morphological and functional consequences to the kidney.

Several factors affect the continence level: patient's age, mental status, intact ureteral sphincter and its preserved innervation, functional length of the urethra, pressure within the pouch, pouch capacity, absence of urinary infection, training to void in a different manner, etc. [11]. The patients first maintain daytime continence which in referent studies reaches 80%–90% one year after the operation [16]. Older patients initially have weakened sphincter tonus which causes major incontinence postoperatively, thus the percentage of patients over 75 who have preserved daytime continence ranges between 56% and 75% whereas nighttime continence is about 25% [3]. Around 30% of patients at this

age have catheters due to urinary retention after a neobladder created. In our series, we had 16% of patients over 70 years, which certainly influenced the percentage of daytime and nighttime continence [17]. Some studies showed that the neobladder shape might affect continence. Therefore, it was concluded that a sphere-shaped neobladder allows larger continence than a neobladder of a different shape, but only in the first 3–6 months, whereas this percentage is identical one year after the operation, irrespective of the pouch shape [18]. In our series, the pouch of ellipsoidal shape was created. "Nerve sparing" procedures certainly affect the continence and improve it [19]. According to referent studies, retention more often occurs in women – even in 25%–40% of operated women due to increased mobility of the urethra, whereas the percentage of daytime continence is around 75%–90% [20]. A smaller percentage of nighttime continence is recorded in women, and according to the results published so far, it ranged between 55%–66% [21]. In our series, one female patient had a urinary catheter placed due to incontinence, and there were no female patients that had a catheter placed due to significant residual urine, i.e. retention. This information largely deviates from the quoted results of major series in which a significantly larger percentage of urinary retention and catheterisation has been mentioned. In our series, the patients had a lower continence rate in the first months, which was expected due to a significantly lower capacity. Between the sixth and the twelfth month after the operation, the capacity of the "Belgrade neobladder" exceeded 350 mL and it still recorded a mild growth which correlated with the constant increase in the daytime continence percentage in this group, even without a major increase of pressure in the neobladder [21]. Since the neobladder capacity shall continue to increase over time, we can understand at this point the advantage of creating a neobladder from a shorter ileal segment, which may result in "optimum capacity" and excellent daytime continence 9–12 months after the operation which may be preserved over time. Achieving nighttime continence is much slower and its proper evaluation is made 12–24 months after the operation. Some studies report that the nighttime incontinence percentage ranges between 20% and 40%. However, some authors presented the results with a high level of nighttime continence of about 90% [3, 11, 13]. In order to increase the nighttime continence level, the patients are advised to void right before going to bed, to avoid sleeping pills and alcohol, and to wake up to an alarm clock 1–2 times during the night and to stop taking large amounts of liquid two hours before going to bed, which we also advised the patients in our series [22]. Although this study did not include the comparison of nycturia prior to operations in all patients, we shall present the information we obtained from a part of the patients within a wider study. The study showed that 47% of the patients experienced nycturia 1–2 times a night even before the operation, so the advice to wake up to an alarm clock every 3–4 hours during the night in order to void in the first months after the operation did not represent any special inconvenience for them. In our series, the nighttime continence percentage was increasing over time. The studies showed that using a 25 mg dose of imipramine hydrochloride right before going to bed might improve the nighttime continence level in 25% of operated patients, although we had no experience in this kind of the treatment [23].

It is a well-known fact that in time, with the neobladder capacity growth and in the absence of any infection, the urinary frequency decreases. Madesbacher et al. [24] have found that the urinary frequency comes down to 6–7 times in 24 hours, twelve months after the operation. After the catheter removal, patients are advised to void every two hours during the day, and every 3 hours during the night, by waking up to an alarm clock in the first three months. Identical recommendations were given by the most experienced urologists in the field of cystectomy [4, 11], which certainly had an effect on a higher urinary frequency in the first several months of the follow-up. In our series, the urinary frequency considerably declined over time, following the neobladder volume growth. This showed that creating a neobladder from a shorter intestinal segment shall not considerably increase the urinating frequency in comparison to patients whose neobladder was created of a standard-length intestinal segment.

The duration of the urine contact with the intestinal mucus, the resorbent surface area, i.e. neobladder volume, residual urine volume, urinary frequency and urinary infection are the predisposing factors for the occurrence of acidosis [4, 25]. In the study that discussed metabolic disorders in patients for whom neobladder was created, the percentage of patients with acidosis was 25%–50% [23, 26]. Metabolic complications occur more often in patients with compromised kidney function. A significant decrease in metabolic complications and acidosis percentage was recorded in the absence of urinary infection and in patients for whom intestinal segment shorter than 45 cm was used for creating the neobladder [3]. This is the point where we came up with the idea of attempting to additionally shorten the intestinal segment used for creating a neobladder thus decreasing the percentage of metabolic complications that are classified as the most frequent subsequent complications, and, at the same time, we attempted to preserve satisfactory capacity and high continence level in patients. The percentage of patients with acidosis in our series was significantly lower than in reference studies and, at the end of the third year, it was only 7%, at which none of the patients used alkaline agents for regulating pH value of arterial blood. Such good results were achieved thanks to the reduced neobladder capacity, i.e. smaller resorbent surface, smaller residual urine volume and seldom occurrence of urinary infections, as proven factors that affect the occurrence of metabolic disorders, primarily acidosis [25–27]. This should be particularly considered in patients for whom antireflux ureter and neobladder anastomosis was created because ureterhydronephrosis may occur, and it can result in permanent failure of kidney function if it is not treated properly results which, by the reduction of kidney buffer system, significantly affects the occurrence of acidosis and its serious consequences. Long-term metabolic acidosis causes mobilization of calcium from the bones, which may

be the cause of reduction of bone marrow density, with consequential hypocalcemia [28]. Reacting to hypocalcaemia, parathyroid gland secretes parathyroid hormone (PTH) in higher concentration attempting to normalize the values of serum calcium. In a few published study results available to us, a direct connection is made between acidosis, bone calcium mobilization and consequential hypocalcemia and the increase of PTH value in blood. These changes become more expressed over time in patients with neobladder created by standard techniques from 45–60 cm of small intestine, and they are directly initially connected with the neobladder capacity, i.e. resorbent surface [29]. In our patients, a slow and mild growth in PTH values in the blood was recorded over time, within reference values [30]. Vitamin B12 absorption is conducted in the terminal ileum, so this process is inversely proportional to the length of the intestinal segment of the ileum from which the neobladder shall be created, and it is in positive correlation with the length of the remaining native part of the ileum [31]. In the studies in which the patients with neobladder were followed for over several years, some authors mentioned the occurrence of vitamin B12 deficiency which, should it last for over an extended period of time, might cause irreversible hematological and neurological sequelae. Some authors reported that around one third of patients after creating a neobladder might develop vitamin B12 deficiency, and this percentage can be even higher particularly in patients for whom the ileum segment longer than 60 cm was used for the creation of a neobladder. The Anticipated time of vitamin B12 deficiency occurrence is three years after the operation [31, 32]. Nieuwenhuijzen et al. [33] stated that around 15% of patients with vitamin B12 deficiency had been operated on in the three-year follow-up period. In the third year of our study we recorded 3% of patients with vitamin B12 deficiency without neurological disorders, which is a significantly better result in comparison to standard techniques of creating a neobladder.

Conclusion

Neobladder created according to the "Belgrade pouch" technique may obtain a high percentage of continence, without increasing the urinary frequency, with neobladder capacity similar to natural urinary bladder and minimal volume of residual urine. This method significantly reduces the percentage of delayed metabolic complications such as acidosis, vitamin B12 deficiency and the increase in PTH levels with the percentage of ureterhydronephrosis occurrence comperable with reference studies. This technique can be applied to both sexes, although certain advantages in terms of reducing the occurrence of retention (in a small number of operated on patients in our studies) have been recorded in women. Depending on the comorbidity and biological age of patients, this technique can also be applied at a later age. It is suitable for the laparoscopic approach, or robot surgery, where we expect shorter operative time due to the shorter sutured length of intestinal segment used for pouch creation.

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Esophageal two-phase supine/standing scintigraphy and balloon dilatation in achalasia: 20 years of experience

Dvofazna scintigrafija jednjaka u ležećem/stojećem položaju i balon dilatacija kod ahalazije: 20 godina iskustva

Zoran Janković*†, Dragan Pucar*†, Radoje Doder†‡, Nenad Perišić‡

Military Medical Academy, *Institute for Nuclear Medicine, ‡Clinic for Gastroenterology and Hepatology, Belgrade, Serbia; †University of Defence, Faculty of Medicine of the Military Medical Academy, Belgrade, Serbia

Abstract

Background/Aim. The most frequent therapeutic method for achalasia is balloon dilatation (BD). Esophageal scintigraphy is potentially the most convenient method for the evaluation of achalasia by quantifying esophageal transit. The aim of this study was to present the results of esophageal scintigraphy (ES) in achalasia treated by BD for a long period of time, from 1997 to 2016, and to set parameters suitable for predicting treatment outcomes. **Methods.** Two-phase ES in anterior projection was performed. The first phase involved swallowing 10 mL of water with 18.5 MBq ^{99m}Tc in a supine position. The second phase was performed in a standing position after swallowing 100 mL of water. The retention index (RI) of esophageal radioactivity was calculated after the imaging using registered esophageal and gastric counts. ES was performed in 52 patients (34 males and 18 females, aged 22–75 years, the median age 44 years) before and after BD. In 31 patients, only two scintigraphies were performed. In the remaining 21 patients, follow-up and repeated scintigraphies were continued including the patients with therapeutic failure and repeated BD. BD was repeated in 8 patients, and those were followed up and evaluated over time. Modified BD was performed with Rigiflex balloon dilators. **Results.** A typical scintigraphic finding in the supine position was passive esophageal filling without the evidence of motility, and in most patients, 43 of them (83%), radionuclide elimination in the stomach was absent. Evaluating values before and long after the first BD, it was shown that certain values of RI indicated remission or relapse of the disease. The RI difference higher than 50% after BD was a reliable indicator of a longer remission. The age and gender of patients had no impact on the outcome of dilatation, but younger patients were with a higher risk of early failure (6 patients during the first year after BD). ES showed that the first BD was initially successful in 50 patients and after the follow-up in 41 (79%) patients. **Conclusion**. A two-phase supine/standing ES is most suitable for diagnosing and the follow-up of achalasia. RI represents an important parameter for the evaluation and prediction of therapeutic response. The RI difference of more than 50% after BD is a reliable predictor of a longer remission. Age and gender have no impact on dilatation outcome, but patients younger than 40 years have a higher risk of early therapeutic failure.

Key words:
balloon dilatation; esophagus; achalasia; prognosis; radionuclide imaging; treatment outcome

Apstrakt

Uvod/Cilj. Najčešća terapijska metoda za ahalaziju je balon dilatacija (BD). Scintigrafija jednjaka je potencijalno najpogodnija metoda za procenu ahalazije kvantifikovanjem ezofagealnog tranzita. Cilj rada je bio da se prikažu rezultati primene scintigrafije jednjaka (SJ) kod bolesnika sa ahalazijom lečenih BD u periodu od 1997. do 2016. godine i pronalaženje parametara pogodnih za predviđanje rezultata lečenja. **Metode**. Dvofazna SJ izvođena je u anteriornoj projekciji. Prva faza je rađena u ležećem položaju gutanjem 10 mL vode sa 18,5 MBq ^{99m}Tc. Druga faza je izvođena u stojećem položaju nakon gutanja 100 mL vode. Posle scintigrafije, izračunavan je indeks retencije (RI) korišćenjem registrovanih impulsa jednjaka i želuca. Kod 52 bolesnika – 34 muškaraca i 18 žena, životne dobi između 22 i 75 godina života, medijana 44 godine, urađena je SJ pre i posle BD. Kod 31 bolesnika urađene su samo po dve scintigrafije. Kod ostalih 21 bolesnika nastavljeni su praćenje i kontrolne scintigrafije, uključujući i bolesnike s neuspehom i ponovljenim BD. Kod 8 bolesnika, koji su dalje praćeni, ponovljena je BD. Modifikovana BD rađena je primenom Rigiflex balon dilatatora. **Rezultati**. Tipičan scintigrafski nalaz u ležećem položaju bio je pasivno ispunjavan

Correspondence to: Zoran Janković, Military Medical Academy, Institute for Nuclear Medicine, Crnotravska 17, 11 000 Belgrade, Serbia. E-mail: zorjan954@gmail.com

je jednjaka bez uočljivog motiliteta i kod većine bolesnika, njih 43 (83%), bila je odsutna eliminacija radionuklida u želudac. Procenjujući vrednosti RI pre prve BD i duže vreme posle nje, pokazano je da određene vrednosti RI ukazuju na remisiju ili relaps bolesti. Razlika vrednosti RI posle BD veća od 50% pokazala se kao pouzdan prediktor dugotrajne remisije. Starost i pol bolesnika nisu uticali na rezultat dilatacije, ali su bolesnici mlađi od 40 godina imali veći rizik od ranog neuspeha (6 bolesnika u toku prve godine posle BD). Nalaz SJ ukazao je na to da je prva BD bila inicijalno uspešna kod 50 bolesnika, a posle perioda praćenja kod 41 (79%) bolesnika. **Zaključak**. Dvofazna scintigrafija jednjaka u ležećem/stojećem položaju je najpogodnija metoda za dijagnostikovanje i praćenje ahalazije. Za procenu i predviđanje terapeutskog odgovora RI je važan pokazatelj. Razlika RI veća od 50% posle BD je pouzdani prediktor duže remisije. Starost i pol bolesnika ne utiču na rezultat dilatacije jednjaka, ali bolesnici mlađi od 40 godina imaju veći rizik od ranog neuspeha lečenja.

Ključne reči:
dilatacija balonom; jednjak; jednjak, ahalazija; prognoza; scintigrafija; lečenje, ishod.

Introduction

Achalasia is a primary esophageal motor disorder characterized by the loss of peristalsis and the failure of relaxation of the lower esophageal sphincter (LES). It is caused by the loss of myenteric neurons, probably as a consequence of a nonreversible autoimmune process [1, 2]. The aim of the treatment is primarily to relieve dysphagia. The most frequent therapeutic method is pneumatic balloon dilatation (BD) which alleviates transit through the LES and leads to a long remission in most patients [3–8]. Some patients are subjected to failed dilatation or a shorter or longer period ending with disease relapse. Clinical success can be evaluated by several methods: follow-up with patient's symptoms (symptom scores), radiography of esophagus, esophageal manometry, and esophageal scintigraphy (ES). ES is potentially the most convenient method because it is safe, simple, quantitative, and with low radiation dose [5, 9–13]. Due to a non-standardized protocol of investigation, ES is underestimated and neglected in diagnostic protocols [14, 15]. Numerous studies have tried to find predictive factors for the clinical success of BD [5, 12, 16–21]. The purpose of this paper was to present the ES results in achalasia treated by BD and to set parameters suitable for predicting the prognosis of the disease and, consequently, the treatment outcomes.

Methods

The data of patients were retrospectively evaluated. The diagnosis of achalasia was confirmed after a clinical evaluation, endoscopy, radiography, and manometry. All patients were evaluated with ES before and after BD. Patients with a previous unassessed dilatation, botulinum toxin, and surgical therapy were excluded. The patients were examined and followed up for a long period of time, from 1997 to 2016.

Modified BD was performed with Rigiflex balloon dilator (Boston Scientific USA), without sedation, up to the pain threshold of patients [4]. In 44 patients, one BD was performed, and in 8 patients, two BD were done due to the relapse of disease (in 7 patients) and dilatation failure (in one patient).

ES was performed in 52 patients (34 males and 18 females, aged 22–75 years, the median age was 44 years) before and after BD. In 31 patients, only two scintigraphies, before and after BD, were performed. In the remaining 21 patients, follow-up and repeated scintigraphies were continued, including the patients with therapeutic failure and repeated BD (follow-up period of 6 months to 17 years, median 26.5 months). BD was repeated in 8 patients, and these were followed up and evaluated over time.

Two-phase scintigraphy was performed. The first phase was in the supine position. A patient swallowed 10 mL of water with 18.5 MBq of ^{99m}Tc-pertechnetate. Imaging was performed with gamma camera Siemens Orbiter in anterior projection with the camera head over the patient (1 image every 4 sec; 16 images were made). After the first phase, the patient stood up, and after moving the head of the camera, it was positioned in front of the standing patient. A glass with 100 mL of water was added to the patient, and he/she swallowed the water with simultaneous imaging: 16 images every 4 sec, and additional 4 images every 4 sec (these images were made after positioning the patient's stomach in the center of the camera field). The recorded images were analyzed visually, and the time-activity curves were constructed. After the imaging, the retention index (RI) of esophageal radioactivity was calculated using the formula RI (%) = E/(E + V) × 100% (E – esophageal activity; V – stomach activity and bowel activity if present). Background activity correction was applied when the ratio E/V or V/E was more or equal to 3 : 1. The presence of gastric activity in supine and standing position before drinking a glass of water was recorded.

BD was successful if the RI value after BD decreased more than 50% from its value before BD. Relapse occurred if the RI value during the follow-up increased more than 50% of its basic value before dilatation or the RI value was higher than 30% of its basic value and with a constant increase on at least two consecutive control scintigrams.

The statistical analysis was done in all patients and in the group of the followed patients. The analysis in the followed patients included one patient with initially failed BD (a total of 21 patients). In the descriptive statistics, medians, frequencies, and ranges were used. Median was used as the only statistical measure of central tendency. The χ^2 test and Fisher's exact test were used to compare categorical variables presented as frequencies. The Mann-Whitney *U* test and Wilcoxon signed ranks test analyzed numeric variables presented as medians. The predictive value of the

RI value after BD was assessed with logistic regression analysis. The result was statistically significant when the *p*-value was less than 0.05. The used statistical software was StatsDirect version 2.7.

Results

In all patients, ES was performed before BD. Scintigraphic findings were typical. In the supine position, the esophagus was passively filling without evidence of motility (Figure 1), and there was no elimination of activity to the stomach after 60 seconds (43 patients) or with minimal elimination in some patients (9 patients). After the patient's transition to a standing position, there were more cases of gastric activity before drinking water (16 patients). After drinking 100 mL of water, variable esophageal retention was recorded (in 27 patients, RI was higher than 50%). In 14 patients, the maximal 100% retention occurred. In 25 patients, retention was less than 50%.

After BD in all patients, esophageal scintigrams obtained in the supine position were similar to the previous before BD (absent motility), with a higher number of patients with visible gastric elimination (21 patients). Images in standing position showed much more patients with gastric elimination before additional water load (39 patients). The great reduction of radionuclide retention was registered after drinking water in 50 patients. Maximal radionuclide retention after BD in successful cases was 26.2%.

In 50 patients, the first BD was successful, while in 2 patients, it was unsuccessful (in one of them, the second successful dilatation was performed, and the patient was followed up for 2 years). The range of RI values in all patients before BD and after BD is presented in Table 1. Age and gender showed no statistically different RI values after BD. In 21 patients, follow-up was continued, including one patient with failed BD.

By evaluating values before and a long time after the first BD (during follow-up), some RI values indicated remission or relapse of the disease. The RI value over 50% before

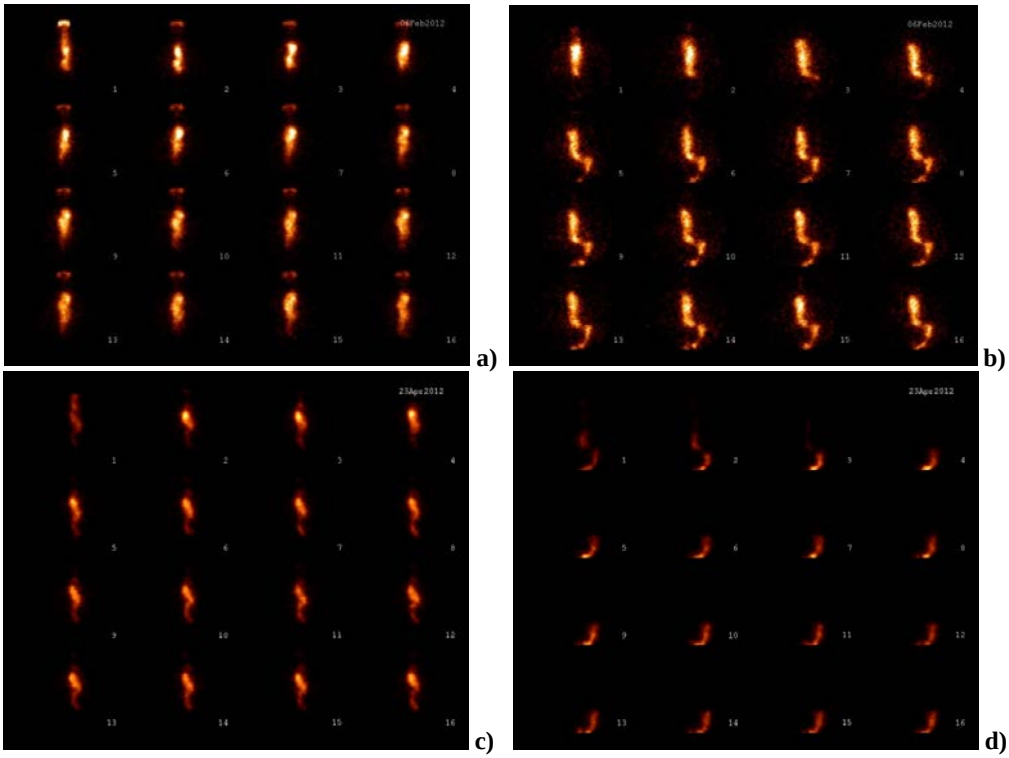

Fig. 1 – The esophageal scintigraphic images of a patient with successful balloon dilatation (BD): scintigrafic images obtained in supine and upright position before BD (a, b) and after BD (c, d).

Table 1

Scintigraphic results in patients with achalasia

Parameter	Before BD	After BD	*p*
Retention index (%)			
all patients (n = 52), median (range)	59.6 (6.5–100)	2.95 (0.2–54.1)	< 0.01[a]
aged > 40 years (n = 29), median	70	2.7	< 0.01[a]
aged <40 years (n = 23), median	58.7	3.2	ns[b]
male (n = 34), median	60.7	2.8	< 0.01[a]
female (n = 18), median	53.85	3.1	ns[b]
Gastric activity (+/-), n	9 / 43	21 / 31	< 0.05[c]
Gastric standing activity (+/-), n	16 / 36	39 / 13	< 0.01[c]

BD – balloon dilatation; [a] – Wilcoxon test; [b] – Mann-Whitney test; [c] – Chi-squared test; ns – non-significant.

BD and the difference of the RI values before and after BD higher than 50% were significantly different in patients with and without successful BD (Table 2). In our study, the RI values lower than 3% were more often in successful BD (7 successful and 3 unsuccessful BD), but the difference was not significant. The age and gender of patients had no statistical significance.

The predictive value of RI before and after BD and the impact of different factors were tested with logistic regression analysis. Variables including the RI values, age, and gender were analyzed in relationship to a therapeutic response (success or failure). The analysis confirmed the high predictive value of 50% RI difference after BD (Table 2) and its power to predict longer remission. An increased number of early dilatation failures in patients younger than 40 years was observed. Initially, failed BD occurred only in 2 patients younger than 40 years. During a one-year follow-up, all four additionally failed BD were registered in patients younger than 40 years. All three patients older than 40 years failed later – 2.5 years, 5 years, and 6 years after BD. These data suggested the impact of age on dilatation outcome, but the statistical significance of age was not confirmed (Table 2). The gastric radioactivity early after BD (the first control ES) was more often registered in the supine position (21/9, $p < 0.05$) (Table 2). The presence of eliminated stomach radioactivity that occurred in a standing position before water drinking was indicative of successful BD (39/16, $p < 0.01$). Analysis of gastric activity in patients on follow-up showed more cases of gastric activity after BD (15/6) but without a statistical difference in patients with successful and unsuccessful dilatation.

In 11 of the followed patients, remission that lasted from 2 months to 3.5 years was registered. In 9 patients during the follow-up period, relapse of the disease occurred (after 6 months to 6 years), and 7 of them were subjected to the second dilatation that was followed up during the next 1 month to 11 years. Of the initial 52 patients, 41 patients were with the first successful BD (Table 3), thus the percentage of success was 79%. The total number of unsuccessful BD one

Table 2

Scintigraphic results in followed patients with achalasia

Parameter	Balloon dilatation (BD), n		*p**
	successful (n = 11)	unsuccessful (n = 10)	
RID (%)			
> 50	8	2	< 0.05
< 50	3	8	
Age (years)			
> 40	3	3	ns
< 40	8	7	
> 40 (first year)	6	0	ns
< 40 (first year)	9	6	
Gender			
male	8	7	ns
female	3	3	
RI after BD			
> 3%	4	7	ns
< 3%	7	3	
GSA after BD			
+	9	6	ns
–	2	4	
	Logistic regression analysis (odds ratio)		*p*
RID 50%	10.67		0.023
RI 3%	3.571		ns
Age	0.875		ns
Gender	1.142		ns

***Fischer's exact test; RID – retention index difference; RI – retention index; GSA – gastric standing activity; ns – non-significant.**

Table 3

Number of successful and unsuccessful first balloon dilatation (SBD and NSBD, respectively) in all patients and in followed patients

Patients	SBD	NSBD	SBD follow-up	NSBD follow-up
Total number (n = 52)	41	11	11	10
male (n = 34)	26	8	8	7
female (n = 18)	15	3	3	3
Age (years)				
> 40 (n = 29)	25	4	3	3
< 40 (n = 23)	16	7	8	7

year after BD was 6, while including the maximal follow-up was 10. After the second BD, a new relapse occurred in two patients (after 1 month and after 1.5 years).

The images of a patient with successful BD are presented in Figure 1. The RI value before dilatation was 70%, and after dilatation was only 0.7%. After BD, spontaneous gastric activity in an upright position was registered.

Discussion

Symptom scores have been used for a long time for therapy assessment, but some authors declined their significance and predictive value [5, 8, 12]. A timed barium esophagogram has been recommended recently [22, 23], but it is a preferentially morphologic method with low functional and physiologic data. Manometry is the basic method of diagnosis, but some authors recommend using it for follow-up with patients [20, 21].

Scintigraphic imaging in the supine position is considered important for diagnosing achalasia (evaluation of motility), but imaging in a standing position with a load of additional water is considered necessary for reliable disease follow-up. Two-phase esophageal supine/standing scintigraphy with additional water load is an absolutely convenient and recommended method for noninvasive diagnostic and therapeutic management of achalasia. If standing position is not possible, a patient can be imaged in a sitting position.

The predictor of successful BD is the achieved great RI reduction (50%) after BD (the greater the difference, the bigger the effect of BD). The effect of the low value of RI was expected to contribute to longer remission but was not statistically confirmed, probably because of the small number of followed patients.

In the total group of patients, the change in gastric appearance after BD was statistically significant, but in the smaller group, with longer follow-up, statistical difference was not found. Gastric activity in supine and standing position were signs of successful therapy effect in first months after BD but without predictive value for a long remission.

The results of Jeon et al. [5] are based on two initial scintigraphies and the follow-up by symptom scores. Results would be more complete if control scintigraphies could be performed. What is unusual in the results of this study is that all 13 failed BD were registered within 6 months after BD. Our results showed that 2 patients failed initially, 4 patients after 6 to 8 months, and the remaining 4 patients after 2.5 to 6 years. The analysis of the esophageal time-activity curve and its use to count functional parameters was the main issue of many studies [5, 9–11]. The problem with that approach is that registered counts and counted parameters highly depend on the variable position of the liquid bolus in the esophagus. Our solution to image and count esophageal and gastric radioactivity was not proposed in published papers. We consider that our method is more reliable for evaluating achalasia. Jeon et al. [5] used a higher diagnostic dose without additional flushing water so a greater unswallowed activity could remain in the mouth.

The failure of BD occurred more often in patients under 40 years of age, but the impact of age was not statistically confirmed, contrary to other studies [3, 17]. All failed BD initially and during one year of follow-up occurred only in patients younger than 40 years (6 patients), but most of these followed younger patients (9 of them) had successful dilatation outcome, and this result caused the negative statistical significance of age.

The patient's gender was not associated with the failure of dilatation and the same appears in most studies [4, 5, 8].

The limitations of the study were the following: this was a retrospective analysis, there were great differences in the period of follow-up, the number of followed patients was low, the RI ratio depended on both esophageal and stomach condition, and the scintigraphic results were analyzed by only one observer.

Inspired by the introduction of high-resolution esophageal manometry (HRM) [24] which defined more subtypes of achalasia we highly suggest combining these two methods for diagnosis and follow-up of achalasia. HRM reveals new data about the normal esophageal function and motility disturbance in achalasia [25, 26]. Therefore, we recommend using scintigraphy as a complementary method in every patient subjected to HRM.

Besides BD, other therapeutic possibilities are being developed [27–32], but this method will remain the most convenient option for most patients.

Conclusion

Esophageal two-phase supine/standing scintigraphy is most suitable for diagnosing and follow-up of achalasia. The RI represents an important parameter for the evaluation and prediction of therapeutic response. The RI difference higher than 50% after balloon dilatation is a reliable predictor of a longer remission. Age and gender have no impact on dilatation outcome, but patients younger than 40 years have a higher risk of early therapeutic failure.

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Adequacy of biopsy samples for epidermal growth factor receptor (EGFR) molecular testing in lung adenocarcinoma

Adekvatnost bioptičkih uzoraka za molekularno testiranje receptora za epidermalni faktor rasta (EGFR) u adenokarcinomu pluća

Dragana Tegeltija*†, Aleksandra Lovrenski*†, Tijana Vasiljević†‡, Bojana Andrejić-Višnjić§

***Institute for Lung Diseases of Vojvodina, Sremska Kamenica, Serbia; ‡Oncology Institute of Vojvodina, Sremska Kamenica, Serbia; University of Novi Sad, Faculty of Medicine, †Department of Pathology, §Department of Histology and Embryology, Novi Sad, Serbia**

Abstract

Background/Aim. Adenocarcinoma of the lung is the most common histological type of lung cancer. The most reliable method in detecting epidermal growth factor receptor (EGFR) mutations is real-time polymerase chain reaction (PCR). It is recommended to sample three to five biopsy samples with a minimum of 200–400 preserved tumor cells. We analyzed the suitability of biopsy samples for EGFR molecular testing in lung adenocarcinoma. **Methods.** This retrospective analysis included 60 patients diagnosed with lung adenocarcinoma at the Institute for Pulmonary Diseases in Sremska Kamenica, Serbia from 2010 to 2015. Biopsy samples were obtained using transbronchial, bronchoscopic, or catheter biopsy procedures. All cases included the identification of morphometric parameters, the concentration of isolated DNA, and EGFR mutations. The proportion of tumors in biopsy samples was assessed in histological sections using computer-aided morphometry. **Results.** Biopsy samples were most commonly obtained by transbronchial biopsy (63%). In 35% of cases, there was either one or two biopsy samples. More than 10% of tumor cells were found in 68% of cases, while the majority of cases (33%) had between 200 and 500 tumor cells and only 8% of cases had between 20 and 50 tumor cells. The average concentration of DNA in all analyzed samples was 5.81 ng/μL and was significantly lower in samples provided by catheter biopsy. Only two cases with mutations were detected, and there was no statistically significant difference between the concentrations of isolated DNA in the wild type and mutated EGFR adenocarcinoma. Invalid results were found in 10% of cases. **Conclusion.** Biopsy samples are suitable for EGFR molecular testing in lung adenocarcinoma.

Key words:
lung neoplasms; adenocarcinoma; biopsy; erbb receptors; mutation.

Apstrakt

Uvod/Cilj. Adenokarcinom pluća je najčešći histološki tip karcinoma pluća. Najpouzdaniji metod za detekciju mutacija receptora epidermalnog faktora rasta (EGFR) je *real time* reakcija lančane polimerizacije (PCR). Preporuka je da se biopsijom uzme tri do pet fragmenata tkiva sa minimalno 200–400 očuvanih tumorskih ćelija. Analizirana je adekvatnost bioptičkih uzoraka tkiva za molekularno testiranje EGFR u adenokarcinomu pluća. **Metode.** Ovom retrospektivnom analizom obuhvaćeno je 60 bolesnika sa dijagnostikovanim adenokarcinomom pluća na Institutu za plućne bolesti Vojvodine u Sremskoj Kamenici, Srbija, u periodu 2010-2015. godina. Bioptički uzorci su dobijeni transbronhijalnom, bronhoskopskom ili kateter biopsijom. Kod svih uzoraka izvršene su morfometrijske analize, određena je koncentracija DNK i prisustvo EGFR mutacije. Udeo tumorskog tkiva u bioptičkom materijalu određen je primenom kompjuterskog programa za morfometrijske analize. **Rezultati.** Bioptički uzorci su najčešće dobijeni transbronhijalnom biopsijom (63%). U 35% uzoraka nalazio se jedan ili 2 bioptička uzorka. U 68% slučajeva, u uzorcima je nađeno više od 10% tumorskih ćelija, dok je najviše uzoraka imalo između 200 i 500 tumorskih ćelija, a samo 8% uzoraka između 20 i 50 ćelija. U proseku je izolovano 5.81 ng/μL DNK, a značajno niža koncentracija je utvrđena u uzorcima dobijenim kateter biopsijom. U samo dva uzorka evidentirana je mutacija EGFR, dok nije bilo razlike u koncentraciji DNK izolovane iz *wild* tip I EGFR-mutiranih karcinoma. Bilo je 10% uzoraka neadekvatnih za testiranje. **Zaključak.** Bioptički uzorci tkiva su adekvatni za molekularno testiranje EGFR u adenokarcinomu pluća.

Ključne reči:
pluća, neoplazme; adenokarcinom; biopsija; erbb receptori; mutacija.

Correspondence to: Bojana Andrejić-Višnjić, University of Novi Sad, Faculty of Medicine, Department of Histology and Embryology, Hajduk Veljkova 3, 21 000 Novi Sad, Serbia. E-mail: bojana.andrejic-visnjic@mf.uns.ac.rs

Introduction

Lung carcinoma (LC) is the leading cause of morbidity and mortality of malignant diseases in the world [1, 2]. Approximately 80% of patients with LC have unresectable tumors at the time of diagnosis. A new approach in the treatment of patients with lung adenocarcinoma (ADC) and epidermal growth factor receptor (EGFR) mutations are associated with sensitivity to the EGFR tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib [3–7].

Surgical samples represent the gold standard for EGFR molecular testing, but formalin-fixed paraffin-embedded biopsy samples, cytological samples, and blood plasma may also be used for this testing. The material obtained by any of these methods is considered adequate for testing [8]. The diagnostic accuracy and adequacy of the biopsy samples depend on their diagnostic modality, and the diameter of the needles used [9–14]. To date, there has been no consensus on the number of tumor cells (TC) necessary for EGFR molecular testing. According to Travis et al. [15], the recommendation for EGFR testing is a minimum of 200 to 400 TC.

Polymerase chain reaction (PCR) is the most widely used and most reliable method for determining EGFR mutations since it requires a minimum amount of the starting material and allows amplification of the desired DNA segment up to a billion times [16]. The PCR method is carried out in three basic steps: denaturation of double-stranded DNA matrix, hybridization of specific oligonucleotides (primers) and DNA matrix, and the extension (elongation) of primers that comprise a single PCR cycle repeated 20 to 45 times, where the desired DNA fragment is multiplied by a million to a billion times. After 40 repeated cycles, the efficiency of the reaction is lost, and the "plateau phenomenon" occurs [17].

The aim of this research was to determine the adequacy of the biopsy samples obtained by different biopsy procedures for EGFR molecular testing in lung ADC.

Methods

Pathohistological analysis and formation of groups

Sixty cases of primary ADC of the lungs diagnosed by biopsy samples were obtained from the records of the Institute for Pulmonary Diseases of Vojvodina from January 2010 to December 2015. The tissue was obtained during transbronchial biopsy (TBB), bronchoscopic biopsy (BB), or catheter biopsy (CB). The original diagnostic hematoxylin and eosin-stained sections were reviewed by two pathologists independently.

The number of biopsy samples (tissue fragments obtained during the biopsy procedure) and the number of TC in the whole biopsy sample were recorded using the Olympus BX43 (Olympus, Tokyo, Japan) light microscope with a magnification of 100X and 400X. All samples were classified into groups according to the number of TC: group I (< 20), group II (21–50), group III (51–100), group IV (101–200), group V (201–500), group VI (501–1000), and group VII (> 1000).

Computer-aided digital morphometry was used to determine the volume densities of tumor tissue (Tvd), non-tumor tissue volume density (NTvd), blood (Bvd), and necrosis (Nvd).

This was performed using the "Olympus DP73" digital camera (Olympus, Tokyo, Japan) that was attached to the Olympus BX43 light microscope. Digital images of the biopsy sample were captured at high power using a 40X lens and then analyzed using the "Image J" software with an installed plugin for the analysis of the number of hits (COST and Analyze). The total biopsy sample, composed of non-tumor tissue (NT) and tumor tissue (T), was digitally covered by a predefined number of hits using the program mentioned above (Figure 1). Given that the volume density of the entire biopsy sample accounts for 100%, based on the obtained data on the number of hits on the entire biopsy (Bih), tumor tissue (Th), non-tumor tissue (NTh), blood (Bh), and necrosis (Nh), Tvd, NTvd, Bvd, and Nvd (in percentages) were calculated using the proportions method (100% : Bih = Tvd : Th; 100% : Bih = NTvd : NTh; 100% : Bih = Bvd : Bh; 100% : Bih = Nvd : Nh).

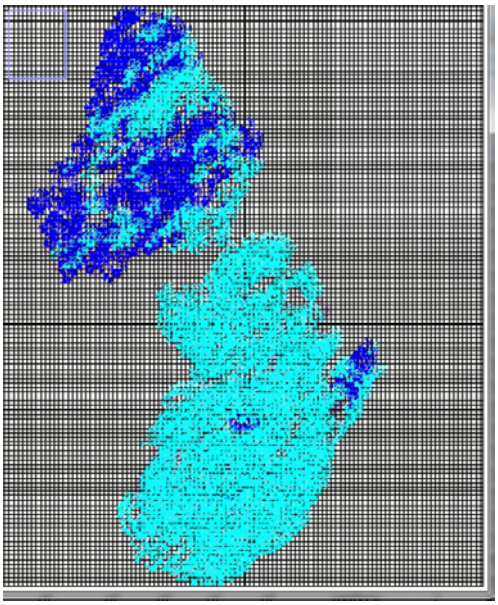

Fig. 1 – Morphometric analysis of biopsy sample (with lung adenocarcinoma tissue and non-tumor tissue) using Image J program [blue – tumor cells (TC); cyan – non-TC; ×40].

Real-time PCR (rtPCR) analysis

Molecular analysis of the EGFR mutation (exon 18-21) was done prospectively for all cases with the cobas EGFR Mutation Test (Roche, Basel, Switzerland) rtPCR. The cobas Sample Preparation Kit *(*Roche, Basel, Switzerland) was used for sample preparation and DNA extraction. Automatic amplification and detection were done on the cobas z 480 Analyzer (Roche, Basel, Switzerland).

Statistical methods

Assessment of correlations and comparisons between the mean values of numerically expressed data groups was performed using the *t*-test and analysis of varience (ANOVA) methods.

Statistical analysis was performed using SPSS 12.0 software.

Results

General characteristics of the patients are shown in Table 1. The median number of biopsy samples verified microscopically was two, with a minimum of 1 and a maximum of 7 samples per slide. More than half of the samples contained one or two biopsy samples. The cases with three, four, and seven samples were rare (20%, 2%, and 2%, respectively), and 4 among 60 analyzed cases could not be classified.

Table 1

Characteristics of patients

Characteristics	Values
Total number, n (%)	60 (100)
Gender, n (%)	
men	35 (58)
women	25 (42)
Age (years), mean ± SD (range)	61.8 ± 8.08 (43–79)
Smoking status, n (%)	
non-smoker	6 (10)
former smoker	10 (17)
smoker	44 (73)

Considering the number of TC, the majority of cases were classified into Group V (33%) and the minority in Group I (0%) and II (8%) (Table 2).

Table 2

Data on type of biopsy performed, and number of tumor cells in one sample

Type of biopsy	Number of cases, n (%)	Tvd, %
TBB	38 (63)	21.63
BB	10 (17)	31.22
CB	12 (20)	21.08
Number of TC		
I (< 20)	0 (0)	0
II (21–50)	5 (8)	6.64
III (51–100)	10 (17)	10.95
IV (101–200)	12 (20)	10.87
V (201–500)	20 (33)	31.63
VI (501–1000)	13 (22)	37.70
VII (> 1000)	0 (0)	0

TBB – transbronchial biopsy; BB – bronchobiopsy; CB – catheter biopsy; TC – tumor cells; Tvd – volume densities of tumor tissue.

Analyzing the biopsy samples, the median value of the Tvd was 24.88%, and there was no statistically significant difference in comparing the Tvd according to the type of the performed biopsy procedure (TBB, BB, CB) ($p = 0.360$). What is perhaps even more significant was a statistically significant difference in the number of TC among the samples obtained during different biopsy procedures when we compared the samples with small Tvd (less than 10%) ($p < 0.001$). In those samples with low Tvd, the biopsy samples obtained during CB had a significantly lower number of TC. Necrosis was recorded in two cases, with Nvd of 20% and 40%, but valid EGFR molecular testing results were obtained. Blood was found in 20 out of 60 cases, and Bvd ranged from 2.25% to 90.72%. In two of the cases containing blood (with Bvd values 11.7 and 71.7), the results of molecular testing were invalid, even after retesting. Out of all analyzed cases, six of them (10%) were invalid even after retesting. The average concentration of DNA was 5.81 ng/μL (range 0.38–19.2 ng/μL), and it was significantly lower in samples provided by CB.

EGFR mutations were detected in two cases, both of them women, one of which was a non-smoker. The tissue in EGFR mutated ADC cases was obtained by TBB, and according to the number of TC, the samples were put into group III. Tvd of one of the mutated cases was 11.59%, and the other had Tvd < 10%. One case had blood (Bvd = 32%), while necrosis was absent in both cases. There was no statistically significant difference in comparing the concentration of DNA in wild type (*wt*) and EGFR mutated ADC ($p = 0.641$). In cases with mutations detected, an average concentration of DNA was 4.53 ng/μL.

Discussion

ADC has been the most common histological type of LC in the last few decades, more frequently in men [18–25]. This fact was also confirmed by the results of our research, in which the ratio of affected men and women was 1.4 : 1. The average age of patients was 61.8 ± 8.08 years. Smoking is one of the most important risk factors for the development of LC [26–29]. The high percentage of active smokers in our research is most likely the consequence of poor socio-economic status, advocating bad lifestyles and bad effects of smoking ban campaigns.

Bronchoscopy is safe and well-tolerated by the patients and has become the mainstay investigation in the evaluation of patients with LC and may be used for molecular biologic analyses to help select therapy and provide prognostic information. The sensitivity and specificity of the biopsy samples depend on the location and distribution of the tumor and the number of samples obtained during the biopsy procedure [30–33]. The amount of tumor tissue obtained by biopsy is small since the obtained tissue contains both tumor and non-tumor cells. Moreover, the amount of tumor tissue depends on the histological type of the tumor and endoscopic findings. The presence of necrosis or the presence of crush artefact (even in a visible endobronchial disease) may cause the failure in achieving the histological diagnosis. In these circumstances, a combination of different cytological and histological procedures provides the optimum diagnostic yield. The number of biopsy samples in published papers varies, although it is recommended to take 3 to 5 samples [25, 31, 32]. Bronchoscopy has been implemented on our Institute since 1960. In this research, we recorded an average of two biopsy

samples (range 1–7) during one procedure, which is a significantly lower number in comparison to the results published.

The majority of biopsy samples contained more than 100 TC [25]. Similar to the above results, more than half of the biopsy samples included in our research had more than 100 TC. There are various methods for the morphometric analysis of biopsy samples. In the analysis of 120 cases, Scarpino et al. [33] used digitized slides where the following items were determined manually: biopsy area, tumor area, TC number, and the total number of cells in the biopsy, followed by determining the percentage of tumor tissue and TC.

In this research, Tvd was determined using the Image J computer program with installed plugins for the analysis of the number of hits (COST and Analyze). Tvd depends on the type of the tested sample and how the samples were obtained. The average Tvd value in our research was 24.88%. After comparing Tvd among the samples obtained with different types of biopsy procedures using the ANOVA method, the obtained difference was not statistically significant ($p = 0.36$). A significant number of biopsy samples (32%) in our research had Tvd less than 10%, which is contrary to the results of Zhu et al. [25] (4.7%).

The concentration of isolated DNA does not differ among the patients with *wt* EGFR and the mutated EGFR ADC [33, 34]. This view was confirmed by the results of our research ($p = 0.641$). In a study by Scarpino et al. [33], there was no statistically significant difference observed among the samples obtained by transthoracic puncture and biopsy (8.0 ng/μL vs. 9.2 ng/μL). Contrary to these results, we have found that the concentration of isolated DNA depends on the type of biopsy procedure and that there is a statistically significant difference between the samples obtained by BB and CB ($p = 0.055$).

The number of biopsies with an insufficient amount of DNA for molecular EGFR testing depends on the quality of the material analyzed. Khode et al. [35] identified an insufficient amount of extracted DNA in 6 (11%) out of 56 cases and recommended resampling. Although blood and necrosis may be the limiting factors for molecular EGFR testing, they were not the exclusion factors in our research, and the percentage of invalid results of 10% was in line with the results of the previous study. Necrosis was recorded in 2 cases only. Blood was present in 20 cases, but only 2 of them had invalid results, even after retesting. The presence of these factors was not considered an absolute limiting factor for exclusion from the EGFR testing.

In detecting mutations, biopsy and cytological samples are used equally with surgical samples, thus eliminating the need for invasive diagnostic procedures [34]. EGFR mutations can also be demonstrated in biopsy samples with a small number of TC [36]. This view was confirmed by the results obtained by Krawczyk et al. [34]. They detected a similar percentage of EGFR mutations in the biopsy samples with TC < 20% and TC ≥ 20% (8.1% vs. 9.2%) [34]. Contrary to these results, Scarpino et al. [33] recorded a smaller percentage of EGFR mutations in biopsy samples with TC < 20% as compared to samples with TC ≥ 20% (19% vs. 25%) ($p > 0.05$) [33]. If the comparison limit is 50% of TC, the difference is statistically significant [24]. Both cases with EGFR mutated ADC in our research had less than 20% of TC, which contributed to the understanding that EGFR mutation can also be determined in biopsy samples with a small number of TC.

In the first major study on the EGFR mutation status of patients from Serbia, EGFR mutations were detected in 42/360 (11.7%) patients with lung ADC [37]. Contrary to these results, we detected EGFR mutations in a significantly smaller percentage (2/60; 3.3%). Deletions in exon 19 are most often detected by applying the Cobas® EGFR Mutation Test [35, 37]. We did not detect this type of mutation, and the results of our research are most likely the consequence of a smaller number of patients involved in the research, as well as a smaller percentage of EGFR mutated lung ADC.

Conclusion

Based on our data presented here, we think that BB is suitable for EGFR molecular testing in lung ADC.

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Colorectal cancer surgery in Serbia 2010–2014: An evaluation of a multicenter registry

Hirurško lečenje kolorektalnog karcinoma u Srbiji od 2010. do 2014. godine: procena registara više centara

Aleksandra Nikolić*, Mina Krnjajić†‡, Velimir Marković†‡, Goran Barišić†‡, Ivan Dimitrijević†‡, Zoran Krivokapić†‡

University of Belgrade, *Institute of Molecular Genetics and Genetic Engineering, †Faculty of Medicine, Belgrade, Serbia; ‡Clinical Center of Serbia, First Surgical Clinic, Department of Colorectal Surgery, Belgrade, Serbia

Abstract

Background/Aim. Surgical registries and databases are especially valuable in monitoring the performances in cancer treatment and detecting potential problems. For Serbian patients with colorectal cancer (CRC), data regarding the treatment received, the factors that may impact the outcome, and whether or not treatment is successful and appropriate are not currently captured. The aim of this study was to establish a collection of a consensus dataset capturing surgical treatment of CRC at multiple public hospitals across Serbia and estimate outcome in CRC patients subjected to surgical treatment in the period 2010–2014. **Methods.** The study encompassed all 52 public CRC surgical units in Serbia. Numerical data on all patients who underwent operative CRC resection were included. An electronic database was created and overseen by the First Surgical Clinic of the Clinical Center of Serbia, Belgrade. Data were collected independently using a specifically designed standardized questionnaire, including the number of operated patients, localization of the primary tumor, type of surgical intervention, type and urgency of surgical intervention, and postoperative mortality. **Results.** A total number of 22,037 colorectal surgical procedures was performed in Serbia in the period 2010–2014 (approximately 4,400 per year). It was shown that 78.5% of the total number of procedures were elective and 21.5% were emergency. The most common cause of emergency surgeries was ileus (3,618 cases, 76.4%), while the less common causes were perforation (899 cases, 18.9%) and bleeding (216 cases, 4.5%). Postoperative mortality during the study period expressed as a percentage of all interventions for CRC was 2.8% on average with a slight increasing tendency. At the end of the study period, 127 doctors were educated for performing colonoscopy. **Conclusion.** The main outcome of this study was the establishment of the necessary preconditions for the multicenter data collection involving large numbers of CRC patients. The study supported the premise that the development of the national database for surgical treatment of CRC is achievable and could provide valuable insight into the routine surgical management of CRC in Serbia, creating a significant resource for further research.

Key words:
colorectal neoplasms; database as topic; general surgery; mortality; serbia; surgical procedures, operative; surveys and questionnaires.

Apstrakt

Uvod/Cilj. Hirurški registri i baze podataka su posebno vredni za praćenje karakteristika lečenja i otkrivanje potencijalnih problema kod karcinoma. Za bolesnike sa kolorektalnim karcinomom (KRK) u Srbiji, podaci o primljenoj terapiji, faktorima koji mogu imati uticaja na ishod, odnosno da li je lečnje uspešno i odgovarajuće ili ne, trenutno nisu sistematizovano obrađeni. Cilj rada bio je uspostavljanje baze usaglašenih podataka o hirurškom lečenju KRK u različitim državnim bolnicama širom Srbije i procena ishoda operativnog lečenja obolelih od KRK u periodu od 2010. do 2014. godine. **Metode.** Istraživanjem su obuhvaćena 52 državna hirurška centra u Srbiji u kojima su sakupljani brojčani podaci o bolesnicima podvrgnutim hirurškoj resekciji KRK. Kreirana je elektronska baza podataka pod nadzorom Prve hirurške klinike Kliničkog centra Srbije, Beograd. Podaci su nezavisno sakupljani korišćenjem posebno sastavljenog standardizovanog upitnika koji je uključio broj operisanih bolesnika, lokalizaciju primarnog tumora, tip hirurške intervencije, urgentnost zahvata i postoperativni mortalitet. **Rezultati.** Ukupno 22 037 kolorektalnih hirurških procedura izvedeno je u Srbiji u periodu od 2010. do 2014. godine (približno 4 400 godišnje) od čega je bilo

Correspondence to: Mina Krnjajić, Clinical Center of Serbia, First Surgical Clinic, Department of Colorectal Surgery, Koste Todorovića 6, 11 000 Belgrade, Serbia. E-mail: minakrnjajic@gmail.com

78,5% elektivnih, a 21,5% urgentnih operacija. Najčešći uzrok urgentnih operacija bio je ileus (3 618 bolesnika, 76,4%), dok su manje česti uzroci bili perforacija (899 bolesnika, 18,9%) i krvarenje (216 bolesnika, 4,5%). Postoperativna smrtnost tokom perioda istraživanja, izražena kao procenat svih KRK intervencija, iznosila je prosečno 2,8% sa diskretnom tendencijom rasta. Na kraju studijskog perioda, 127 lekara je bilo obučeno za obavljanje kolonoskopije. **Zaključak.** Glavni ishod istraživanja bilo je uspostavljanje neophodnih preduslova za multicentrično sakupljanje podataka koji uključuje veliki broj obolelih od KRK. Studija je podržala pretpostavku da je razvoj nacionalne baze podataka hirurškog lečenja KRK dostižan i da može obezbediti značajan uvid u rutinsko hirurško lečenje KRK u Srbiji i doprineti uvećanju resursa za buduća istraživanja.

Ključne reči:
kolorektalne neoplazme; baze podataka; hirurgija, opšta; mortalitet; srbija; hirurgija, operativne procedure; ankete i upitnici.

Introduction

Colorectal cancer (CRC) is the third commonest malignancy and a common cause of cancer death worldwide [1, 2]. The incidence of CRC in Serbia is approximately 40/100,000 in men and 20/100,000 in women [3].

Radical surgery with curative intent is the treatment of choice in the majority of colorectal cancer patients [4, 5]. The fundamental surgical principles are removal of the major vascular pedicle feeding the tumor along with its lymphatics, making a tumor-free margin, and *en bloc* resection of any surrounding organs or structures in direct contact with the tumor. To date, the primary modality of treatment for colon cancer is surgery with postoperative chemotherapy in selected cases, even though preoperative chemotherapy in advanced colon cancer patients is undergoing investigation [6]. In rectal cancer, there are several treatment strategies, depending on the tumor stage regarding the depth of penetration and lymph node involvement [7]. In early-stage rectal cancer, a less invasive technique such as local excision may be sufficient as opposed to the majority of cases where radical surgical resection is the treatment of choice. With the increase of the tumor stage, the probability of cure with surgery alone diminishes. In advanced-stage rectal cancers (advanced T3, T4, and N+ cases), preoperative chemoradiotherapy has been a well-established step in treatment for many years. Adjuvant chemotherapies have been shown to increase the cure and survival rates, and they are recommended for patients with lymph node metastases (stage III) and for selected metastasis-free patients (stage II) but with unpromising prognostic features, such as poorly differentiated tumors, lymphovascular or perineural invasion by tumor cells.

Cancer registries are the fundamental source of objective cancer data and are thus indispensable for the evaluation of the cancer burden and design of effective cancer control plans [8]. Surgical registries and databases are especially valuable in monitoring the performance in cancer treatment and detecting potential problems. The detailed recording of outcomes in a registry or database is a powerful tool in the analysis of surgical performance and can indicate areas that deserve further study from a clinical perspective. Data collection outside of clinical trials is beneficial, and its importance is increasingly recognized. There are a growing number of registries and databases across the spectrum of diseases. These efforts are of most value when multiple sites combine efforts maximizing patient numbers and hence increasing the statistical power of any analysis. Documenting and analyzing variations in care across different sites and treatment settings may help understand how they impact patient outcomes. For Serbian patients with colorectal cancer (CRC), data regarding the treatment received, the factors that may impact the outcome, and whether or not treatment is successful and appropriate are not currently captured.

Data that describe surgical activity and performance in CRC treatment and outcome of colorectal surgery are currently unavailable on the national level in Serbia. A nationally organized screening program for CRC was introduced in Serbia in 2013, and it was based on a previously implemented National program for CRC prevention that started in 2009, in accordance with European guidelines for quality assurance in CRC screening and diagnosis [9–11].

The aim of this study was to establish a collection of a consensus dataset capturing surgical treatment of CRC at multiple public hospitals across Serbia and estimate outcome in patients subjected to surgical treatment in the period 2010–2014.

Methods

The study has encompassed all 52 public surgical units in Serbia in which colorectal surgery is performed (Figure 1). Numerical data on all patients who underwent operative CRC resection from 2010 to 2014 were included. The electronic da-

Fig. 1 – Map representing distribution of public colorectal surgical units included in the study.

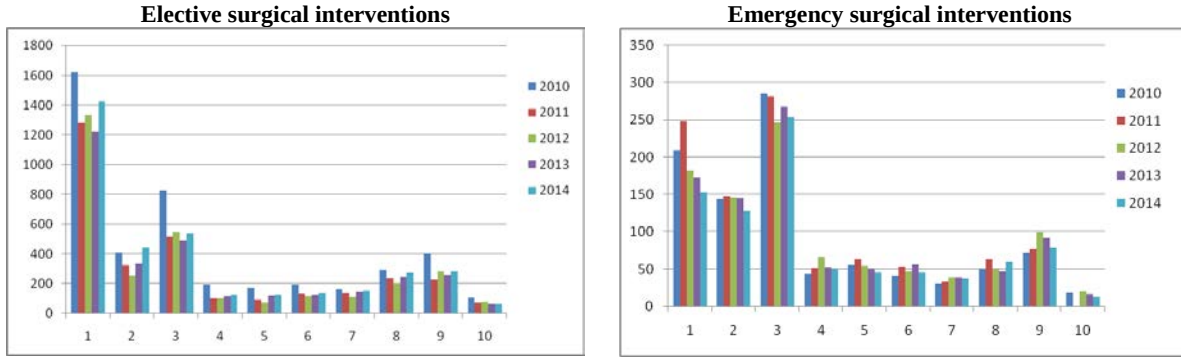
tabase was created and overseen by the First Surgical Clinic of the Clinical Center of Serbia, Belgrade. Data were collected independently using a standard questionnaire specifically designed for this purpose and completed by colorectal surgeons annually. Surgical procedures were recorded, and for each procedure, the number of patients subjected to it was entered under the predefined classification. The following dataset was included in the questionnaire: localization of the primary tumor, type of surgical intervention, type and urgency of surgical intervention, and postoperative mortality. Postoperative mortality was defined as death following colorectal surgery occurring during hospitalization or within 30 days of discharge. The total number of procedures was registered for each year and the entire five-year period.

Results

The study included all CRC cases managed operatively in 52 colorectal surgical units across Serbia during five years. Trends in standards of surgical care were recorded through data collection from each center. The dataset was tested initially on paper by participating clinicians, and feedback was obtained regarding clarity of the dataset and improvements made, after which an electronic version was created. This project relied on individual sites collecting and entering accurate data. According to the results of the database entries, 22,037 colorectal surgical procedures were performed in Serbia in the period 2010–2014 (approximately 4,400 per year). Out of the total number of surgical procedures, 78.5% were elective and 21.5% were emergency.

The distribution of procedures by tumor site is represented in Figure 2. The most common cause of emergency surgeries was ileus (3,618 cases, 76.4%), while the less common causes were perforation (899 of 18.9% cases) and bleeding (216 of 4.5% cases).

Surgical procedures were similarly distributed throughout the study period, with primary tumor resection in combination with stoma slightly more frequent (43.4%) than stoma (32.8%) and primary tumor resection in combination with anastomosis (23.8%) (Figure 3).



1 – *Ca recti*
2 – *Ca junct. coli rectosigmoidis*
3 – *Ca coli sigmoidis*
4 – *Ca coli descedentis*
5 – *Ca coli lienalis*
6 – *Ca coli transversi*
7 – *Ca flexurae coli hepaticae*
8 – *Ca coli ascedentis*
9 – *Ca caeci*
10 – *Ca* double localization

Fig. 2 – Distribution of elective and emergency surgical procedures by tumor site.
Ca – *carcinoma*.

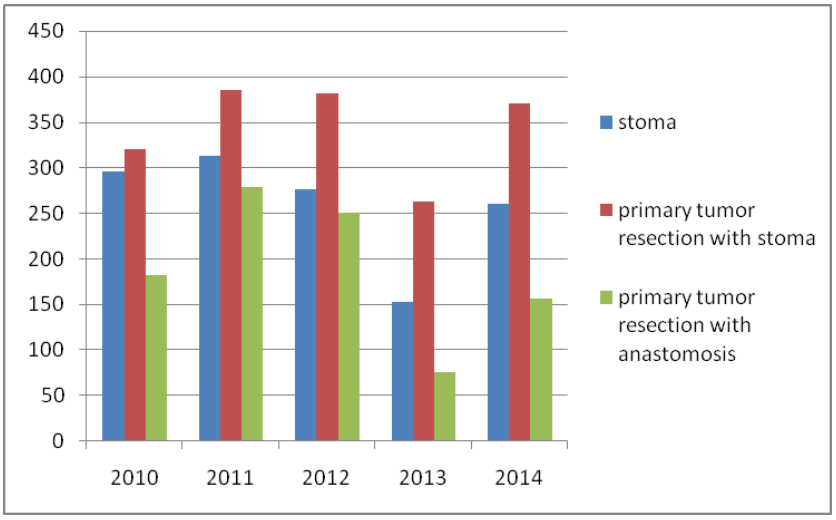
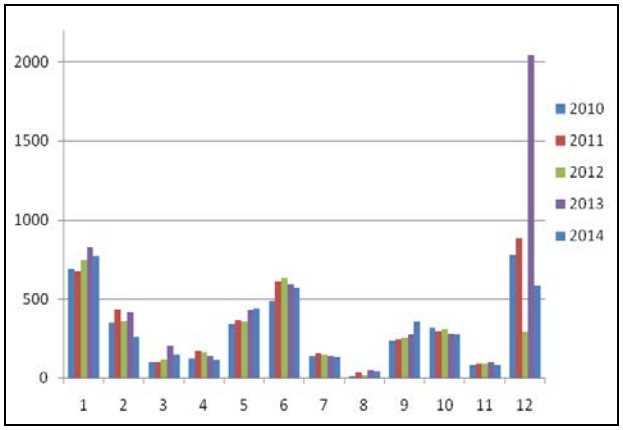


Fig. 3 – Distribution of surgical procedures by study years.

Types of radical surgical procedures were also similarly distributed over the study years, except for colonoscopic polypectomies that have shown a significant increase in 2013 (Figure 4). At the end of the study period, 127 doctors were educated for performing colonoscopy with 55 instruments available.



1 – Right hemicolectomy
2 – Left hemicolectomy
3 – Atypical colon resection
4 – Subtotal colectomy
5 – High anterior rectal resection
6 – Low anterior rectal resection with colorectal anastomosis
7 – Low anterior rectal resection with coloanal anastomosis
8 – Low anterior intersphincteric resection
9 – Hartmann procedure
10 – Miles procedure
11 – Local tumor excision
12 – Colonoscopic polypectomy

Fig. 4 – Distribution of radical surgical procedures by study years.

The distribution of types of palliative surgical procedures was also similar during the study period, with stoma lacking primary tumor resection being the most commonly used procedure (40%) (Figure 5).

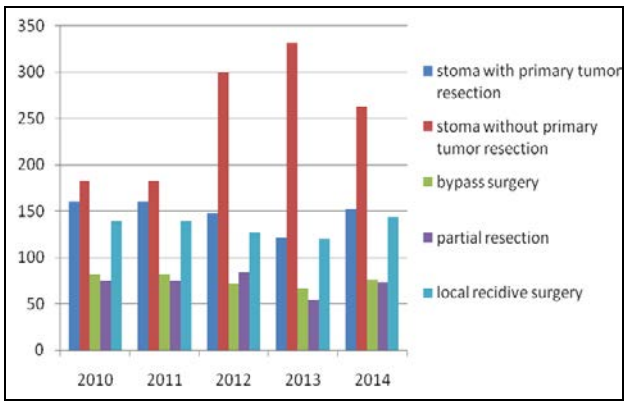


Fig. 5 – Distribution of palliative surgical procedures by study years.

Trends in chemoradiotherapy for CRC changed over the study period, with the more common use of perioperative treatment and an increase in polychemotherapy application (Figure 6).

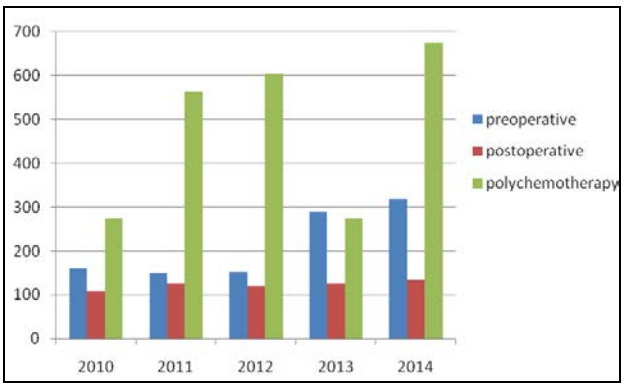


Fig. 6 – Application of chemoradiotherapy over the study period.

Postoperative mortality during the study period was expressed as a percentage of all interventions for colorectal malignancies, with a mean value of 2.8% and a slight increasing tendency (Figure 7).

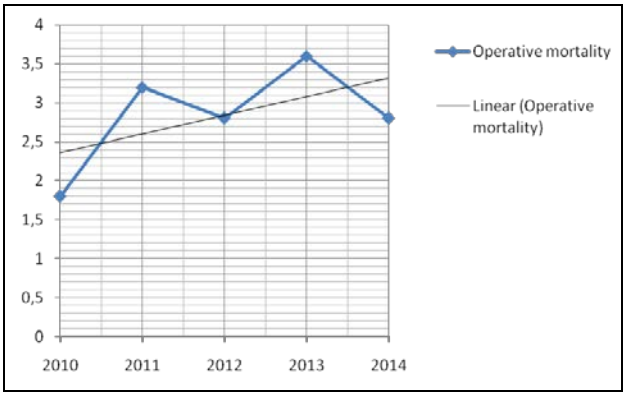


Fig. 7 – Postoperative mortality observed over the study period.

Discussion

The total number of patients who underwent operative CRC resection in Serbia from 2010 to 2014 was included, as well as several other numerical data regarding the surgical treatment of CRC (type of procedure, tumor localization, and applied chemoradiotherapy). The database was established, enabling each of the participating centers to contribute by entering the previously defined dataset. In order to avoid variations in the nomenclature, categories of required data were also predefined. The dataset was limited to the information regarding surgical care of CRC, and neither demographic data nor clinical information were included. The study was designed in such a way because this survey was focused only on gaining basic information on colorectal surgical procedures. The main problem in the data collection process was the fact that the data were collected retrospectively, at the end of the calendar year by a single surgeon per center who performed this task on a volunteer basis. This probably led to minor errors in the registered number of procedures. However, the obtained distribution of the certain surgical procedures did not vary significantly from the expected, neither

did it vary significantly between the centers included in the study.

Recorded trends in standards of surgical care showed the expected distribution, except for colonoscopic polypectomies that have shown a significant increase in 2013 (Figures 2–5). This fact was due to the significant improvements in infrastructure and the training of professionals. In the year 2013, 55 colonoscopes were introduced in the practice in different surgical units across Serbia, while 127 doctors were educated for performing colonoscopy. The distribution of tumor localization and types of surgical procedures were similar during the study period. Trends in treatment changed, with the more common use of perioperative treatment and increase in polychemotherapy application. The growing trend of polychemotherapy application in CRC patients over the analyzed period was a direct consequence of the increased availability of different conventional and targeted chemotherapeutic agents (Figure 6). These improvements, in combination with the introduction of the screening program, represent an excellent basis for further development of colorectal surgical care in Serbia.

Postoperative mortality during the study period showed a tendency to increase, but with the mean value of 2.8%, it remains within the range of values observed for other populations [12–14]. Postoperative mortality might have escalated in the analyzed period because the number of centers and surgeons performing surgical interventions in colorectal cancer in Serbia, who have not yet been provided with extensive training and practice, significantly increased. The future improvement in colorectal surgical care in Serbia will mostly rely on the education and training of professionals. Hence, the postoperative mortality rate is expected to decrease in the next period. It should also be noted that operative mortality may quite often be the direct result of preexisting comorbidity and not always the direct result of the surgical procedure.

The purpose of this study was to describe surgical activity and performance in CRC treatment in Serbia through a national registry and investigate the outcome of colorectal surgery on a national level. The main benefit of the study was the establishment of the system for data collection and preliminary information based on which future database can be developed. Considering the large number of CRC cases in Serbia and the fact that patients are often referred to different centers for repeated surgical interventions, there is a growing need for creating an online national database with the possibility of entering individual information for each surgical case.

Conclusion

The main benefit of this study was the establishment of the necessary preconditions for the multicenter data collection involving large numbers of CRC patients. The study supported the premise that developing the national database for surgical treatment of CRC is achievable and should be established in Serbia. It can provide valuable insight into the routine surgical management of CRC and create a significant resource for further research.

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ORIGINAL ARTICLE

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What contributes the most to the breast cancer patients' quality of life during therapy – clinical factors, functional and affective state, or social support?

Šta najviše doprinosi kvalitetu života pacijentkinja tokom terapije karcinoma dojke – klinički faktori, funkcionalni i emocionalni status ili socijalna podrška?

Ivana Novakov*, Svetlana Popović-Petrović*†, Sonja Ilinčić-Zlatar*, Milanka Tatić*†, Mirjana Ševo‡

***Oncology Institute of Vojvodina, Sremska Kamenica, Serbia; †University of Novi Sad, Faculty of Medicine, Novi Sad, Serbia; ‡Institute for Pulmonary Diseases of Vojvodina, Sremska Kamenica, Serbia**

Abstract

Background/Aim. As significant advances in cancer treatment have occurred over the past decades, the crucial questions in oncology nowadays are not related only to the treatment of the illness but also to the quality of life (QOL) of patients. The aim of our study was to explore which set of determinants (clinical, functional, affective, or social) has the greatest impact on explaining QOL in women who live with the diagnosis of breast cancer. **Methods.** The research was conducted on 64 women (with a mean age of 58.36 ± 11.30) while undergoing radiation therapy at the Oncology Institute of Vojvodina, Serbia. Quality of Life Instrument – Breast Cancer Patient Version (QOL-BC) questionnaire was used for evaluation of physical, psychological, social, spiritual, and general well-being, the Upper Extremity Functional Index (UEFI) was applied for the assessment of the upper extremity function, the Depression, Anxiety, and Stress Scale-21 Items (DASS-21) was used for measuring symptoms of depression, anxiety, and stress, the Medical Outcomes Survey, Social Support Survey (MOS-SSS) served for evaluation of social support; demographic and clinical data of patients were also collected. **Results.** Analysis of Variance (ANOVA) with repeated measures [$F(2.03, 127.80) = 20.24$, $p < 0.001$] showed that in our sample, physical QOL was significantly better from all other domains, while social QOL was significantly lower from both physical and psychological aspect. A hierarchical regression analysis [$F(8, 55) = 7.16$, $p < 0.001$, $R^2 = 0.51$] showed that patients who received adjuvant chemotherapy and experienced high levels of stress and poor social support usually had diminished general QOL. Introduction of affective-related variables [$\Delta R^2 = 0.16$, $p(\Delta F) < 0.01$] and social support [$\Delta R^2 = 0.05$, $p(\Delta F) < 0.05$] led to a significant increase in proportion of explained variance over and above the clinical and functional variables. **Conclusion.** Our results indicate that psychological and social resources are more important in predicting QOL compared to clinical and functional factors. At the same time, the social, psychological, and spiritual well-being of patients is significantly worse compared to the physical QOL, meaning that there is still much left to be done regarding the progress from a purely somatic to a holistic approach in the treatment of breast cancer.

Key words:
breast neoplasms; drug therapy; neoadjuvant therapy; psychology; social support; quality of life; radiotherapy; surveys and questionnaires

Apstrakt

Uvod/Cilj. Pošto je tokom decenija došlo do značajnog napretka u lečenju raka, ključna pitanja u onkologiji danas se ne odnose samo na lečenje bolesti, već i na kvalitet života pacijentkinja. Zbog toga je cilj ovog istraživanja bio da se ispita koji skup determinanti (klinički faktori, funkcionalno stanje, afektivni status ili socijalna podrška) u najvećoj meri doprinosi predviđanju kvaliteta života kod žena koje žive sa dijagnozom raka dojke. **Metode.** U istraživanju su učestvovale 64 žene (prosečne životne dobi od 58,36 ± 11,30 godine) tokom zračne terapije na Institutu za onkologiju Vojvodine. Upitnik *Quality of Life Instrument – Breast Cancer Patient Version* (QOL-BC) primenjen je za procenu fizičkog, psihičkog, socijalnog, duhovnog i opšteg kvaliteta života, *Upper Extremity Functional Index* (UEFI) je upotrebljen za procenu funkcije gornjih ekstremiteta, *Depression, Anxiety, and Stress Scale-21 Items* (DASS-21) korišćen je za procenu simptoma depresije, anksioznosti i stresa, *Medical Outcomes Survey, Social Support Survey* (MOS-SSS) služio je za procenu

Correspondence to: Ivana Novakov, Oncology Institute of Vojvodina, Put doktora Goldmana 4, 21 204 Sremska Kamenica, Serbia. E-mail: novakov.ivana@onk.ns.ac.rs

socijalne podrške, a prikupljeni su i demografski i klinički podaci pacijentkinja. **Rezultati.** Primenom testa analize varijanse (ANOVA) sa ponovljenim merenjima [$F(2{,}03, 127{,}80) = 20{,}24$, $p < 0{,}001$] nađeno je da je fizički kvalitet života pacijentkinja bio značajno bolji u odnosu na ostale domene, dok je socijalni kvalitet života bio značajno lošiji u odnosu na fizički i psihološki aspekt. Hijerarhijska regresiona analiza [$F(8, 55) = 7{,}16$, $p < 0.001$, $R^2 = 0{,}5$] pokazala je da su ispitanice koje su primile adjuvantnu hemioterapiju, i iskusile povišen nivo distresa i lošiju socijalnu podršku, imale smanjen generalni kvalitet života. Uvođenje varijabli povezanih sa afektivnim stanjem [$\Delta R^2 = 0.16$, $p(\Delta F) < 0.01$] i socijalnom podrškom [$\Delta R^2 = 0.05$, $p(\Delta F) < 0.05$] dovelo je do značajnog porasta u proporciji objašnjene varijanse, povrh kliničkih i funkcionalnih faktora. **Zaključak.** Naši rezultati ukazuju na to da psihološki i socijalni resursi imaju važniju ulogu u predviđanju kvaliteta života u poređenju sa kliničkim i funkcionalnim faktorima. Istovremeno, socijalno, psihološko i duhovno blagostanje pacijentkinja bilo je značajno lošije u poređenju sa fizičkim kvalitetom života, što znači da još mnogo toga treba da bude preduzeto u cilju napretka od dominantno somatskog, ka holističkom pristupu u lečenju karcinoma dojke.

Ključne reči:
dojka, neoplazme; lečenje lekovima; lečenje, neoadjuvantno; psihologija; socijalna podrška; kvalitet života; radioterapija; ankete i upitnici.

Introduction

Breast cancer (BC) is considered the most common type of cancer in women both worldwide [1, 2] and in Serbia [3]. As significant advances in cancer treatment have occurred over the past decades, and the survival rate is getting increasingly extended, crucial questions in oncology nowadays are not related only to the treatment and control of the illness but also to the emotional and psychological well-being and the quality of life (QOL) of patients. Knowing that breast cancer treatment is a very demanding and stressful process, often accompanied by uncertainty, assessing QOL is very important since patients are faced with a wide range of unpleasant experiences, which presumably tend to diminish different aspects of their functioning, both in the short term and in the long run.

Previous research, which focused on QOL as an important outcome measure, showed that clinical and demographic variables such as stage of the disease, adjuvant chemotherapy, marital status, and educational level might be important determinants of QOL [4]. Some authors found that single women and those with lower levels of education had poorer scores on QOL measures [5]. Younger age has also been found to predict lower QOL in breast cancer patients [5–7]. Furthermore, receiving chemotherapy [8], additional comorbid conditions [9, 10], and arm problems due to axilla surgery [11–13] are likely to affect the deterioration of QOL in patients treated for breast cancer. Axillary lymph node dissection, which results in several upper-limb symptoms and certain restrictions of daily activities, may be related to compromised body image, impaired functioning, and decreased QOL [14, 15]. One study that included a wide range of demographic and clinical factors revealed that arm dysfunction, comorbidity, and age were some of the strongest predictors of QOL among breast cancer patients [13]. The same study indicated that marital, educational, and employment status were significantly related to QOL, but to a lesser extent compared to previously mentioned variables [13].

However, findings regarding socio-demographic and clinical data are not always consistent. While some authors, for example, have stated that application of chemotherapy or radiotherapy significantly affects patients' QOL [16, 17], others have found no alterations in QOL regarding tumor severity and treatment type [18, 19]. In addition, some authors have shown that socio-demographic and treatment variables were only to a moderate extent related to QOL, while psycho-social variables showed a more convincing and consistent relationship with QOL measures [20]. Thus, age, chemotherapy, and time passed from the diagnosis were not related to any of the QOL outcomes, whilst psycho-social factors such as partner relationships, sexual functioning, body image, coping strategies, etc. showed a significant connection to QOL and well-being [20].

Given that the experience of breast cancer and its treatment is perceived as intensively stressful, facing the diagnosis can lead to psychological reactions such as denial, fear, uneasiness, anger, sadness, despair, and hopelessness. Patients with breast cancer diagnosis are at high risk of developing psychiatric comorbidities, especially depression and anxiety. In one recent study, it has been shown that, from 152 breast cancer patients, 38.2% could be classified as depressed, while 32.2% were found to be anxious [21]. Other authors have found that almost 50% of women with breast cancer manifested depression, anxiety, or both, one year following the diagnosis; 25% of them reported symptoms two, three, and four years after the diagnosis, and finally, 15% of women had symptoms five years following the diagnosis [18]. Furthermore, it has been found that 41% of newly diagnosed breast cancer patients experienced high levels of distress, while 11% had a major depressive disorder [22]. These findings clearly suggest that the affective state of breast cancer patients is at apparent risk and should be taken into account as an important factor in the better understanding of QOL determinants.

In addition, it is known that adequate social support is of outstanding importance when it comes to dealing with cancer diagnosis and demanding oncological treatment. Some studies have found that social support (which involves tangible support, emotional informational support, affectionate support, and positive social interaction) was related to better QOL and acted as a moderator between symptoms of depression and QOL [23]. It has also been shown that a decrease in social support quantity and quality is

associated with increased symptoms of depression, stress, and negative effect in breast cancer patients [24]. According to the previous findings, social support quality is a more important predictor of well-being than social support quantity [25]. What's more, the quality and quantity of social support among women with breast cancer were shown to be related to posttraumatic growth, which is considered to be one of the positive well-being outcomes [26, 27].

Although there is a large body of literature regarding breast cancer patients' QOL, it remains unclear which group of factors has the strongest impact on different QOL domains, especially when it comes to women who live with the diagnosis of breast cancer in Serbia. Therefore, the main aim of this research was to offer a more comprehensive understanding of the contribution that different clusters of factors may have on diverse aspects of QOL in breast cancer patients. More specifically, the aim of our study was to explore which set of determinants (such as clinical factors, functional condition, affective state, and social support) had the greatest impact in explaining specific domains of QOL (physical, psychological, social, and spiritual) in patients. As some authors emphasize [13], a comprehensive approach to understanding the QOL in breast cancer patients could be useful to all members of the medical team by highlighting which problems should be prioritized and addressed in order to improve different aspects of life satisfaction in patients.

Methods

Study design and procedure

This cross-sectional research was conducted with the approval of the Ethics Committee of the Oncology Institute of Vojvodina, Serbia. Based on the principles of voluntary participation, the data were collected from patients at the beginning of their entry or stay at the Clinic for Radiotherapy within the Oncology Institute of Vojvodina. All participants were informed about the main goals of the research. Before filling in the questionnaires, all participants signed informed consent. The assessment period lasted approximately 45 min to 1 h, which also included a short break for refreshment. A research assistant was available all the time while participants were filling in the questionnaires, helping them in the case of need and reminding them to check if they had answered all the questions, due to which the problem of missing data has been overcome.

Sample

The research was conducted on 64 women, from 33 to 79 years [mean (M) age = 58.36, standard deviation (SD) = 11.30 years], while undergoing radiation therapy at the Oncology Institute of Vojvodina. Patients took part in the research within the period of 2 to 13 months following the breast cancer surgery (M = 5.39, SD = 2.62 months). Criteria for inclusion in the study were that participants had never been diagnosed with breast cancer before (or any other malignant disease) and that the presence of metastatic changes was not detected at the time of psychological assessment. In terms of educational level, 26.6% of participants completed elementary school, 54.7% completed secondary school, 7.8% had a college degree, while 11% had a university degree. Women mainly reported that they live in a city (64.1%). Besides, 26.6% of participants were employed, 25% unemployed, and 48.4% were retired. Considering marital status, 71.9% of participants had a partner, while 28.1% were single, divorced, or widowed. Finally, 85.9% of women had children. The data about clinical variables assessed in our research are presented in Table 1.

Table 1
Clinical variables in the study population

Clinical variables	Patients, n (%)	
	yes	no
Neoadjuvant chemotherapy	12 (18.8)	52 (81.3)
Adjuvant chemotherapy	32 (50)	32 (50)
Nonmalignant comorbid conditions	40 (62.5)	24 (37.5)

Instruments

A demographic and clinical data questionnaire was designed in order to collect data about the age of the participants, their educational level, working, marital, and birth status, as well as the place of residence. Information about clinical data, such as the number of months that passed after the surgical treatment, receiving neoadjuvant and/or adjuvant chemotherapy, and finally, the presence of any nonmalignant comorbid conditions, was also assessed.

Quality of Life Instrument – Breast Cancer Patient Version (QOL-BC) [28] scale is based on earlier versions of the QOL instrument, constructed by researchers at the City of Hope National Medical Center. The scale consists of 46 items representing four domains – physical, psychological, social, and spiritual well-being. Items are presented on a 10-point scale (from 0 = worst to 10 = best outcome). Participants are instructed to indicate the degree to which they agree or disagree with every statement. The physical aspect of QOL includes information about nausea, constipation, appetite, menstrual changes/fertility, sleep, aches/pain, and fatigue. Psychological well-being includes a sense of usefulness, happiness/satisfaction, control/coping, anxiety/depression, concentration/memory, overall perception of QOL, changes in appearance and self-concept, distress of diagnosis and treatment, and finally, fear of recurrence/tests. Social well-being refers to family distress, personal relationships, support, employment, home activities, sense of isolation, financial burden, and sexuality. The spiritual aspect contains a sense of hope and life purpose, (positive) spiritual change, religious/spiritual activity, and experience of uncertainty [29–33].

The Upper Extremity Functional Index (UEFI) [34] is a 20-item self-reporting scale. The instrument is used as a measure of upper extremity dysfunction. Items refer to

everyday activities such as cleaning, dressing, driving, lifting a bag, preparing food, etc. Participants should report if they have any difficulties related to listed activities. The answers are rated on a five-point Likert scale (from 0 = extreme difficulty to 4 = no difficulty performing the task). The maximum possible score is 80. The higher the score, the better the upper extremity function. The instrument has shown good reliability [34].

Depression, Anxiety, and Stress Scale - 21 items (DASS-21) [35] is an instrument that contains three self-report scales which measure the emotional states of depression, anxiety, and stress. Each of the three scales consists of 7 items presented on a 4-point Likert scale, ranging from "do not apply to me at all" to "applied to me very much or most of the time". The depression scale covers dysphoria, hopelessness, depreciation of life, self-devaluation, lack of interest, anhedonia, and apathy. The anxiety scale includes information about autonomic nervous system arousal, tremor, situational anxiety, and subjective experience of anxious affect. The stress scale assesses difficulty relaxing, nervous arousal, and being easily agitated, irritable, and impatient. Three separate scores can be calculated by summing the items which belong to the same subscale [35].

Serbian translation of The Medical Outcomes Study Social Support Survey - Serbian translation (MOS-SSS) [36] is prepared according to the original version of MOS-SSS [37]. This scale was developed within The Medical Outcomes Study [38], whose goal was to explore the QOL in patients suffering from chronic diseases such as hypertension and diabetes. The scale consists of 19 items whose measure perceived social support. Items are classified into 4 subscales: 1) *emotional support* (the availability of a person who understands us and our problems, who is ready to listen, and with whom we can share our worries) / *informative support* (availability of a person whom we can ask for advice or information), 2) *instrumental or practical support* (availability of a person who can help us when we are sick), 3) *affective support* (availability of a person who shows us love and attention), and 4) *positive social interactions* (availability of a person with whom we can enjoy and relax) [36]. Participants are asked how often every type of support has been available to them when needed. Items are presented on a five-point Likert scale (from 1 = never to 5 = always). In addition, the scale contains one question which refers to structural social support. Here, participants should write how many close friends and relatives they have with whom they can feel comfortable. To this question, participants should respond by writing a number [36].

Statistical analysis

For statistical data processing, the IBM SPSS Statistics 21.0 software was used. After considering the descriptive data and correlation analysis for all variables in our research, we examined whether there were any statistically significant differences in the representation of four domains of QOL in our sample. Following the equalization of means according to the number of items by every subscale, and after checking for normality of distributions (we considered as normal data the measuring according to George and Mallery [39]), the One-way ANOVA with repeated measures was performed for QOL across four subscales. The Mauchly's test [$\chi^2(5) = 43.29$, $p < 0.001$] indicated a violation of sphericity, therefore, the Greenhouse-Geisser method was used for correction.

Besides, we tested whether there were some important differences between the participants in the four domains and overall QOL regarding demographic data. Due to the small sample size (and thus assumed compromised normality), we decided to use nonparametric tests (Mann-Whitney U-test and Kruskal-Wallis test).

In order to answer our main research question – which sets of variables are the most important in predicting different aspects of QOL in breast cancer patients – a hierarchical regression analysis was applied. Since some of the predictors correlate highly with one another (Table 2), the data were checked for multicollinearity. For all predictors, the obtained VIF value was in an acceptable range, indicating that the problem of multicollinearity was not present. Furthermore, the data were analyzed for heteroscedasticity, which could also be excluded. Five hierarchical regression analyses were performed, with four

Table 2

Correlations between measures of quality of life (QOL), depression, anxiety, stress, social support (quantity and quality) and upper extremity function

Variables	Age (1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
Physical QOL (2)	0.10										
Psychological QOL (3)	-0.14	**0.66****									
Social QOL (4)	-0.03	**0.47****	**0.52****								
Spiritual QOL (5)	**-0.25***	0.07	0.22	-0.24							
General QOL (6)	-0.13	**0.77****	**0.95****	**0.63****	**0.32****						
Depression (7)	0.07	**-0.30***	**-0.51****	**-0.56****	0.05	**-0.52****					
Anxiety (8)	-0.00	**-0.42****	**-0.53****	**-0.61****	0.11	**-0.56****	**0.75****				
Stress (9)	-0.15	**-0.43****	**-0.58****	**-0.50****	0-.04	**-0.60****	**0.73****	**0.80****			
Social support quantity (10)	-0.14	0.03	0.06	0.09	0.24	0.12	-0.09	-0.02	-0.02		
Social support quality (11)	-0.03	**0.30***	**0.31***	0.22	0.20	**0.36****	**-0.31***	-0.17	-0.23	0.18	
Upper extremity function (12)	-0.19	**0.36****	**0.45****	**0.46****	-0.01	**0.47****	**-0.58****	**-0.65****	**-0.53****	**0.34***	0.18

***p* < 0.05; ****p* < 0.01.**

domains and the overall QOL as criterion variables, while eight predictors were the same across all models and were always introduced in the same order. The first block of predictors consisted of clinical variables, such as receiving neoadjuvant and/or adjuvant chemotherapy and the presence of any nonmalignant comorbid conditions. Functional status, i.e., upper extremity function, was entered in the second step of the model. The third block consisted of the variables related to the affective state – indicators of depression, anxiety, and stress. In the final step, the perceived quality of social support was introduced.

Results

The descriptive statistical indicators and Cronbach's alpha coefficients for continuous variables in our research are presented in Table 3. Values of skewness and kurtosis indicate that distributions of data measured by different scales do not deviate importantly from normal [39]. Cronbach's alpha coefficients demonstrated that the reliability of the entire QOL-BC instrument was good, and the same went for the psychological QOL subscale. Physical and social QOL subscales manifested acceptable reliability, while the spiritual domain had questionable internal consistency (probably due to one reversely scored item, which turned out to compromise the reliability of this subscale). The reliability of subscales from DASS-21 varied from acceptable to good, while MOS-SSS and UEFI showed excellent reliability.

The Pearson's correlation was used in order to determine the relationship between variables. In Table 2, we can see that the age of participants correlated significantly only with spiritual QOL [r = -0.25, $p < 0.05$] (the older the person, the lower the spiritual aspect of QOL). Furthermore, it could be noticed that physical, psychological, social, and general QOL correlated significantly with the majority of other variables, such as depression, anxiety, stress, quality of social support, and indicators of functional status. However, it is important to note that correlation coefficients whose value is less than 0.5 are not of greater clinical significance. Finally, the spiritual aspect of QOL and social support expressed through quantity manifested low and nonsignificant correlations with almost all the above-mentioned variables. Therefore, the latter was not included in the hierarchical regression analysis.

Repeated measures ANOVA showed that the overall difference between the means on four aspects of QOL was statistically significant [$F(2.03, 127.80) = 20.24$, $p < 0.001$, $\eta p^2 = 0.24$]. Furthermore, we were interested in exploring which subscales differ one from another, therefore, the Bonferroni *post hoc* test was performed. The physical QOL was significantly better than all other domains. The psychological aspect was significantly worse than the physical but better than the social segment. The social QOL was significantly lower from both the physical and psychological domain, while the spiritual aspect was worse only compared to the physical QOL. Means for all subscales and the results of pairwise comparisons are shown in Figure 1.

Table 3

Descriptive statistics and reliability coefficients for all scales

Variables	Mean	Standard Deviation	Skewness	Kurtosis	Cronbach's α
Physical QOL	59.61	12.48	-0.51	-0.35	0.72
Psychological QOL	140.05	34.48	-0.32	-0.05	0.88
Social QOL	50.00	13.94	-0.64	0.15	0.71
Spiritual QOL	43.73	12.62	0.00	-0.15	0.64
General QOL	293.39	55.31	-0.52	0.43	0.89
Depression	5.11	4.77	1.06	0.32	0.88
Stress	6.34	4.77	0.63	-0.20	0.89
Anxiety	3.75	3.64	1.06	0.51	0.77
Social support quality	82.17	11.54	-1.71	2.13	0.97
Upper extremity function	59.92	15.84	-0.65	-0.21	0.95

QOL – quality of life.

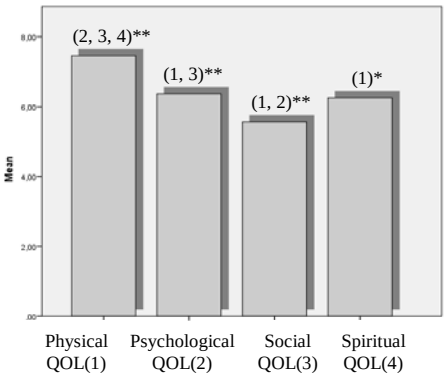

Fig. 1 – Means for physical, psychological, social, and spiritual quality of life (QOL) and results of pairwise comparisons ($p < 0.001$; * $p = 0.01$).**

When examining the marital status, no significant differences were found on physical, psychological, social, or general QOL. However, the Mann-Whitney U-test for independent samples showed that women who had a partner differed significantly on spiritual well-being (Mann-Whitney U = 279.5, $p = 0.04$), achieving better scores (mean rank = 35.42) compared to single women (mean rank = 25.03).

Results of the hierarchical regression analysis presented in Table 4 show that for physical QOL, all steps, except the first one, resulted in statistically significant models. The introduction of upper extremity function in the second step, $F(4.59) = 4.00$, $p < 0.01$, led to statistically significant increase in explained variance [$\Delta R^2 = 0.14$, $p(\Delta F) < 0.01$] with adjuvant chemotherapy ($p = 0.03$) and functional status ($p < 0.01$) as significant predictors. However, with the inclusion of depression, anxiety, and stress symptoms, functional status lost its significance, and adjuvant chemotherapy remained the only relevant predictor ($p = 0.04$). In the last step, a significant increase in explained variance occurred due to the introduction of social support quality [$\Delta R^2 = 0.05$, $p(\Delta F) < 0.05$]. The whole model [$F(8.55) = 3.64$, $p < 0.01$] explains around 35% of criterion variance, with adjuvant chemotherapy ($p = 0.03$) and perceived quality of social support ($p = 0.05$) being the only significant predictors. Our results indicated that women who did not receive adjuvant chemotherapy and had the perception of better social support were more likely to have increased scores in the physical domain of QOL.

As for the psychological aspect of QOL, in Table 4, it can be seen that all steps, except the first one, yield significant models. In the second step, where the introduction of functional status occurred, significant increase in the proportion of explained variance was detected [$\Delta R^2 = 0.20$, $p(\Delta F) < 0.001$] with adjuvant chemotherapy ($p = 0.01$) and upper extremity function ($p < 0.01$) as significant predictors. However, in the next step, when variables related to the affective state were entered, a significant increase in explained variance occurred again [$\Delta R^2 = 0.16$, $p(\Delta F) < 0.01$] with adjuvant chemotherapy ($p < 0.01$) and stress ($p = 0.03$) being now the only significant predictors. No further increase in explained variance was detected with the introduction of perceived quality of social support. The complete model [$F(8.55) = 6.78$, $p < 0.001$] explains around 50% of criterion's variance. Adjuvant chemotherapy ($p < 0.01$) and stress ($p = 0.03$) again turned out to be the only significant predictors. In other words, those women who underwent adjuvant chemotherapy and experienced higher levels of stress are more likely to have worse psychological QOL.

Results of the hierarchical regression analysis for social QOL, which are presented in Table 5, demonstrated again that significant models were obtained in all steps, except in the first one. Statistically significant model [$F(4.59) = 4.81$, $p < 0.01$] and relevant increase in the proportion of explained variance were obtained in the second step [$\Delta R^2 = 0.19$, $p(\Delta F) < 0.001$] when functional status was introduced, with upper extremity function as the only significant predictor ($p < 0.01$). When depression, anxiety and stress were entered [$F(7.56) = 6.25$, $p < 0.001$], another increase in the proportion of explained variance occurred [$\Delta R^2 = 0.19$,

Table 4

Hierarchical regression for physical and psychological quality of life (QOL)

Physical QOL				Psychological QOL		
Model	β	*p*	Predictors	β	*p*	Model
1	0.10	0.44	Neoadjuvant chemotherapy	0.02	0.86	1
F(3, 60) = 1.48	-0.26	0.04	Adjuvant chemotherapy	-0.32	0.01	F(3, 60) = 2.35
$p = 0.23$, $R^2 = 0.07$	0.04	0.77	Comorbid conditions	-0.04	0.74	$p = 0.08$, $R^2 = 0.10$
2	0.06	0.58	Neoadjuvant chemotherapy	-0.02	0.88	2
F(4, 59) = 4.00	-0.26	0.03	Adjuvant chemotherapy	-0.32	0.01	F(4, 59) = 6.40
$p < 0.01$, $R^2 = 0.21$	0.17	0.18	Comorbid conditions	0.11	0.34	$p < 0.001$, $R^2 = 0.30$
$\Delta R^2 = 0.14$	0.40	0.00	Upper extremity function	0.47	0.00	$\Delta R^2 = 0.20$
$p(\Delta F) < 0.01$						$p(\Delta F) < 0.001$
3	0.08	0.48	Neoadjuvant chemotherapy	0.02	0.86	3
F(7, 56) = 3.37	-0.24	0.04	Adjuvant chemotherapy	-0.31	0.00	F(7, 56) = 7.04
$p < 0.01$	0.16	0.21	Comorbid conditions	0.08	0.44	$p < 0.001$
$R^2 = 0.30$	0.21	0.17	Upper extremity function	0.18	0.19	$R^2 = 0.47$
$\Delta R^2 = 0.08$	0.14	0.45	Depression	-0.15	0.34	$\Delta R^2 = 0.16$
$p(\Delta F) = 0.10$	-0.20	0.39	Anxiety	0.02	0.93	$p(\Delta F) < 0.01$
	-0.27	0.20	Stress	-0.39	0.03	
4	0.10	0.36	Neoadjuvant chemotherapy	0.03	0.74	4
F(8, 55) = 3.64	-0.24	0.03	Adjuvant chemotherapy	-0.32	0.00	F(8, 55) = 6.78
$p < 0.01$	0.10	0.40	Comorbid conditions	0.04	0.70	$p < 0.001$
$R^2 = 0.35$	0.18	0.22	Upper extremity function	0.16	0.24	$R^2 = 0.50$
$\Delta R^2 = 0.05$	0.23	0.21	Depression	-0.08	0.62	$\Delta R^2 = 0.03$
$p(\Delta F) < 0.05$	-0.23	0.30	Anxiety	-0.01	0.97	$p(\Delta F) = 0.08$
	-0.26	0.20	Stress	-0.39	0.03	
	0.24	0.05	Social support - quality	0.18	0.08	

Table 5

Hierarchical regression for social and general quality of life (QOL)

Social QOL				General QOL		
Model	β	*p*	Predictors	β	*p*	Model
1	0.11	0.37	Neoadjuvant chemotherapy	0.04	0.72	1
F(3, 60) = 1.19	-0.18	0.15	Adjuvant chemotherapy	-0.27	0.04	F(3, 60) = 1.71
p =0.32, R^2 = 0.06	-0.11	0.40	Comorbid conditions	-0.07	0.59	p = 0.17, R^2 = 0.08
2	0.08	0.50	Neoadjuvant chemotherapy	0.005	0.97	2
F(4, 59) = 4.81	-0.18	0.12	Adjuvant chemotherapy	-0.26	0.02	F(4, 59) = 6.21
p < 0.01, R^2 = 0.25	0.04	0.73	Comorbid conditions	0.09	0.43	p < 0.001, R^2 = 0.30
ΔR^2 = 0.19	0.46	0.00	Upper extremity function	0.49	0.00	ΔR^2 = 0.22
$p(\Delta F)$ < 0.001						$p(\Delta F)$ < 0.001
3	0.07	0.48	Neoadjuvant chemotherapy	0.04	0.72	3
F(7, 56) = 6.25	-0.16	0.12	Adjuvant chemotherapy	-0.25	0.01	F(7, 56) = 6.79
p < 0.001	0.10	0.38	Comorbid conditions	0.07	0.53	p < 0.001
R^2 = 0.44	0.09	0.52	Upper extremity function	0.20	0.15	R^2 = 0.46
ΔR^2 = 0.19	-0.26	0.12	Depression	-0.11	0.51	ΔR^2 = 0.16
$p(\Delta F)$ < 0.01	-0.44	0.03	Anxiety	-0.04	0.86	$p(\Delta F)$ < 0.01
	0.09	0.63	Stress	-0.38	0.04	
4	0.08	0.44	Neoadjuvant chemotherapy	0.06	0.55	4
F(8, 55) = 5.48	-0.16	0.12	Adjuvant chemotherapy	-0.26	0.01	F(8, 55) = 7.16
p < 0.001	0.08	0.48	Comorbid conditions	0.01	0.90	p < 0.001
R^2 = 0.44	0.08	0.56	Upper extremity function	0.17	0.20	R^2 = 0.51
ΔR^2 = 0.005	-0.23	0.18	Depression	-0.01	0.95	ΔR^2 = 0.05
$p(\Delta F)$ = 0.49	-0.46	0.03	Anxiety	-0.07	0.72	$p(\Delta F)$ < 0.05
	0.09	0.63	Stress	-0.38	0.03	
	0.08	0.49	Social support - quality	0.25	0.02	

$p(\Delta F)$ < 0.01]. However, functional status was no longer relevant, and only symptoms of anxiety significantly predicted social QOL (p = 0.03). The introduction of social support in the last step did not lead to the significant increase in variance explained. When all predictors were included [F(8.55) = 5.48, p < 0.001], the model explained around 44% of criterion's variance, with anxiety remaining again the only significant predictor in the model (p = 0.03). In other words, women who experienced stronger symptoms of anxiety were more likely to have diminished social QOL.

For spiritual QOL, no significant models in any of the four steps were obtained.

Finally, hierarchical regression analysis for general QOL (Table 5) yields statistically significant models in all steps, except in the first one. In the second step [F(4.59) = 6.21, p < 0.001], significant increase in the proportion of explained variance occurred [ΔR^2 = 0.22, $p(\Delta F)$ < 0.001] due to the introduction of the functional status, with adjuvant chemotherapy (p = 0.02) and upper extremity function (p < 0.01) as significant predictors. The third step of the analysis brought again the significant increase in the variance explained [ΔR^2 = 0.16, $p(\Delta F)$ < 0.01], where stress (p = 0.04), along with the adjuvant chemotherapy (p = 0.01), turned out to be a statistically significant predictor, while functional status lost its significance. In the last step [F(8.55) = 7.16, p < 0.001], further increase in explained variance occurred due to the introduction of social support [ΔR^2 = 0.05, $p(\Delta F)$ < 0.05], with adjuvant chemotherapy (p = 0.01), stress (p = 0.03), and social support (p = 0.02) being significant predictors. The coefficient of determination indicated that the proportion of explained variance of the criterion was around 51%. Results showed that patients who received adjuvant chemotherapy and experienced high levels of stress and poor social support tend to have diminished general QOL.

Discussion

Since QOL nowadays is one of the core concepts in cancer treatment, a better understanding of factors that may contribute to its improvement is of great importance. Therefore, the main goal of our research was to explore which sets of variables contribute the most to the prediction of various aspects of QOL in breast cancer patients.

Although the process of oncological treatment is very challenging and involves overwhelming subjective reactions to its side effects, our results demonstrated that breast cancer patients, paradoxically, reported that their physical aspect of QOL was significantly better compared to all other domains. Psychological and spiritual aspects were significantly diminished compared to physical QOL. However, the social segment seems to be the most compromised. This finding once again sets light on the well-known fact that the experience of breast cancer diagnosis and its treatment largely exceeds physical level and disrupts not only psychological and spiritual but also social well-being. Perhaps disease-related reactions of family members, available support, personal relationships, sexuality, work, finances, etc. are the most threatened segments in our socio-cultural background that frequently remain in the shadow of concern about the physical health of the patients. This finding points to the fact that our healthcare system still focuses the most on the physical aspects of the disease, while other domains might remain overlooked, meaning that additional changes are needed in the direction of the holistic approach to breast cancer treatment. The scarce outcome in

the social aspect of QOL might also be culturally-specific and it might be the result of inadequate information. Unfortunately, it might also be the result of prejudices that are still widespread about malignant diseases in our society, as well as of yet traditionally burdened cultural context in which women live, and the specific socio-economic situation in this region.

Our results demonstrated that, at the time of assessment which took part during the radiation therapy, the previously received adjuvant chemotherapy was an important determinant of physical, psychological, and general QOL, manifesting the reverse relationship with QOL indicators. This finding is pretty much expected, and it goes in line with both previous empirical results [40, 41] and generally known subjective reports of patients about their experiences during the treatment. Chemotherapy may lead to treatment-related somatic symptoms and changes in body image, resulting in adverse relations with different aspects of QOL. Although it was expected that neoadjuvant chemotherapy and additional health problems would affect the quality of patients' life, our results demonstrated that only adjuvant chemotherapy was related to QOL measures, at least in this period of assessment. It may be that recently experienced adverse chemotherapy effects mask previously felt discomfort. However, clinical factors included in the analysis were never sufficient by themselves to get a significant prediction of any QOL aspect.

When it comes to the contribution of functional status, our findings indicate that its inclusion always led to significant models and an increase in the proportion of explained variance. The better the upper limb function, the better physical, psychological, social, and general QOL is. These findings are strongly in accordance with the previous literature [42, 43], knowing that upper extremity disfunction and related limitations (with or without lymphedema) are one of the most limiting complications following breast cancer treatment. At first sight, it seems that functional status together with information about received adjuvant chemotherapy may constitute a satisfying model for the prediction of different aspects of QOL. Nevertheless, whenever symptoms of depression, anxiety, and stress were introduced, functional status lost its predictive significance, indicating that the emotional state of participants might be more powerful in predicting QOL in breast cancer. However, this could also be the result of the fact that the impaired affective state of patients is strongly interrelated to (or even possibly the consequence of) the compromised function of upper limbs, and consequently, restricted daily activities.

Our findings demonstrated that variables related to the emotional state of participants significantly predicted psychological, social, and general QOL, over and above clinical and functional factors. Heightened levels of distress during the treatment predict the worst psychological and general QOL, while symptoms of anxiety are significant in predicting poor social well-being. The notion that symptoms of distress or affective disorders have the reverse relationship with different indicators of QOL is anticipated and goes in line with previous literature [44-47]. It is expected for symptoms of anxiety and distress to intensify in transition times of diagnosis and treatment course, coexisting with decreased QOL indicators [48]. Nevertheless, from our results, it can be seen that symptoms of depression did not significantly predict any of the QOL measures, which appears to be unanticipated at first glance. However, if we notice that the majority of QOL-BC items, especially those contained in the psychological, and partially social subscale, are focused on (health) anxiety, fear (of recurrence), stress, and sense of control, it is reasonable that symptoms of anxiety and stress would be dominant predictors of QOL. Another less expected finding is that anxiety symptoms are the only significant predictors of social QOL, while perceived social support is not. It is known that an increased level of anxiety can affect individuals' social and work functioning [49, 50]. Moreover, it is possible that anxiety is strongly bonded to the social subscale of QOL-BC, whose items are aimed at worries related to finances, disrupted activities at home and work, compromised sexuality, concern for the health of close family members, etc. Probably all those facets of the social aspect of QOL are more saturated with uncertainty (which is the core component of vulnerability to anxiety) than with the quality of social support measured with MOS-SSS.

Another less anticipated result is that perceived quality of social support significantly predicts physical and general QOL, but not psychological and social aspects, which would be more expected. Quality of social support might be important for physical QOL, probably due to emotional/informative and instrumental/practical support, which could be crucial for coping with physical symptoms, difficulties, and limitations. An additional important observation is that, at least in this period of assessment, social support matters more in explaining the physical QOL than the presence of functional complications, or some clinical factors such as, for example, the existence of comorbidity. The earlier research has already shown that social support is substantial for health-related QOL in breast cancer patients [23, 26]. Thus, one longitudinal study indicated that positive emotional and informational support provided by a partner is especially important in maintaining health-related QOL [51]. Our results showed not only that social support is relevant for physical and general QOL but also that it is quality, not quantity of support that matters, which is in accordance with previous findings [25].

In earlier studies, a significant relationship had been found between religiosity/spirituality and better physical and functional status, reduced symptoms of psychopathology, greater emotional well-being, and improved social support [52–55]. However, our study did not show such results. No significant correlations between the spiritual aspect of QOL and levels of stress, anxiety, depression, social support (quantity and quality), and functional status were detected. What's more, from all QOL-BC subscales, the spiritual aspect manifests the lowest correlation with the total score and no significant correlations with the other three domains, meaning that it might be somehow the most distinctive construct. It also seems that clinical, functional, affective, and social factors analyzed in our study are not so prominent

in explaining spiritual QOL in breast cancer. However, in review on spirituality and well-being of cancer patients, Visser et al. [56] point out that although most of the studies report positive relation between spirituality and well-being, the majority of them utilized spirituality questionnaires which contained items related to well-being itself, possibly leading to an artificial increase in the observed relationship. The spirituality subscale of QOL-BC does not contain related items to other well-being domains, so this could be one of the plausible explanations for low and nonsignificant correlations with other examined variables. On the other hand, as spirituality is a multidimensional concept, its different aspects (i.e., sense of meaning in life and active religious practices) may correlate differently with other physical and psychosocial variables. And yet, our research showed that age and marital status could be relevant when it comes to the spiritual aspect of QOL. Obtained results indicate that older patients and those who are single manifest lower spiritual QOL, which is inconsistent with previous studies showing that younger age is a risk factor for poor QOL in breast cancer patients [5-7]. It is probable that the process of aging, loneliness, possible loss of a partner (a significant part of single women in our sample were widowed) and dependence, bring to the foreground existential issues and loss of meaning, with which patients have difficulty coping. Hence, those women undergoing breast cancer treatment who are older and single are especially vulnerable in the context of uncertainty, hope, the meaning of life, and the capacity to grow after the crisis. These results could help target and include vulnerable patients in supportive programs for fostering spiritual well-being, as an important resource for adjustment and healing [57].

Given that our study is cross-sectional, the observation of temporal changes in QOL during and after the treatment would be recommended for future research. Therefore, the longitudinal design with a larger sample and more complex statistical approach is highly desirable in order to overcome some of the disadvantages of our study. Moreover, the inclusion of a wider range of variables, such as coping mechanisms, doctor-patient communication, type of surgical intervention, etc., would contribute to a more sophisticated understanding of this topic. Our results generally support the notion already detected in other studies that psychological and social resources are more important in predicting different aspects of quality of life in breast cancer patients, compared to clinical, demographical, and functional factors [20, 58].

Conclusion

While adjuvant chemotherapy turned out to be a powerful predictor of the physical, psychological, and general quality of life, clinical factors by themselves could not explain the quality of life outcomes, whilst functional status always lost its significance with the inclusion of psychological and social support measures, indicating that the latter are superior in determining different quality of life domains. At the same time, the social, psychological, and spiritual well-being of our patients is significantly worse compared to the physical quality of life, meaning that there is still much left to be done in the process of moving the focus from a purely somatic to a holistic approach in the treatment of breast cancer.

Additionally, it seems that for different aspects of patients' well-being during the treatment, training in coping skills, with a focus on reducing symptoms of distress and affective disorders, as well as fostering social and communicational skills, would be a target of highest priority for psycho-oncologists.

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Adverse drug reactions associated with sunitinib therapy: characteristics and risk factors

Neželjena dejstva sunitiniba: karakteristike i faktori rizika

Snežana Mugoša*†, Zoran Džamić‡, Majda Šahman-Zaimović*†, Nevenka Lukovac-Janjić§

University of Montenegro, *Faculty of Medicine, §Institute for Oncology and Radiotherapy, Podgorica, Montenegro; †Institute for Medicines and Medical Devices, Podgorica, Montenegro; ‡University of Belgrade, Faculty of Medicine, Clinic for Urology, Belgrade, Serbia

Abstract

Background/Aim. Kidney tumors account for 2–3% of all tumors. Renal cell carcinoma (RCC) is the tenth most common malignancy. Sunitinib is used as the first treatment line in patients with a good and intermediate prognosis. The aim of this study was to analyze the risk factors, frequency, and adverse drug reactions (ADRs) of sunitinib in patients with metastatic RCC. **Methods.** The retrospective study included 170 patients treated at the Clinic for Oncology of the Clinical Center of Montenegro, Urology Clinic of the Clinical Center of Serbia, and Clinic for Oncology of the Clinical Center Niš. As a data source, we used patient medical histories and/or electronic patient records. ADRs were characterized by using Rawlins and Thompson classification. Each ADRs severity was assessed in accordance with the World Health Organization criteria. Causality was assessed using the Naranjo probability scale. **Results.** ADRs of sunitinib occurred in 152 (89.4%) patients. ADRs were 89% type A and 11% type C. Disorders of the blood and lymphatic system, gastrointestinal disorders, and disorders of the skin and subcutaneous tissue were the most common manifestations of ADRs of sunitinib. Causality assessment was most commonly classified as certain (60%). Serious ADRs occurred in 4.5% of patients. Most patients recovered without consequences. The most common manifestations of ADRs were: leukopenia, hypothyroidism, thrombocytopenia, diarrhea, stomatitis, asthenia, and hypertension. All ADRs were expected. The number of concomitant medications and the duration of therapy proved to be the most significant risk factors for ADR to sunitinib. **Conclusion.** Our study shows that the incidence of ADRs of sunitinib in patients with kidney cancer is high. The ADRs were mostly moderate and mild in intensity and occurred as a consequence of the pharmacological action of the drug. It is necessary to conduct continuous education of medical oncologists in the field of monitoring safe drug use, as well as patients on sunitinib therapy, in order to improve their awareness of the sunitinib ADRs and the risk factors that lead to them, with the aim of reducing their frequency.

Key words:
drug-related side effects and adverse reactions; kidney neoplasms; sunitinib.

Apstrakt

Uvod/Cilj. Tumori bubrega čine 2–3% svih tumora. Karcinom bubrežnih ćelija nalazi se na desetom mestu najčešćih maligniteta. Kao prva terapijska linija kod bolesnika sa dobrom i intermedijarnom prognozom koristi se sunitinib. Cilj rada bio je analiza faktora rizika, učestalosti ispoljavljivanja i karakteristika neželjenih dejstava sunitiniba kod bolesnika sa metastatskim karcinomom bubrega. **Metode.** Retrospektivnom studijom je bilo obuhvaćeno 170 bolesnika lečenih na Klinici za onkologiju Kliničkog centra Crne Gore, Urološkoj klinici Kliničkog centra Srbije i Klinici za onkologiju Kliničkog centra Niš. Kao izvor podataka koristili smo istorije bolesti i/ili elektronske kartone bolesnika. Neželjena dejstva su klasifikovana prema *Rawlins and Thompson* klasifikaciji, težina prema kriterijumima Svetske zdravstvene organizacije, a uzročno-posledična povezanost korišćenjem Naranjo skale. **Rezultati.** Neželjena dejstva sunitiniba ispoljila su se kod 152 bolesnika (89,4%). Neželjena dejstva tipa A ispoljila su se kod 89%, a tipa C kod 11% bolesnika. Najčešće su se ispoljili poremećaji krvi i limfnog sistema, gastrointestinalni poremećaji i poremećaji kože i potkožnog tkiva. Uzročno-posledična povezanost između leka i neželjenog dejstva najčešće je klasifikovana kao sigurna (60%). Značajna neželjena dejstva imalo je 4,5% bolesnika. Većina bolesnika se oporavila bez posledica. Najčešća neželjena dejstva bila su: leukopenija, hipotireoza, trombocitopenija, dijareja, stomatitis, astenija i hipertenzija. Sva zabeležena neželjena dejstva bila su očekivana. Najznačajniji faktori rizika od

Correspondence to: Snežana Mugoša, Institute for Medicines and Medical Devices of Montenegro, Bulevar Ivana Crnojevica 64A, 81 000 Podgorica, Montenegro. Email: snezana.mugosa@cinmed.me

nastanka neželjenih dejstava sunitiniba bila su broj istovremeno korišćenih lekova i trajanje terapije. **Zaključak.** Naše istraživanje pokazuje da je učestalost neželjenih dejstava sunitiniba kod bolesnika sa karcinomom bubrega visoka. Neželjena dejstva su uglavnom bila umerena i laka po intenzitetu i nastala su kao posledica farmakološkog dejstva leka. Potrebno je sprovesti dodatnu edukaciju medikalnih onkologa iz oblasti praćenja bezbedne primene lekova, a takođe i bolesnika koji su na terapiji sunitinibom, sa ciljem unapređenja njihove informisanosti o neželjenim dejstvima sunitiniba i faktorima rizika koji do njih dovode, kako bi se njihova učestalost smanjila.

Ključne reči:
lekovi, neželjeni efekti i neželjene reakcije; bubreg, neoplazme; sunitinib.

Introduction

A significant increase in the incidence of renal cell carcinoma (RCC) has been observed in the last 50 years, including cancers detected at an early stage of the disease, which is explained by the increasing use and improvement of diagnostic procedures, as well as the increasing impact of the growing presence of risk factors such as smoking, obesity, and hypertension [1]. Sunitinib, an oral multitargeted tyrosine kinase inhibitor, is used as the first-line treatment in patients with a good and intermediate prognosis, while patients with a poor prognosis are treated with temsirolimus. The therapeutic success of this drug depends on the three most important factors: the dosage of the drug, the length of therapy, and the adverse drug reactions (ADRs) that the drugs cause [2]. The most serious adverse reactions (ADRs) associated with sunitinib, some with fatal outcomes, are renal failure, heart failure, pulmonary embolism, gastrointestinal perforation, and hemorrhages [3]. The most common ADRs (≥1/10) of any grade included decreased appetite, taste disturbance, hypertension, fatigue, gastrointestinal disorders, skin discolouration, and palmar-plantar erythrodysesthesia syndrome. These ADRs are usually expected to decrease during the treatment.

However, there are ADRs that require additional management due to the metabolic pathway of the sunitinib (by cytochrome P450 3A4) and its pharmacological and toxicological characteristics. Furthermore, sunitinib is intended for long-term use. Therefore, it is very important to consider any problems related to ADRs of the drug that could, among other things, be the reason for the inevitable discontinuation of the drug and adversely affect the comfort of patients during treatment.

The aim of this study was to establish the criteria for detection of ADRs of sunitinib, to analyze these ADRs and risk factors for their development in order to provide recommendations for their prevention, and thus to ensure optimal benefit from sunitinib treatment.

Methods

Study design and patients selection

The retrospective study included 170 patients treated at the Clinic for Oncology of the Clinical Center of Montenegro, Urology Clinic of the Clinical Center of Serbia, and Clinic for Oncology of the Clinical Center Niš during the six-month period, from April to October 2018.

Inclusion criteria were the following: patients of both sexes with metastatic RCC treated with sunitinib in first-line therapy, performance status 0–2. Severely ill patients with performance status > 2 were excluded from the study.

As a data source, we used patient medical histories and/or electronic patient records.

At the very beginning of sunitinib therapy, the expected ADRs were explained to patients. Patients usually had check-ups with a medical oncologist at intervals of 15 days and more often if necessary. Each time a medical report was written. The report contained information about the problems reported by a patient, e.g. skin changes, changes in mucous membranes, headache, etc., and also information about other ADRs noted by the medical oncologist, based on available laboratory and other parameters (e.g. thrombocytopenia, leukopenia, hypothyroidism).

Data on the demographic characteristics of patients, underlying disease, therapy, laboratory, and other available data were entered into the computer database.

Definition and classification of ADRs

Definition of ADRs according to the World Health Organization (WHO) was used in this research [4].

ADRs were characterized by using Rawlins and Thompson [5] classification. Each ADR severity was assessed in accordance with the WHO criteria [4]. The causality relationship between the drug and the effect was established using Naranjo's ADR probability scale [6]. ADRs were classified by criteria suggested by Meyboom et al. [7] as type A ("drug actions"), type B ("patients reactions"), and type C ("statistical").

In addition, the level of intervention was attributed, using a four-level scale: Level 1 – no change in the treatment; Level 2 – dose adjustment or drug stop, no additional treatment required; Level 3 – dose adjustment or drug stop, additional treatment required; Level 4 – transfer to intensive care unit [8]. Each ADR was also classified according to the system organ class, according to the Medical Dictionary for Regulatory Activities (MedDRA) classification of ADRs, as recommended by the WHO [9].

Statistical analysis

Data contained in medical histories and patient records, indicating possible ADRs of sunitinib, were entered into a computer database. Descriptive statistical methods (arithmetic mean, median, standard deviation) and methods for testing statistical hypotheses (*t*-test, Mann-Whitney test, χ^2-test, and Fisher's test of exact probability) were used for the analysis of primary data. Statistical hypotheses were tested at the level of statistical significance (alpha level) of 0.05.

Results

The study included 170 respondents who received sunitinib, 97 (57.1%) from Belgrade, 44 (25.9%) from Podgorica, and 29 (17.1%) from Niš.

The mean age of all subjects in the study was 61.8 ± 9.2 years. The youngest respondent was 24 and the oldest 84 years old. Out of all respondents included in the study, 70.6% were male and 29.4% were female.

Adverse drug reactions of sunitinib occurred in 152 (89.4%) patients (Table 1).

Adverse reactions were present in 84.5% of patients from Belgrade, 97.7% from Podgorica, and 93.1% from Niš. There was a statistically significant difference in the frequency of ADRs in relation to the city (accurate probability test; $p = 0.043$).

The total number of ADRs was 467. Table 2 shows the characteristics of sunitinib ADRs.

The most common certain ADRs were haematological toxicity (leukopenia, thrombocytopenia, and anemia), as well as gastrointestinal system disorders (nausea, diarrhea). The most common probable ADRs were general disorders (asthenia, malaise, myalgia) and endocrine system disorders (primarily hypothyroidism). The most common possible ADRs were loss of appetite, hypertension, headache, and epistaxis.

Serious ADRs, which occurred in 4.5% of patients, included severe skin reactions and severe forms of diarrhea. One patient died due to a possible ADR of sunitinib (renal failure characterized as a possible ADR).

Table 1

Demographic and clinical data of the patients included in the study

Data	Patients without ADRs	Patients with ADRs
	n = 18	n = 152
Age (years), mean ± SD	62.0 ± 7.9	62.5 ± 9.3
Sex, n (%)		
male	14 (77.8)	106 (69.7)
female	4 (22.2)	46 (30.3)
Occupation, n (%)		
employed	2 (11.1)	44 (28.94)
unemployed	7 (38.9)	52 (34.2)
retired	9 (50.0)	56 (36.8)
Education level, n (%)		
elementary	7 (63.6)	4 (36.4)
college	87 (66.9)	43 (33.1)
undergraduate	19 (59.4)	13 (40.6)
graduate	18 (69.2)	8 (30.8)
Comorbidities, n (%)		
endocrine system	2 (13.3)	26 (18.1)
central nervous system	0 (0)	4 (2.8)
gastrointestinal system	0 (0)	4 (2.8)
respiratory system	1 (6.7)	2 (1.4)
cardiovascular system	6 (40)	86 (59.3)
Risk factors for RCC, n (%)		
smoking	11 (100)	82 (65.1)
malignancy history	4 (50)	21 (26.3)
abuse of analgesics	0 (0)	0 (0)
chronic kidney disease	1 (5.6)	7 (4.7)
Disease onset, n (%)		
hematuria	4 (36.4)	41 (35)
back pain	3 (27.3)	17 (14.5)
without difficulty, by accident	2 (18.2)	39 (33.3)
other	2 (18.2)	20 (17.1)
Prevalence of metastases, n (%)		
initially metastatic disease	2 (11.1)	38 (25)
more than 2 metastatic sieves	13 (72.2)	102 (67.1)
Number of drugs, mean ± SD	4.9 ± 1.6	2.1 ± 1.1
Duration of therapy (months), mean ± SD	3.9 ± 2.5	7.4 ± 5.4

ADRs – adverse drug reactions; RCC – renal cell carcinoma; SD – standard deviation.

Table 2

Characteristics of detected adverse drug reactions (ADRs) associated with sunitinib

Characteristics of ADRs	ADRs, n (%)
Type	
A	416 (89.1)
B	0 (0.0)
C	51 (10.9)
Causality	
certain	276 (59.1)
probable	84 (18.0)
possible	98 (21.0)
Level of intervention	
level 1 (no change in dose)	416 (89.1)
level 2 (dose changed or drug stopped)	9 (1.9)
level 3 (drug stopped + additional therapy)	39 (8.0)
level 4 (transfer to intensive care unit)	3 (1)
Severity	
serious	21 (4.5)
non serious	446 (95.5)
Outcome	
death	1 (0.2)
recovery with consequences	4 (0.9)
recovery without consequences	462 (98.9)
Reported by	
patient	234 (50.1)
treating physician	233 (49.9)

The most common manifestations of ADRs were: leukopenia (40%), hypothyroidism (34%), thrombocytopenia (31%), diarrhea (20%), stomatitis (17%), asthenia (17%), and hypertension (16%).

Grades 1–2 ADRs were the most frequent. The frequency of grades 3 and 4 toxicities was relatively low (< 10%).

All ADRs were expected (as described in the Summary of Product Characteristics).

Table 3 shows the prevalence of involved organic systems where ADRs occurred, according to the MedDRA classification.

Table 3

Presentation of adverse drug reactions (ADRs) in different organ systems

Organ system disorders	ADRs, n (%)
Disorders of the blood and lymphatic system	123 (26.3)
Nervous system disorders	18 (3.9)
Gastrointestinal disorders	98 (21)
Respiratory. thoracic and mediastinal disorders	11 (2.4)
Musculoskeletal and connective tissue disorders	9 (1.9)
Eye disorders	9 (1.9)
Endocrine disorders	52 (11.1)
Vascular disorders	25 (5.4)
Skin and subcutaneous tissue disorders	61 (13.1)
General disorders and administration site conditions	35 (7.5)
Laboratory tests	24 (5.1)
Other	2 (0.4)
Total	467 (100)

Discussion

The number of studies where the frequency of adverse reactions to sunitinib was monitored and analyzed is scarce. In our study, we have found that the incidence of sunitinib ADRs in patients treated for RCC was 89%. This data shows that the frequency of ADRs in our study was slightly higher compared to the other studies in which the frequency of ADRs of this drug was about 80% [10]. In a study that comprised 1,073 patients receiving sunitinib, the incidence of ADRs was 82.1% [10].

There are several reasons for such a high incidence of sunitinib ADRs: the different incidence of ADRs in literature can be explained by differences in methodology, the definition of ADRs, classification, algorithms for causality assessment of ADRs, etc. [11]; we have included "possible" ADRs in the total frequency of ADRs, unlike, e.g., some authors [12, 13] who listed only “certain” and “probable” ADRs, thus we may have included some false-positive results; all potential ADRs listed in the Summary of Characteristics of sunitinib were checked, all data contained in medical histories and temperature lists were used, including

laboratory findings, X-ray examinations, ECG, etc.; the population of patients with RCC is comprised mainly of elderly patients, with frequent comorbidities. Numerous previous studies have shown that both age and comorbidity affect pharmacokinetics, i.e., resorption, distribution, metabolism, and excretion of drugs from the body, which makes these patients more sensitive to the occurrence of ADRs [14–18]. The population of the patients included in the study generally receive a large number of drugs at the same time, which turned out to be a significant risk factor for the occurrence of ADRs. In a study of 9,000 Italian patients, mostly over the age of 60, Carbonin et al. [19] showed that the incidence of ADRs increased from 1.2% in patients receiving one drug to 10% in those receiving nine drugs, and to about 50% in patients receiving more than 10 drugs.

Numerous studies [20–24] have shown that the female sex is a risk factor for the occurrence of ADRs, although there is no reliable explanation for this in the literature. Some authors believe that lower body weight and surface area and degree of glomerular filtration, as well as higher fat content, are the reason for the higher frequency of ADRs in the female population [25, 26]. In our study, we did not obtain a statistically significantly higher incidence of sunitinib ADRs in female patients.

When it comes to the causality assessment of ADRs, we obtained the highest prevalence of "certain" ADRs in our study, which differs significantly from the data obtained in similar studies [24, 27–29]. In some studies [27, 29], over 50% of the reported adverse reactions were classified as “possible” and less than 10% as “certain”. In contrast, Classen et al. [20] describes 62% of "certain" ADRs and 0.7% of "probable" ADRs. The reason for the high prevalence of "certain" ADRs in our study stems from the definition of "certain" ADRs, which is that the relationship between the drug and the resulting symptoms and/or signs is established with certainty only if identical clinical and/or laboratory finding occurs on re-exposure to a drug (drug rechallenge). Given that the most common adverse reactions were hematological toxicity (leukopenia, thrombocytopenia, and anemia), as well as gastrointestinal system disorders (nausea, diarrhea) and that these adverse reactions recurred in each cycle of chemotherapy, it is clear that they were classified as "certain" ADRs.

ADRs were 89% type A and 11% type C in our study, which was in accordance with the data obtained by Classen et al. [20]. Given the mechanism of occurrence of these types of ADRs, the prevalence we obtained was expected. In some studies, however, type B reactions accounted for one-third of registered ADRs [30, 31]. Adverse reactions observed with intensive monitoring most often manifested as disorders at the level of the blood and lymphatic system, gastrointestinal disorders, skin and subcutaneous tissue disorders, and endocrine disorders, which is in line with the safety profile of sunitinib [3].

In our study, the data showed that 50% of patients themselves notice the ADRs of sunitinib and report it to their medical oncologist, while the remaining 50% of ADRs are recognized by the oncologist. Numerous studies on informing patients about the ADRs of the drug they take say that additional measures are needed to improve patient awareness, with the aim of accomplishing better compliance and reducing the risk of ADRs [32–34].

Many studies have shown that the percentage of preventable ADRs is high and ranges over 50% [35–39]. This can be achieved by the following methods: knowing the pharmacological characteristics of the drug (including pharmacokinetic and pharmacodynamic properties), as well as the profile of ADRs that the drug can cause, understanding drug interactions when using multiple drugs, avoiding prescribing drugs with the same or similar ADRs profile, dosing drug according to age, body weight, and organ function, medical history taking, which includes pharmacological history, systematic monitoring of ADRs, patients full awareness about all potential ADRs, and precautions when using the drug [40].

The main limitation of the study was the small number of patients in the group without ADRs compared to the other group of patients with ADRs, which makes a large difference in the size of the groups. This implies the necessity to continue this research with more patients in order to increase the power of the study.

Conclusion

Our study shows that the incidence of ADRs of sunitinib in patients with kidney cancer is high. All reported ADRs were expected and described in the Summary of Product Characteristics. The ADRs were mostly moderate and mild in intensity and occurred as a consequence of the pharmacological action of the drug. A lower percentage of ADRs occurred as a result of long-term exposure to the drug. It is necessary to conduct continuous education of medical oncologists in the field of the safe use of drugs monitoring, as well as patients on sunitinib therapy, in order to improve their awareness of the ADRs of sunitinib and the risk factors that could lead to ADRs occurrence in order to reduce their frequency.

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Frailty in family practice/primary health care: Care and management

Sindrom senilne astenije u praksi porodičnog lekara/primarnoj zdravstvenoj zaštiti: nega i upravljanje

Hakan Yaman

Independent scholar, (*former* Akdeniz University, Faculty of Medicine, Department of Family Medicine, Antalya, Turkey), Antalya, Turkey

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Ključne reči:
krhkost; stare osobe, 80 i više godina; primarna zdravstvena zaštita; zdravstvena zaštita, organizacija.

Introduction

Health systems in different parts of the world are waiting for a troubled future due to the rapid ageing of the population. Health systems which are traditionally designed to care for acute health problems are forced to cover problems of co-morbidity with reduced physical and mental functions. The Frailty syndrome is now challenging health care providers like other health problems of ageing populations, which mainly cannot be treated curatively [1, 2].

Studies on the operational definition of frailty continue, and the balance between physical, psychological, social, and environmental factors and the health deterioration of ageing people are considered [3–7].

The loss of physiological reserves and decrease of resistance to stressors leads to frailty. Even a small health problem can trigger a deterioration of the inner balance of the organism [2]. Complaints like involuntary body weight loss, weakness, tiredness, slowness, walking speed reduction, inactivity, and balance problems do frequently accompany physical frailty [5–7]. Frailty also affects other dimensions of the human being. The mental domain can present mental frailty with cognitive, mood or motivational consequences. Cognitive frailty should be distinguished from physical frailty and dementia [5, 8, 9]. Social frailty depends on the loss of social or general resources, self-care skills, and social behaviours and activities that an individual needs [5, 10, 11].

Frailty generally increases with age. Women are more affected than men, but men are dying more of this condition [12–14]. Lower education, poverty, chronic diseases, and poor health status is more common in frail elderly [15, 16]. Further, loss of appetite, anorexia, smoking, low alcohol consumption, chronic disease, excessive low and high body mass index are associated with frailty [3].

The prevalence of frailty shows different results [17, 18]. According to a systematic review of 21 cohorts and about 61,500 individuals published in 2012, the prevalence was 10.7% (ranging from 4.0% to 59.1%) according to studies [12].

Frailty is now accepted as a geriatric syndrome [3], which has become an essential problem for health services. Unnecessary hospital admissions, emergency service visits, and intensive care units lead to overutilization of health and social care resources [5, 7]. Weak health infrastructure, rehabilitation, and geriatric clinical services in developing environments are enforcing this problem. Early diagnosis and proactive management of frailty are warranted to relieve the burden of this condition to the individual, their family, community, and health system [19–21]. Family physicians are in a unique position to contribute to the management of this increasing problem in the community. Besides the attributes as health leaders in the community, they have the opportunity to scan other members of the family and assess dimensions such as social frailty. Additionally, they will assist patients and their caregivers in supporting and guiding them through the health system [3].

Besides dozens of developed Frailty scales to diagnose Frailty in community, hospital, and research setting, two main criteria exist in literature: the Phenotype of Frailty (frailty phenotype; FP) and Frailty Index (FI) models. First of them is predominantly physical (involuntary body weight loss, exhaustion, slow walking speed, weak handgrip strength, and low physical activity), while the other one is

Correspondence to: Hakan Yaman, Uncalı Mh. 1262. Sk. No: 15, Oksuzoglu Konakları, A Blok Kat: 2 Daire 5, Konyaaltı, 07070 Antalya-Turkey. Email: hakanyam@yahoo.com

based on the concept of frailty due to multiple diseases (accumulation of deficits due to time) [15, 18].

Since a single frailty assessment tool if reliable and valid could measure only the domain of Frailty, other domains of ageing-related problems need to evaluated with a comprehensive geriatric assessment tool. Data retrieved from these examinations will enable health care providers in the individual's health planning process [2, 3, 6, 18, 22].

A smooth, practical, and short, comprehensive geriatric evaluation toolkit is preferred under this circumstances, and the Aging-Friendly Primary Health Care Toolkit developed by the World Health Organization might be a right choice in this situation [20, 23, 24].

Looking deeper into the five criteria of FP shows walking speed to be highly predictive for frailty. Walking speed predicts chronic disability, length of stay in long-term care institution, falls, and survival [3, 25]. However, in order to increase sensitivity and selectivity, the combination of walking speed and handgrip strength has been reported as the most sensitive and selective combination [3]. Two measures are likely to be readily applicable in family medicine. The United Kingdom (UK) Geriatric Society (BGS) recommended a rapid gait test (4 meter), time-dependent stand-up and go test or PRISMA-7 questionnaire (sensitivities of 83%, 93% and 99%, and specificities of 83%, 62% and 64%, respectively) to detect candidates for comprehensive geriatric assessment [2, 5, 26–28]. Despite the limitations of the scales, a systematic review published in 2012 recommends Tilburg Frailty Indicator and SHARE-FI [5, 29] for use in the family medicine environment. The FI instrument is recommended for research matters [5, 30, 31]. In the last few years, the electronic Frailty Index (eFI), which has been tested in family medicine in the UK and has been working on electronic health records, is seen as promising [27].

To trigger the screening process, the evaluation of risk factors for frailty will be more efficient (i.e. advanced age, gender, socioeconomic status, educational level, functional disability, multiple drug use, nutritional problems, weight loss, presence of co-morbidity). In case of a suspicious risk factor walking speed, handgrip strength test, stand-up and go test or PRISMA-7 can be applied for further screening [26, 28]. Any further positive screening is an indication for referral to a geriatric team or a community-based geriatric care service, which coordinates and provides evidence-based interventions to the frail patient [3, 5, 32]. In low-resource settings and emerging health systems with fast ageing populations, the family physician with available resources has to cover the management need of frail patients. They will benefit by the utilisation of a short geriatric assessment tool, recommending preventive measures (ie. vaccines and chemoprophylactic agents), and intervening with evidence-based interventions against frailty manage these conditions [23, 24].

Evidence-based interventions against frailty

Physical activity and exercise are beneficial for frailty. Nine of 14 interventional community-based studies decreased frailty in a systematic review. Most components of physical activity (i.e., strength, balance, coordination, flexibility and endurance exercises) and their combination with each other and prehabilitation interventions were found to be effective [33, 34]. Systematic reviews and meta-analyses evaluating multicomponent physical activities showed positive effects on frailty [33–36], physical functioning and daily activities of living [37–40] and reduction on falls by improving walking, balance, and strength [39, 41]. Positive effects of physical activity on cognition, emotion and social network were found [42, 43]. Physical exercise provides improvements in mobility, balance, functional capacity and frailty [36].

Multi-component training programs, which last 30–45 minutes a day and are practised not less than five months and three days a week, are recommended for individuals with health problems. Concerning the intensity, an exercise carried out 2–3 times a week and lasting for at least 3–6 months are effective [6]. However, this needs to be validated with high-quality studies with a valid definition of frailty [44]. Exercise interventions made as a group are reported to be better, and the combination of exercise and nutrition interventions are also useful. However, further studies are needed [45].

There is a relationship between nutrition and nutrients in frailty. Carotenoid and vitamin-rich foods, as well as sufficient protein intake, have been evaluated. If healthy people, a protein intake of 25–30 g per meal or daily, 1.2 g / kg body weight are recommended [46]. Protein consumption is vital in elderly individuals, and inadequate protein intake causes loss of muscle, strength and bone mass, which contributes to the development of sarcopenia and frailty [47]. But this is not the case for middle-aged individuals; protein intake should be restricted to combat cancer [48]. In a systematic review covering 5,615 subjects in 30 randomised controlled trials, vitamin D supplementation (25 [OH] D) also provided a slight significant increase in general strength. The intake of 25 [OH] D in people over 65 years of age with a blood value below 30 nmol/L is recommended [49]. A target of at least 75 nmol/L is recommended to increase calcium absorption and enable healthy functioning of the parathyroid gland [50]. Positive effects of creatine on muscle ageing were observed with strength studies. Creatine increases lean body mass and was especially beneficial in the development of upper and lower body strength. Due to the increase in energy stores, the strength exercises are carried out with a higher volume, and a higher level of adaptation is achieved. It is expressed that it contributes to protein synthesis with the osmotic effects it has created. Also, it is believed that it reduces oxidative stress and inflammation. In light of these benefits, it is recommended to add creatine to the strength exercises [51, 52].

Social support

Advancing stages of frailty and the existence of social dependence call for the evaluation of social support. A clear analysis of the family, caregiving condition, mobility, and dependency needs to be clarified. Advanced care directives should be discussed with patients with an expected restriction in later life (i.e., dementia, cancer, etc.). Other discussions with

the patient and with the relatives may include care and caregiver requirements, self-care issues, support institutions [5, 53] and caregivers health needs. Polypharmacy is frequently observed in individuals with frailty. The issue of inadequate drug utilisation, drug interactions or adverse effects needs to be addressed. The screening tool for the prescribing of elderly individuals (STOPP), the screening tool for the right treatment (START) and the Beers criteria are recommended to address these issues [54–56].

Care models of frailty

The transfer and adoption of the successful interventions offered to frail individuals require intensive efforts. There are many models implemented for this. Here are some of the models that are tested or implemented to the extent possible by countries.

Integrated care

Health services are traditionally designed to address some acute health problems. The increase in the number of multiple diseases in a single person or the lack of curative treatment will complicate the care process. In this case, the integration of fragmented service structures should be increased in order to guide the patient through the health system. The integration is a process of combination of social and health services with elderly patients. An interdisciplinary team serving here is associated with financial, administrative and clinical care. Thus, it is aimed to solve all procedures related to the patient under the same roof and at the same institution [57, 58]. Two services for frailty are PRISMA and SIPA, which are integrating the complex health care process of frail people [59].

Shared care

This model provides close cooperation between the family physician and the specialist consultant. It is expected that the relevant clinician providing the consultation will be close to primary care. In Canada, traditionally, cooperation in mental health in family medicine can be an example of shared care [60]. In Canada, a shared care service for geriatric care in family medicine was mentioned in a study [61].

Home care service

There are studies conducted to maintain the care of the patients at home. We provide services to resolve acute and subacute health problems at home. There are also programs where the family doctor is involved in this process. Here, he/she intervenes to the patient's general health problems and acts as a coordinator of health care [59].

Specialized family medicine clinics

In cases where there is a highly specialist shortage, family physicians are specialising in specific fields (i.e., the Family Clinic Memory Clinic developed in Ontario) [59, 62].

Comprehensive geriatric assessment

It is performed interdisciplinary and very detailed, if possible. All members of the team make their evaluations (geriatric, family physician, nurse, social worker, psychologist, physiotherapists, occupational therapists, dietician, etc.). The findings are discussed in team meetings. A decision on care and hospitalisation plans and complex care issues are given during this meeting. The patient is evaluated periodically, and their requirements are reviewed and the care plan updated [23, 63, 64].

All-inclusive care for the elderly (PACE)

The care service is provided in the home environment, to avoid the adverse effect of hospital stays. Services such as daycare service, case management with the interdisciplinary team, integration of finance, transportation service, care house, palliative care are offered [65].

Acute care model of the elderly (ACE)

Acute care model is an interdisciplinary care model directed by a geriatrist. It is one of the most frequently offered care models. It is a patient-centred care service in a home-like environment (in a hospital) [66].

Community nursing services

In a setting where resources are limited, a community nurse can take care of a patient. Tasks such as early diagnosis of patients, coordination of care and case management are undertaken. The follow-up of patients is done after the care plans are prepared. Case management includes services such as active case detection, assessments, coordination, and linking between health and social services are provided [20].

Conclusion

The management of frailty, which has no clear treatment, is based on addressing the multi-factor structure of frailty. Evidence-based management strategies are recommending the intervention of exercise and nutrition or both in combination as the first-line approach. The interventions recommended need a unique care structure which is provided by geriatric teams or community-based teams. In low-resource settings, direct intervention by family physicians with their available resources could be expected. However, the primary role of the family physicians lies in their unique role as first – contact persons, communicators, coordinators and family-oriented care providers. Using a brief comprehensive geriatric assessment tool (i.e., The WHO Aging Friendly Primary Health Care Toolkit and derivates) in combination with frailty screening tests will help identify candidates for further geriatric assessment and frailty management. In low resourced settings, recommendations concerning lifestyle changes (i.e., exercise and nutrition) could be prescribed directly to the patients.

Conflict of interest

Author declares that he has no conflict of interest.

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CASE REPORTS

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A rare case of primary pleural synovial sarcoma

Redak slučaj primarnog sinovijalnog sarkoma pleure

Nebojša Marić*†, Aleksandar Nikolić*, Vlado Cvijanović*†, Aleksandar Ristanović*, Nataša Vešović*, Dejan Stojković*, Vanja Kostovski*, Ljubinko Djenić*, Stevan Čičić*

***Military Medical Academy, Clinic for Cardiothoracic Surgery, Belgrade, Serbia; †University of Defence, Faculty of Medicine of the Military Medical Academy, Belgrade, Serbia**

Abstract

Introduction. Pleural synovial sarcoma (SS) is a rare type of mesenchymal tumor, that can easily be misdiagnosed. **Case report.** We presented a case of primary monophasic SS of the pleura in a middle-aged woman who initially presented with dyspnoea and a large pleural effusion. Computed tomography (CT) scans showed a large, well-demarcated right lung tumor mass. After a blind closed biopsy of the pleura, the tumor was misdiagnosed as adenocarcinoma and treated with chemotherapy but without response. The correct diagnosis was established after surgery and histological and immunohistochemical analyses. The diagnosis was fulfilled with cytogenetic analysis showing the typical translocation t (X,18). The tumor was completely extirpated during surgery. CT of the chest done four, and positron emission tomography done six months after surgery showed encapsulated reactive pleural effusion without tumor rest or relapse. In contrast, a CT scan done nine months after surgery showed an extrapulmonary soft-tissue mass in contact with the lower right lobe highly suspicious of tumor relapse. Surgery was performed, and the described mass was extirpated, but histological analysis showed no presence of malignant tissue. CT scan performed three months later showed no signs of the disease relapse. **Conclusion.** Considering that pleural SS can easily be misdiagnosed, immunohistochemical as well as cytogenetic analysis should always be performed in order to reach the proper diagnosis.

Key words:
cytogenetics; diagnosis; immunohistochemistry; pleural neoplasms; sarcoma, synovial; thoracic surgical procedures; treatment outcome.

Apstrakt

Uvod. Sinovijalni sarkom (SS) pleure je redak oblik mezenhimalnih tumora čija dijagnoza lako može biti propuštena. **Prikaz bolesnika.** Prikazali smo slučaj primarnog monofaznog SS kod sredovečne žene koji se ispoljio u vidu otežanog disanja uz veliki pleuralni izliv. Kompjuterizovanom tomografijom (KT) viđena je velika, jasno ograničena tumorska masa u desnom plućnom krilu. Slepom biopsijom pleure postavljena je pogrešna dijagnoza adenokarcinoma na osnovu čega je sprovedena hemioterapija, ali bez terapijskog odgovora. Ispravna dijagnoza postavljena je posle hirurške intervencije i histoloških i imunohistohemijskih analiza uklonjenog tumora. Dijagnoza je upotpunjena citogenetskom analizom kojom je pokazano prisustvo tipične translokacije t (X,18). Tumor je kompletno uklonjen tokom operacije. Urađeni su KT grudnog koša posle 4 meseca i pozitronska emisiona tomografija posle 6 meseci od operacije i nađen je reaktivni inkapsuliranog pleuralni izliv bez recidiva tumora. Nasuprot tome, KT grudnog koša, urađena 9 meseci posle operacije, pokazala je ekstrapulmonalnu mekotkivnu masu u kontaktu sa donjim desnim režnjem pluća koja je bila sumnjiva na recidiv tumora. Hirurškom intervencijom je uklonjena opisana masa, a histološkom analizom isključeno je postojanje malignog tumorskog tkiva. Primenom KT grudnog koša tri meseca kasnije nisu nađeni znaci recidiva bolesti. **Zaključak.** Dijagnoza pleuralnog SS lako se može propustiti, pa je, u cilju postavljanja ispravne dijagnoze, uvek potrebno sprovesti imunohistohemijske i citogenetske analize tumorskog tkiva.

Ključne reči:
citogenetika; dijagnoza; imunohistohemija; pleura, neoplazme; sarkom, sinovijalni; hirurgija, torakalna, procedure; lečenje, ishod.

Introduction

Synovial sarcoma (SS) is a rare form of mesenchymal neoplasm and represents around 10% of all soft tissue tumors [1–3]. Pulmonary SS is uncommon as well as SS of the pleura, which is more often the consequence of metastatic disease from another primary soft tissue tumor [4]. Primary SS of the pleura is infrequent and represents less than 1% of all

Correspondence to: Aleksandar Nikolić, Military Medical Academy, Clinic for Cardiothoracic Surgery, Crnotravska 17, 11 000 Belgrade, Serbia. E-mail address: anikolic8@yahoo.com

primary pulmonary malignancies [1]. To the best of our knowledge, no cases of primary pleural SS in Serbia have been published so far.

Case report

A 56-year old woman presented with dyspnea which initially appeared three months earlier but at that period was insignificant, and the patient did not seek medical help. The patient had no previous chronic diseases and was never a smoker. There were no malignant diseases in her family history.

At presentation, besides dyspnea, she had absent breath sounds in the right lung field. The rest of her physical exam was unremarkable. Chest radiography revealed large right-sided pleural effusion and computed tomography (CT) of the chest also visualized a well-demarcated tumor in the posterior part of right hemithorax, 116 × 87 × 106 mm in size, more probably of pleural than of pulmonary origin, with compressive atelectasis and without mediastinal lymphadenopathy (Figure 1, A and B). Thoracentesis was performed, and 1,400 mL of serous fluid was evacuated. Cytological analysis of the fluid showed a lymphocytic type of pleural effusion and microbiological analysis demonstrated that the evacuated fluid was sterile. No malignant cells were identified. No pathological findings were revealed during bronchoscopy. The patient subsequently underwent blind closed biopsy of the pleura and histological analysis of the sample discovered non-microcellular adenocarcinoma, epidermal growth factor re-

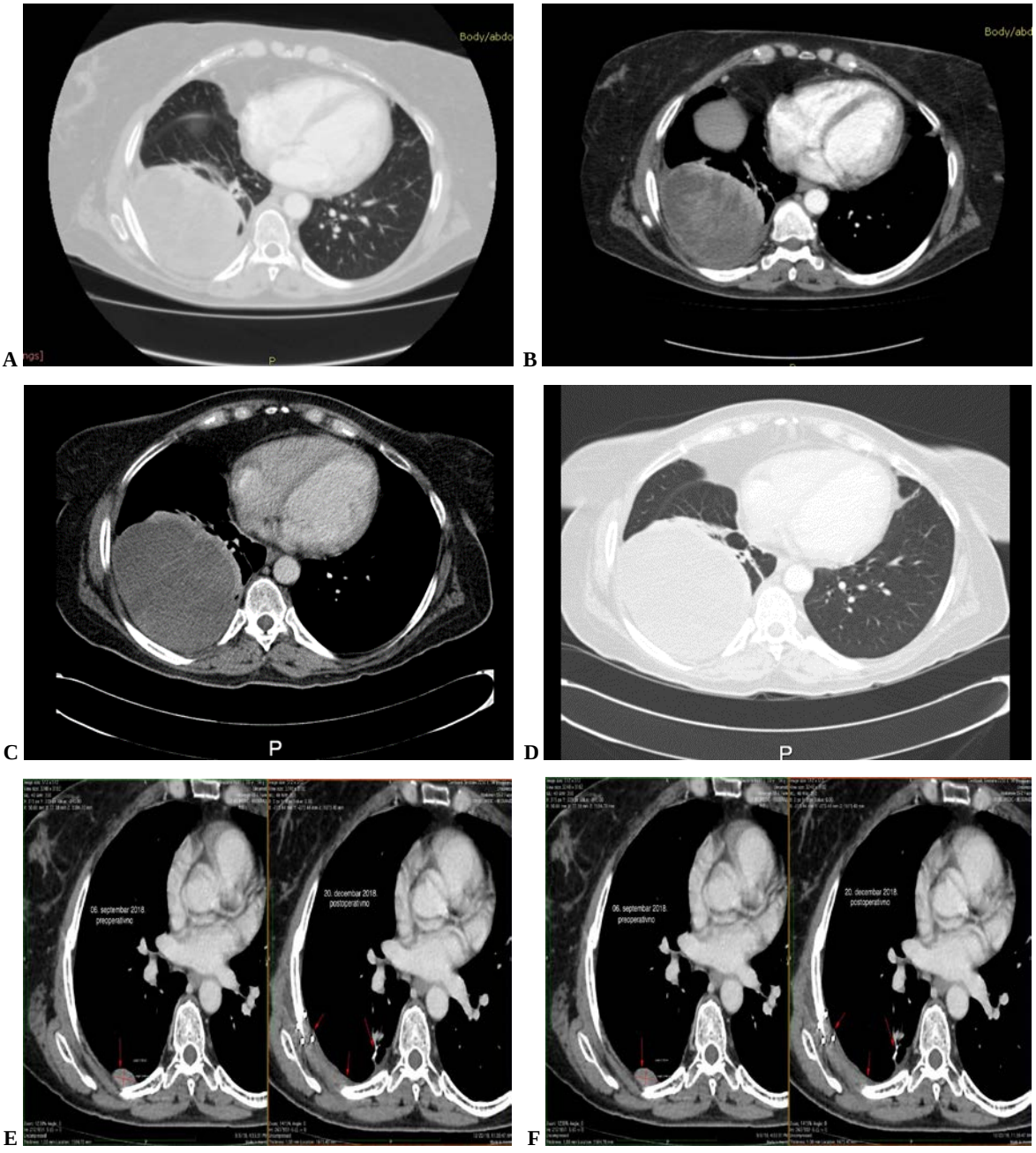

Fig. 1 – Chest computed tomography: A, B) At presentation; C, D) After initial chemotherapy; E) Nine months after first surgery; F) After second surgery.

ceptor (EGFR) wild type, anaplastic lymphoma kinase negative, programmed cell death-ligand 1 negative.

According to all of the aforementioned, the disease was considered to be in the IV stage, and three cycles of paclitaxel/carboplatin (Taxol-CBDCA) chemotherapy protocol were indicated. The CT scan, which was performed after chemotherapy, showed the progression of the disease. A non-homogenous, partially necrotic tumor in the lower right lobe, measuring 120 × 120 × 140 mm, was visualized. It infiltrated the pleura and was followed by compressive atelectasis and right pleural effusion, without mediastinal lymphadenopathy (Figure 1, C and D). The change of the chemotherapy protocol was indicated, and the patient underwent three cycles of cisplatin (Gemzar®-CDDP) protocol. The following thoracic CT scan showed no difference compared to the previous one.

After a studious reevaluation of the case, it was decided to perform a surgical intervention.

Namely, although the diagnosis was established by blind biopsy of the pleura, the minute insight of the CT scans suggested that the diagnosis might have been made by biopsying the tumor itself. This, in combination with no mediastinal lymphadenopathy, led to the conclusion that the patient is most probably in the IIIA (T4 No Mx) stage of the disease when surgery is indicated. Furthermore, since the tumor showed no response to chemotherapy, we considered the possibility of some other form of malignancy which was another reason to perform surgery.

The explorative right anterolateral thoracotomy was performed. Intraoperatively, a large tumor originating from the pleura was seen, localized posteriorly and above the diaphragm, measuring 120 × 120 × 140 mm (Figure 2). It was well-demarcated, partially solid and partially cystic, adherent to the lower right lobe, diaphragm, and posterior thoracic wall but without infiltration of these structures from which it was easily detached during resection. Tumor was completely extirpated. The postoperative course was uneventful.

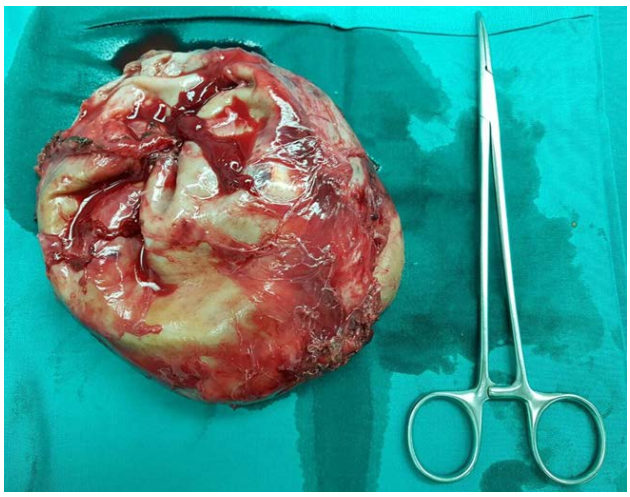

Fig. 2 – The extirpated tumor.

The histological analysis showed a poorly differentiated/undifferentiated malignant mesenchymal neoplastic proliferation sarcoma. Since in the sample obtained preoperatively during blind closed biopsy acinar structures of adenocarcinoma were seen, carcinosarcoma was considered as a potential differential diagnosis. Histology was reevaluated, and immunohistochemical analyses were performed. According to the immunohistochemical analysis [positive for vimentin, TLE-1, B cell lymphoma 2 (Bcl2) and CD99 and negative for cytokeratin 7, 8, 19, 20, epithelial membrane antigen (EMA), LCA, desmin, FLI-1, STAT 6, S100, CD 34, p53], the final diagnosis of high grade malignant mesenchymal monophasic SS of pleura was established. Genetic fluorescent *in situ* hybridization analysis identified t (X,18) translocation.

The CT scan of the chest done four months after surgery and positron emission tomography done 6 months after surgery showed reactive encapsulated pleural effusion without tumor rest or relapse. In contrast, a chest CT scan performed nine months after surgery visualized an extrapulmonary soft-tissue mass by the posterior part of the seventh right rib, which was highly suspicious of tumor relapse (Figure 1E). Thoracotomy was performed and atypical resection of the lower right lobe with the extirpation of the described mass was made. Histological analysis showed no malignant tissue but revealed the presence of well-demarcated organized pleural effusion. Therefore, there was no tumor relapse. Three months later, another CT scan was performed, and no signs of relapse were observed, meaning that one year after the first surgery, the patient was disease-free (Figure 1F).

Discussion

SS usually presents in younger people in soft tissues near large joints, but cases of primary SS localized in other atypical regions have also been reported [1]. SS of the pleura is extremely rare, and according to our knowledge, less than 40 cases have been described in the literature so far.

SS is histologically classified into four types: biphasic, monophasic fibrous, monophasic epithelial, and poorly differentiated [1]. In this report, the patient had a poorly differentiated monophasic primary SS of the pleura. Monophasic SS most often occurs in people between 33 and 69 years old, which is in accordance with the age of our patient [4].

SS of the pleura usually presents with dyspnoea, which was the case in our patient but can also present with thoracic pain, cough, and haemoptysis. Chest radiography and CT most frequently reveal pleural effusion or well-demarcated, sometimes large, soft tissue mass.

The diagnosis of SS is verified by histological and immunohistochemical analysis [1]. In order to confirm the diagnosis of primary pleural SS, primary extrathoracic localization must be excluded with CT and physical exam [1, 4].

Pleural SS can be easily misdiagnosed as malignant mesothelioma, adenocarcinoma, or carcinosarcoma [1]. In this report, the tumor was initially misdiagnosed as adenocarcinoma according to the result of histology of the sample gained by preoperative blind closed biopsy of the pleura. Misdiagnosis led to inadequate treatment and disease progression. At that point, the decision to perform surgery was made, and subsequently, the histology of the tumor taken intraoperatively enabled reaching the correct diagnosis. Having in mind the struc-

tures of adenocarcinoma seen in the preoperative sample, the initial diagnostic consideration after postoperative histological analysis, which revealed the presence of sarcoma, was that the intraoperative material was the sarcoma component of carcinosarcoma. Further immunohistochemistry used to differentiate SS from other pleural neoplasms established the diagnosis of poorly differentiated monophasic SS of the pleura. Immunohistochemically, in the majority of cases, SS are positive for cytokeratin, EMA, bcl-2, and vimentin and negative for S-100, desmin, smooth muscle actin, and vascular tumor markers [3]. The presence of bcl-2 differentiates SS from pleural mesothelioma. In the reported case, although the majority of the typical immunohistochemical findings were present, it was unusual that the tumor was EMA and cytokeratin negative. Dennison et al. [1] also reported a case of primary pulmonary SS negative for cytokeratin and EMA.

Nowadays, cytogenetic analyses are used to help confirm the diagnosis of SS. Translocation t (X,18) characteristic for SS and found in around 90% of cases was also confirmed in our patient [4, 5].

The optimal treatment regimen of SS is still not defined. It requires a multidisciplinary approach combining surgery, chemotherapy, and radiotherapy, with surgery being the treatment of choice [6, 7]. Adjuvant radiotherapy is usually advised after incomplete resection or extensive resection of large tumors [5, 6]. Neoadjuvant chemotherapy can be used preoperatively in order to reduce the size of large tumors [8]. SS are chemosensitive to ifosfamide and doxorubicin [9]. Radiotherapy, chemotherapy, and radiofrequency thermal ablation may be alternative treatment options for inoperable cases [10]. Furthermore, other options, like combined hyperthermia and chemoradiotherapy in patients with advanced inoperable primary pleural SS, have also been described [11].

Pulmonary SS is an aggressive tumor, and the prognosis of the disease is poor, with a 5-year survival rate of around 50% [1]. The main prognostic factor is the possibility for complete surgical resection [1]. Recurrences are frequent, and patients often require repeated surgical interventions. According to the literature data, the disease-free period was 2 to 14 months after surgical resection of primary pleural SS [12]. In the presented case, the tumor was completely extirpated and easily detached from the surrounding tissue, which might be of good prognostic value. One year after surgery, no recurrence of the tumor was discovered.

Conclusion

Primary pleural SS is a very rare tumor that can easily be misdiagnosed. Immunohistochemistry should always be performed as well as cytogenetic analysis whenever possible, in order to reach the proper diagnosis. Due to a small number of cases presented in the literature, there is no gold standard therapy for SS, but complete surgical resection should be performed whenever possible.

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CASE REPORT

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The importance of identifying environmental factors for stuttering treatment in monozygotic twin girl

Značaj utvrđivanja sredinskih faktora za kreiranje tretmana mucanja kod jednojajčane bliznakinje

Sanja M. Dimoski*, Vesna J. Tepšić Ostojić†‡

***University of Belgrade, Faculty of Special Education and Rehabilitation, Belgrade, Serbia; †Military Medical Academy, Clinic for Psychiatry, Belgrade, Serbia; ‡University of Defence, Faculty of Medicine of the Military Medical Academy, Belgrade, Serbia**

Abstract

Introduction. Stuttering is a speech disorder and its etiology is an interplay of genetic and environmental factors. Despite the absence of definite etiology understanding, there are numerous available treatments for stuttering. For some adult patients, the contemporary concept includes psychotherapist involvement concomitant with speech therapist. **Case report.** A 24-year-old girl, who is a monozygotic twin, has been stuttering from early childhood, while her twin sister has never exhibited a speech disorder. Since the role of genetic factors was evident (father stuttered too), the focus of this report was on environmental factors of physical and psychological development (slow development), as well as family psychodynamics (divorce of parents in early adolescent period and criticizing from her father forstuttering). The patient, as well as her family members, denied the significance of the symptoms, which could also explain the absence of early treatment. **Conclusion.** Unfavorable conditions of psychological development, as well as family psychodynamics could explain speech therapy starting at the age of twenty-four and being insufficient for symptom overcoming. Psychotherapy is indicated in the integrative part of treatment in this case of speech disorder.

Key words:
family; genetics; parent-child relations; psychotherapy; risk factors; speech disorders; stuttering; twins.

Apstrakt

Uvod. Mucanje je govorni poremećaj čija etiologija podrazumeva međudejstvo genetskih i sredinskih faktora. Uprkos odsustvu konačnih odgovora o etiologiji mucanja, postoji više različitih terapijskih metoda. U nekim slučajevima lečenja odraslih bolesnika, savremeni model lečenja mucanja, pored logopeda, uključuje i angažovanje psihoteraputa. **Prikaz bolesnika.** Prikazana je jednojajčana bliznakinja stara 24 godine, koja muca od ranog detinjstva, dok njena sestra bliznakinja nikada nije imala govorni poremećaj. S obzirom na to da je uticaj genetskih faktora bio očigledan (otac muca), fokus prikaza je usmeren na sredinske faktore fizičkog i psihičkog razvoja (sporiji razvoj), kao i na porodičnu psihodinamiku (razvod roditelja u ranom adolescentnom periodu i kritikovanje od strane oca zbog mucanja). Članovi porodice, kao i sama pacijentkinja, negirali su značaj simptoma, čime se može tumačiti i odsustvo ranog tretmana. **Zaključak.** Nepovoljni uslovi psihološkog razvoja kao i porodična psihodinamika mogu biti objašnjenje za početak logopedskog tretmana tek u 24. godini, kao i za njegove nedovoljno dobre rezultate. Psihoterapija je indikovana kao deo integrativnog tretmana kod govornog poremećaja ove pacijentkinje.

Ključne reči:
porodica; genetika; roditelj-dete odnosi; psihoterapija; faktori rizika; govor, poremećaji; mucanje; blizanci.

Introduction

Stuttering is a speech fluency disorder with the etiology that implies the interaction of genetic and environmental factors [1, 2]. Evidence is consistent in reporting higher prevalence rates in families with a history of stuttering [3, 4]. Available literature [5, 6] suggests that 70–80% of variance can be explained by genetic factors. Studies focusing on the environmental factors of psychological development in the onset and maintenance of stuttering are rare [7]. Effective treatment is needed since stuttering is a disorder affecting not only speech fluency but also mental health, social functioning and quality of life, effective treatment is needed [8, 9]. Some researchers suggest using an individualized

Correspondence to: Sanja M. Dimoski, University of Belgrade, Faculty of Special Education and Rehabilitation, Visokog Stevana 2, 11 000 Belgrade, Serbia. E-mail: sanja.dimoski@gmail.com

approach for every person who stutters in an attempt to understand the specific set of etiological factors, onset conditions and indications for certain modalities of treatment [10]. Other authors suggest a "smart tailored" approach to adult stuttering – innovative treatments that are adapted for subtypes in adults who stutter [11]. Holistic therapeutic approach addresses speech as well as emotions and attitudes of people who stutter in the social context [12]. Therefore, integrative programs including speech-language pathologists and psychologists, psychiatrisst, psychotherapists may better serve adults who stutter [7, 13]. Some researchers suggest that some people who stutter could benefit from psychological counseling/psychotherapy that focuses on mental health issues associated with stuttering [14-16].

Case report

A 24-year-old girl was monozygotic twin stuttered from early childhood but whose treatment did not start before the age of 24 when speech therapy started. Other female twin did not stutter and has never had this speech disorder. The father of the twins also had the same speech problem in adulthood. Since the presence of genetic factors was evident, the study focused on environmental factors that might be associated with stuttering persistence and the late-onsent of speech therapy. Structured interview based on Berger's list of basic biographic data [17] was applied. The unstructured interview with the patient was focused on the family psychodynamics, her attitudes to the disorder and her family attitudes towards stuttering. The interview with her mother consisted of questions covering biographic data, possible illnesses, family psychodynamics and information related to daughter's stutter and the course of development.

Early psychophysical status showed some physical consequences of childbirth. Upon birth, the patient was significantly bluish, with slightly sprained shoulder and had weaker status after birth compared with her twin sister. Although she had a normal weight for a twin pregnancy (2,850 g), she lost significant weight immediately after delivery (2,200 g) and had slower weight gain in the next few months. She began to walk first, while her sister was the first to talk. At age 7, she had a short period of enuresis.

The twins were born in a complete family and they grew up in an extended family with parents and grandparents in their father's family house. In the preschool period, the patient was more attached to her grandmother, and other family members reinforced this relationship saying she was her grandmother's granddaughter. Members of the father's family also used to emphasize she was "theirs" because of her speech difficulties being similar to her father's. When she was 12 years old, her parents divorced, and the twins continued to live with their mother. The parents' marriage was characterized by emotional distance and a lack of communication, the atmosphere in the home was quiet and cold.

For the first time, the patient became aware of stuttering when she was about 9 years old and her mother took her to a speech therapist. According to her mother, both psychologist and speech therapist concluded that the cause of stuttering was her attempt to imitate her father trying in that way to become closer to him. For her, this was not accepted as a valid cause of stuttering and she stopped treatment after two or three appointments. After the divorce of her parents, her father neglected her in her early teen ages. In this period, stuttering intensified in her father's presence, but with specific relation towards her, which reflected in teasing and insulting jokes about her stuttering. The mother offered very scanty information about her stuttering. The mother's attitude towards her daughter's problem was missing as well as the stuttering (either that of the danghter or her father) was not perceived as a problem or was ignored in general. In the patient's opinion, the family was ashamed of her disorder. When she was about 20 years old, her problems with speech increased. Stuttering was more frequent and she developed strong jaw spasms developed when she tried to pronounce a word. That was the same period when she started to be more frequently in contact with her father. She decided to seek professional help, this time on her own. Realizing that she had a speech disorder that needed treatment a long time ago and that she would have to be in therapy for a long period of time, was very distressing for her. Because of that and the limited success od speech therapy, the patient decided to see a psychotherapist too.

The patient was referred to a psychiatrist and psychologist. After a clinical interview, and psychological exploration, it was concluded that psychiatric treatment was not indicated. Psychological exploration showed that what stood out about patient was above all her relationship with and toward people. She easily established and maintained harmonic relationships by being pleasant, warm and well-intentioned. Good self control and a capacity for self-management helped her achieve her goals and tasks easily. However, the patient had a tendency to worry too much and to perceive problems bigger than they objectively were. She easily became anxious and put up with more than other people would in her circumstances. She rarely got mad which contributed to her harmonious relationships. On the other hand the absence of rage as a result of its inhibition potentiated her anxiety. Excessive rage inhibition was likely a possibility because she cared deeply about good relations and acceptance by others, frequently at the cost of her own needs. She was at risk of being over-defensive concerning other unpleasant emotions since it was of the utmost importance for her to be happy, satisfied and well accepted.

Discussion

We described a case of a 24-years-old monozygotic twin girl who has been stuttering since early childhood, while her twin sister has never exhibited a speech disorder. Given that age is one of the strongest risk factors of stuttering, it is important to emphasize that this study examined a later beginning of stuttering. Although the disorder begins in a wide range of ages, results of research so far show that in about 65% the stuttering poeple appears

before the age of 3, and even in 85% until the age of 3.5. Children older than 4 are faced with a relatively smaller risk of stuttering. Since the role of genetic factors was evident (father stuttered too), the focus of this report was on environmental factors of psychological development, as well as family psychodynamics.

The function of the stutter maintenance could be to bring her closer to her father and her father's family (grandmother). Although more accepted by the father's family because of her stutter, she was at the same time additionally humiliated by her father because of it. In her overall development, the patient has shown a strong orientation towards social relations. This is contrary to studies [18, 19] that show that people who stutter, especially those whose stutter is a long-standing problem (as it was with our examinee), are socially withdrawn and develop social anxiety. Our patient was very expressive and therefore received the encouragement she needed so much in the interaction with her environment. Like a more vulnerable twin but at the same time strongly oriented to relations with others, the patient developed social relationships that were compensation for her deficit and protected her from rejection as a weaker twin. More oriented towards others and at the same time attached to her grandmother as the source of emotional security, she was exposed to greater pressure to position herself as the one who belongs to her father's family comparing with her twin sister too. Therefore, she was under greater risk to adopt her father's symptom, which was stuttering, than her sister was.

The maintenance of stuttering as well as treatment avoidance has enabled patient, as well as her family, to deny the symptoms. This has notably lowered the possibility of a successful overcoming of the stutter [20]. Namely, as several studies show [2, 20–23], children can successfully overcome stuttering in a high degree (70%–87%), while the lack of treatment with age carries a risk, where that the stuttering can become a permanent speech disorder. Her family members favored and ridiculed her stuttering at the same time. The father's stuttering was never treated as a disability, this the same attitude was kept toward the patient's condition. This attitude was equally shared by her mother, which altogether resulted in the negligence of the need for professional help. On a personal level, the patient denied her symptom as well. In the case of our patient, it is the lack of treatment of stuttering in early childhood. It is noticeable that she did not start to painfully realize her speech disorder before her early twenties. Her compensatory mechanisms which kept her social relations at the high level, as well as her tendency to suppress unpleasant emotions started to weaken with the intensification of her symptomatology. Our findings on the examinee are in linethe with research that shows that persons who stutter, compared to the control group, have elevated neuroticism, which is characterized by anxiety, emotional instability, stress vulnerability [24].

The same research shows that these individuals have an elevated level of agreeableness – they are kinder, warmer, and more thoughtful, which are tendencies that characterize our examinee. The existence of these tendencies, particularly expressed in our examinee, can explain the absence of her social anxiety and social isolation. Although her symptoms maintained at the same level for years, they began to worsen in the period in which she started to have more frequent contact with her father. This was at the same time of actualization of her early experiences and confrontation with her father's essential negligence and harassment. This probably resulted with the release of hostile emotions, which could explain her initiative to meet with a psychologist. The fact that the worsening of her speech problem symptoms incited her to seek help implied that she had a good internal motivation. However, the unfavorable conditions of her psychological development, as well as the family's psychodynamics concerning her stuttering resulted in speech therapy being not enough for the symptoms overcoming. Adequate treatment for a patient's stuttering must include psychotherapy, as well as speech therapy, since clear indications for psychotherapy have been determined. Recommended focus in psychotherapy would be to gain insight into the meaning of her symptoms and their denial, which would lead to a clearer self-perception and the development of more authentic relationships, above all with her father.

Conclusion

This case report detected, besides evident genetic factors, clear environmental factors concerning psychological development and family psychodynamics which could explain the onset and maintenance of stuttering. Same factors were responsible for the long-term problem denial and the treatment starting at the age of 24. It is reasonable to suppose that psychotherapy as a part of integrative treatment and focused to gain insight into the meaning of symptoms could be beneficial in treatment of speech disorder.

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CASE REPORT
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Primary breast angiosarcoma in postmenopausal women with a picture like Kasabach-Merritt syndrome – A case report

Primarni angiosarkom dojke kod žene u postmenopauzi sa slikom nalik na Kasabach-Merritt-ov sindrom

Mirjana Ćuk*†, Radoslav Gajanin‡§, Radmil Marić*†, Veljko Marić*†, Svjetlana Todorović*, Milena Vasić Milanović‖

***University of East Sarajevo, Faculty of Medicine, Foča, Republic of Srpska, Bosnia and Herzegovina; †University Hospital, Center for Laboratory Diagnosis, Foča, Republic of Srpska, Bosnia and Herzegovina; ‡University of Banja Luka, Faculty of Medicine, Banja Luka, Republic of Srpska, Bosnia and Herzegovina; §University Clinical Center, Department of Pathology, Banja Luka, Republic of Srpska, Bosnia and Herzegovina; ‖Public Health Hospital, Zvornik, Republic of Srpska, Bosnia and Herzegovina**

Abstract

Introduction. Primary angiosarcoma of the breast (PAB) is a very rare tumor and accounts for 0.04% of all breast malignant tumors and most commonly occurs in young women. Kasabach-Merritt syndrome (KMS) is described as consumption coagulopathy with thrombocytopenia, and without adequate therapy almost certainly leads to a very fast lethal outcome. The literature contains only a few cases of PAB associated with thrombocytopenia or with KMS and there are no clear defined protocols for the treatment of these patients, which requires the presentation of as many cases as possible. **Case report.** We presented a rare case of 60-year-old postmenopausal woman with metastatic PAB grade III associated with a picture like KMS (thrombocytopenia and anemia without the coagulation factor disorder with massive bleeding in the tumor). Mastectomy was performed without the extirpation of the axillary region. After the surgery, improvement with anemia and thrombocytopenia was noticed. One month after the surgery, supportive and symptomatic therapy was administered, as well as bisphosphonate therapy, but with temporary improvement. Deplasmated erythrocytes and methylprednisolone were added during the another hospital stay, but prominent symptoms of general weakness along with the progression od thrombocytopenia were noted. Fibrinogen and coagulation factors were within reference values all the time. The patient died four months after the surgery. **Conclusion.** PAB in postmenopausal women is a very rare tumor, and may be associated with anemia and thrombocytopenia without other laboratory parameters for KMS. Anemia and thrombocytopenia are refractory to standard treatment protocols, and also significantly reduces the quality of life of these patients.

Key words:
angiosarcoma of the breast; breast neoplasms; diagnosis; histological techniques; immunohistochemistry; kasabach-merritt syndrome; postmenopause; thrombocytopenia; treatment outcome.

Apstrakt

Uvod. Primarni angiosarkom dojke je vrlo redak tumor koji čini 0,04% svih malignih tumora dojke, a najčešće se javlja kod mladih žena. Kasabach-Merritt-ov sindrom (KMS) je opisan kao potrošna koagulopatija sa trombocitopenijom i bez adekvatne terapije gotovo sigurno dovodi do veoma brzog letalnog ishoda. Literatura sadrži samo nekoliko slučajeva primarnog angiosarkoma dojke povezanih sa trombocitopenijom ili sa KMS-om i nema jasno definisanih protokola za lečenje tih bolesnika, što zahteva prezentaciju što većeg broja slučajeva. **Prikaz bolesnika.** Prikazali smo redak slučaj žene u postmenopauzi, starosti 60 godina, sa metastatskim primarnim angiosarkomom desne dojke trećeg stepena udruženim sa slikom sličnom KMS (trombocitopenija i anemija bez poremećaja faktora koagulacije, sa masivnim krvarenjem u tumoru). Urađena je mastektomija, bez odstranjivanja aksilarne regije. Posle hirurške intervencije došlo je do poboljšanja, ali su registrovani anemija i trombocitopenija. Mesec dana posle hirurgije ordinirana je suportivna i simptomatska terapija, kao i terapija bifosfonatima, ali sa privremenim poboljšanjem. Terapiji su dodati deplazmatisani eritrociti i

Correspondence to: Mirjana Ćuk, University of East Sarajevo, Faculty of Medicine, Department of Pathology, Studentska 5, 73 300 Foča, Republic of Srpska, Bosnia and Herzegovina. E-mail: akicuk@yahoo.com, mirjana.cuk@ues.rs.ba

metilprednizolon tokom ponovnog hospitalnog lečenja, ali je došlo do izraženih simptoma opšte slabosti zajedno sa registrovanom progresijom trombocitopenije. Fibrinogen i faktori koagulacije su sve vreme bili u okviru referentnih vrednosti. Bolesnica je umrla četiri meseca posle hirurške intervencije. **Zaključak.** Primarni angiosarkom dojke kod žena u postmenopauzi je vrlo redak tumor i može biti povezan sa anemijom i trombocitopenijom bez drugih laboratorijskih parametara tipičnih za KMS. Anemija i trombocitopenija su otporne na standardne protokole lečenja, a takođe značajno smanjuju kvalitet života tih bolesnika.

Ključne reči:
dojka, angiosarkom; dojka, neoplazme; dijagnoza; histološke tehnike; imunohistohemija; kasabach-merritt sindrom; postmenopauza; trombocitopenija; lečenje, ishod.

Introduction

Primary angiosarcoma of the breast (PAB) is a very rare, soft tissue tumor, and makes less than 0.04% of all malignant breast tumors and about 1% of all soft tissue breast tumors [1]. The etiology of this tumor is unknown. It usually occurs in the third and fourth decade of life as opposed to other malignant tumors of the breast [2]. Secondary angiosarcoma of the breast is more likely to occur in elderly women patients as a result of previous radiotherapy and mastectomy [3]. Kasabach-Merritt syndrome (KMS) is described as consumption coagulopathy with thrombocytopenia, first detected in children with benign tumors of vascular genesis, and recently has also been described in adults usually in association with malignant tumors of vascular genesis [4].

Case report

We presented a 60-year-old postmenopausal woman who was admitted to our hospital because of the pain in the lumbar region of the spine. Eighteen months ago, the patient noticed swelling and induration in the right breast, without pain and without nipple discharge from. Previous surgical procedures and radiation were denied. When examining the right breast, it was enlarged, occupied by a tumor mass of the largest diameter of 15 cm with the presence of fluctuations in the tumor. The skin of the breast above the tumor was livid and tense. Physical findings in the left breast and both axillary regions were neat. In laboratory findings, accelerated sedimentation of 50/87 values was noticeable, elevated values of lactate dehydrogenase (LDH) – 303 U/L (normal values less than 241 U/L) as well as moderate anemia and mild thrombocytopenia: white blood cells (WBC) – 4.6 × 10^9/L [reference range (rr) 4.5–11 × 10^9/L]; red blood cells (RBC) – 3.14 × 10^{12}/L (rr 4.1–5.1 × 10^{12}/L); haemoglobin (Hgb) – 88 g/L (rr 120–160 g/L); hematocrit (Hct) – 0.27 (rr 0.356–0.470); platelets (PLT) – 122 × 10^9/L (rr 150–400 × 10^9/L). Other laboratory findings were within the limits of the reference values: fibrinogen – 7.2 µmol/L (rr 5.9–11.7 µmol/L); coagulation time – 430 sec (rr 10–18 min); bleeding time – 110 sec; protrombine time – 12 sec (rr 12.3 ± 0.18 sec); prothrombine index – 100% (rr 70%–130%); international normalized ratio (INR) – 1 (rr 0.9–1.2); aspartate aminotransferase (AST) – 19 U/L (rr 8–43 U/L); alanine aminotransferase (ALT) – 18 U/L (rr 7–55 U/L); creatinine – 111 µmol/L (rr 60–110 µmol/L); urea – 6.2 mmol/L (rr 2.9–8.2 mmol/L); glucose – 4.95 mmol/L (normal values less than 5.6 mol/L); cholesterol – 4.85 mmol/L (rr 3.5–5 mmol/L); triglyceride – 1.61 mmol/L (normal values less than 1.7 mmol/L); electrolytes (Na^+ – 143 mmol/L (rr 135–145 mmol/L); K^+ – 4.7 mmol/L (rr 3.5–4.5 mmol/L); Cl^- – 100 mm/L (rr 98–108 mmol/L); Ca^{2+} – 1.31 mmol/L (rr 2.2–2.5 mmol/L)). Core tumor biopsy resulted in fragments of necrotic detritus and blood. Computed tomography (CT) confirmed metastatic deposits in the lungs, liver, spinal vertebrae and ribs. During hospitalization, the right breast was rapidly enlarged with severe pain and tension of the skin. Because of the skin tear above the tumor, mastectomy was performed without the extirpation of the axillary region. Intraoperatively, rupture of the tumor node occured with evident abundant central bleeding area. By macroscopic examination, the tumor tissue mostly appeared to be coagulated blood, dilapidated, and only on the periphery underneath the skin there was a narrow area of grayish-white, highly vascularized tumor tissue of firm consistency. A microscopic examination of the tumor tissue recorded a colorful picture. The tumor was composed of solid areas of atypical spindle cells, capillary vessels of irregular shape incorporated in collagen stroma, and focal papillary proliferation of atypical, spindle and polygonal tumor cells with necrosis zones (Figure 1). Mitoses were numerous, more than 80 to 10 high-power fields. Positive immunohistochemical reaction was present on endothelium specific antibodies: CD31, CD34 and factor VIII, and negative on S100. A definitive diagnosis was set: Angiosarcoma of the breast, grade III (Figure 2). Preoperatively and postoperatively, the patient was given two doses of deplasmated erythrocytes. After the surgery, improvement with anemia and thrombocytopenia was noticed: WBC – 3.3 × 10^9/L; RBC – 3.37 × 10^{12}/L; Hgb – 92 g/L; Hct – 0.28; PLT – 156 × 10^9/L. This patient was presented to Oncology Advisory Board one month after the surgery with laboratory findings of blood count: WBC – 3.5 × 10^9/L; RBC – 2.74 × 10^{12}/L; Hgb – 81 g/L; Hct – 0.23; PLT – 104 × 10^9/L. Supportive and symptomatic therapy was indicated, as well as bisphosphonate therapy. Because of the progressive anemia and thrombocytopenia, the patient was admitted to the hospital again very soon. Deplasmated erythrocytes and methylprednisolone were ordinated. Blood count was as follows: WBC: 3.2 ... 4.4 ... 4.7... 4.4 ... 5.1 ... 6.2 × 10^9/L; RBC: 1.84 ... 2.07 ... 2.50 ... 2.64 ... 2.76... 3.32 × 10^{12}/L; Hgb: 57 ... 58 ... 74 ... 76 ... 78 ... 95 g/L; Hct: 0.18 ... 0.19 ... 0.23 ... 0.24 ... 0.25 ... 0.31; PLT: 87 ... 89 ... 74 ... 58 ... 64 ... 67 × 10^9/L. After the improvement of anemia, the patient was sent to home treatment with recommendation to continue with supportive,

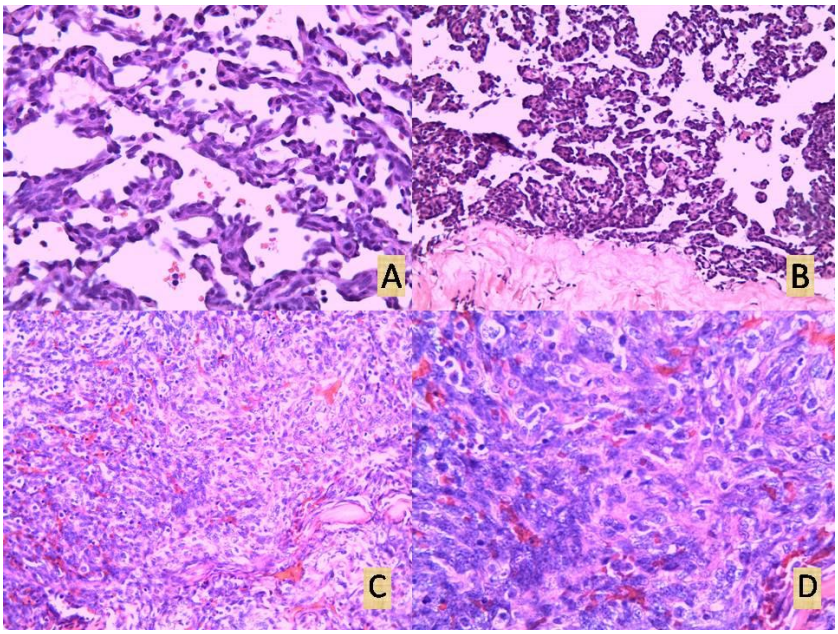

Fig. 1 – Angiosarcoma of the breast: A) area with capillary histopathological picture [hematoxylin and eosin (H&E) stain, ×400]; B) area with papillary histopathological picture (H&E stain, ×200); C) area with a solid histopathological picture (H&E stain, ×200); D) area with a solid histopathological picture (H&E stain, ×400).

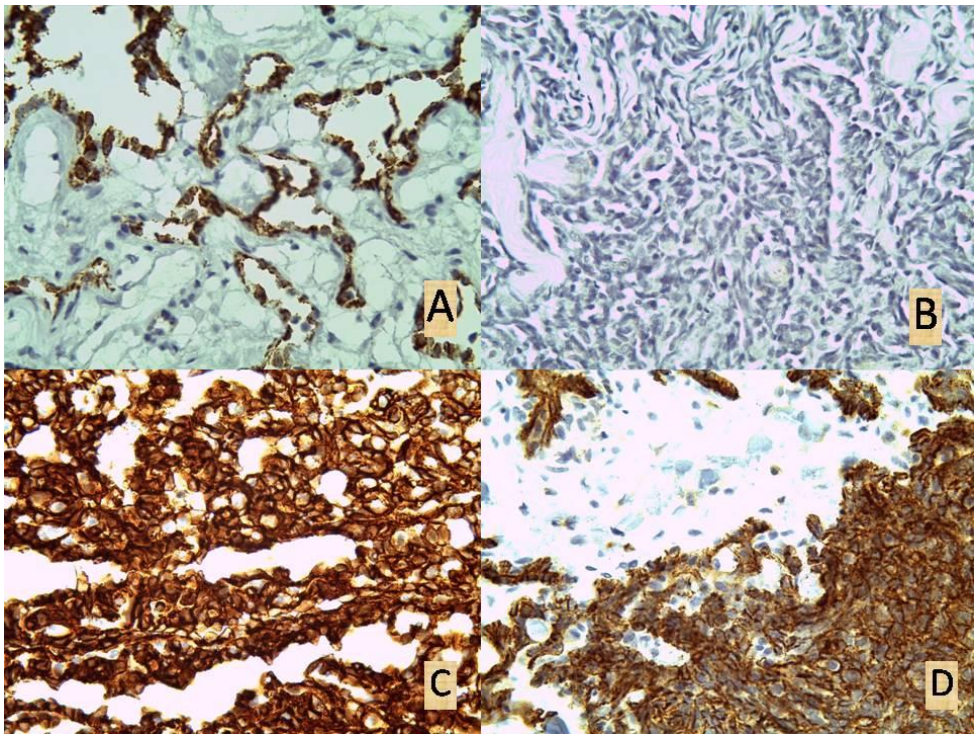

Fig. 2 – Angiosarcoma of the breast: A) Von Willebrand (F VIII), ×400; B) S 100, ×400; C) CD34, ×400; D) CD31, ×400.

symptomatic and oral corticosteroid therapy. However, the patient visited us again because of the prominent symptoms of general weakness. During the hospitalisation on a daily basis, the patient was given deplasmated erythrocytes and methylprednisolone. The blood count was as follows: WBC: 5.6 ... 5.8 ... 5.0 ... 5.0 ... 5.1 × 10^9/L); RBC: 1.34 ... 1.71 ... 1.69 ... 2.07 ... 2.09 × 10^{12}/L; Hgb: 41 ... 51 ... 52 ... 60 ... 62 g/L; Hct: 0.13 ... 0.16 ... 0.16 ... 0.19 ... 0.19; PLT: 59 ... 53 ... 51 ... 44 ... 39 × 10^9/L. The improvement with anemia and progression of thrombocytopenia was noticed. Fibrinogen and coagulation factors were still within reference valus. The patient died four months after the surgery.

Discussion

PAB is a very rare tumor and most commonly occurs in young women. Yin et al. [5] have shown that high grade PAB occurs at a significantly younger age compared to intermediate and low grade PAB, 24:35:41, respectively [4]. Diagnosis of PAB is most commonly rapid and accurate thanks to modern imaging techniques, and is confirmed by a definitive histopathological analysis on core biopsy samples. Although the core biopsy has become the gold standard in breast tumor diagnosis, in cases like ours in which over 90% of the tumor mass is necrotic with massive central bleeding, this method is quite limited in obtaining representative samples. The diagnosis of primary angiosarcoma may be challenging even in cases without massive necrosis and bleeding. Differential diagnosis of low grade angiosarcoma includes benign vascular lesions: hemangiomas, papillary endothelial hyperplasia, and diffuse dermal angiomatosis [6, 7]. The main differential diagnostic problem in high grade angiosarcoma is sarcomatoid carcinoma. In setting up the diagnosis of sarcomatoid carcinoma, the most important parameter is the negativity of tumor cells to vascular markers, while the cells of the epithelioid subtype of PAB can express cytokeratin receptors on the surface [8].

Progressive thrombocytopenia in vascular tumors is often a sign of consumption coagulopathy associated with KMS. KMS is characterized by: rapidly enlarging vascular anomaly established, hypofibrinogenaemia, consumption coagulopathy, thrombocytopaenia, prolonged prothrombin time and activated prothrombin time, presence of d-dimer and fibrin split products with or without microangiopathic haemolytic anemia. Management of KMS includes high dose of corticosteroids and interferon alpha. Other therapeutic modalities include compression of the lesion, arterial embolization with bleomycin, laser therapy, sclerotherapy, radiotherapy and chemotherapy with vincristine, doxorubicin, paclitaxel and use of drugs such as pentoxyfylline, dipyridamole and propranolol. An unrecognized syndrome, without adequate therapy, almost certainly leads to a very fast lethal outcome [9, 10].

Conclusion

PAB in postmenopausal women is a very rare tumor, and may be associated with anemia and thrombocytopenia without other laboratory parameters for KMS. Anemia and thrombocytopenia are refractory to standard treatment protocols, and also significantly reduces the quality of life of these patients. The literature contains only a few cases of PAB associated with thrombocytopenia or with KMS and there are no clearly defined protocols for the treatment of these patients, which requires the presentation of as many cases as possible.

Conflict of interest

The authors have no conflicts of interest to declare.

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