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According to data from the World Health Organization (WHO), in 2024, 1.3 million people were infected with the human immunodeficiency virus (HIV), 40.8 million people were living with confirmed HIV infection, and approximately 630,000 people died from HIV-related diseases. The WHO marks World Acquired Immunodeficiency Syndrome (AIDS) Day on December 1 each year. The theme of this year's slogan, "Overcoming disruption, transforming the AIDS response," highlights the need to strengthen international cooperation in order to end AIDS by 2030.

Prema podacima Svetske zdravstvene organizacije (SZO), tokom 2024. godine virusom humane imunodeficijencije (HIV) inficirano je 1,3 miliona ljudi, 40,8 miliona ljudi je živelo sa potvrđenom HIV infekcijom, a oko 630 000 ljudi umrlo je od bolesti povezanih sa ovim virusom. SZO obeležava Svetski dan borbe protiv sindroma stečene imunodeficiejncije (*acquired immunodeficiency syndrome* – AIDS) 1. decembra svake godine. Tema ovogodišnjeg slogana je „*Od prevazilaženja izazova do unapređenog odgovora na AIDS"* i ukazuje na potrebu jačanja međunarodne saradnje kako bi se AIDS okončao do 2030. godine.

Dear Authors, Editors, Peer Reviewers, and Readers of the Vojnosanitetski pregled,
I thank you for your cooperation and support in the last year
and wish you all the best in the coming 2026!

Merry Christmas and Happy New Year!

Cordially,
Editorial staff of the *Vojnosanitetski pregled*

***Poštovani autori, urednici, recenzenti i čitaoci Vojnosanitetskog pregleda*,**
Uz zahvalnost na saradnji i podršci u protekloj godini,
želimo vam sve najbolje u nastupajućoj 2026. godini!

Srećna Nova godina i božićni praznici!

Srdačno,
Redakcija *Vojnosanitetskog pregleda*

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Influenza surveillance in the Autonomous Province of Vojvodina, Serbia, over ten consecutive seasons (2015/16–2024/25): epidemiological trends, fatal case characteristics, and vaccination

Nadzor nad gripom u Autonomnoj pokrajini Vojvodini, Srbija, tokom deset uzastopnih sezona (2015/16–2024/25): epidemiološki trendovi, karakteristike smrtnih ishoda i vakcinacija

Mioljub Ristić*†, Aleksandra Patić*‡, Nataša Nikolić*‡, Gordana Kovačević*, Vladimir Petrović*†

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Abstract

Background/Aim. Influenza represents a significant public health concern. The aim of this study was to analyze indicators of influenza activity over ten consecutive seasons in the Autonomous Province of Vojvodina (APV), Serbia, including age-specific and weekly distribution, and characteristics of influenza-related mortality. In addition, influenza vaccination trends in APV over the past ten seasons were assessed. **Methods.** A retrospective study was conducted using data from sentinel surveillance of influenza-like illness (ILI) and acute respiratory infections (ARI) among outpatients, as well as severe acute respiratory infection (SARI) and acute respiratory distress syndrome (ARDS) among hospitalized patients with fatal outcomes, along with data on the number of individuals vaccinated against influenza in APV. Laboratory confirmation of influenza-related deaths was performed at the Virology Center, Institute of Public Health of Vojvodina, Novi Sad, Serbia. **Results.** ILI and ARI incidence varied seasonally, peaking typically between surveillance weeks 5 and 10. The 2020/21 season showed markedly low influenza virus activity, while the 2021/22–2024/25 seasons displayed patterns typical of influenza seasons. Throughout the surveillance period, children aged 0–4 and 5–14 exhibited the highest incidence rates. Over the ten seasons, 202 laboratory-confirmed influenza-associated deaths were recorded, predominantly among men aged 24–64 years, occurring mainly during winter months. None of the patients had been vaccinated. The most common influenza A subtype was A (H1N1)pdm09 (59.4%). Fatal cases were almost evenly distributed between SARI (49.0%) and ARDS (51.0%), with ARDS being more common in adults aged 24–64 years. No statistically significant difference in the distribution of fatal outcomes was observed between the pre- and post-coronavirus disease 2019 periods (p = 0.6870). Influenza vaccination coverage among high-risk populations remained low, peaking at 17.5% among individuals ≥ 65 years in the 2020/21 season. Among healthcare workers, coverage reached over 40.0% in 2020/21 but declined to 20.0–25.0% in the subsequent seasons. **Conclusion**. Integrated sentinel and hospital-based surveillance encompasses monitoring of outpatient, severe, and fatal influenza cases. A significant increase in influenza vaccination coverage among high-risk groups could reduce hospitalization rates, the frequency of complications, and influenza-related deaths.

Key words:
covid-19; epidemiology; influenza, human; respiratory tract infections; serbia; vaccination; virology.

Apstrakt

Uvod/Cilj. Grip predstavlja značajan javnozdravstveni problem. Cilj rada bio je da se analiziraju indikatori aktivnosti virusa gripa tokom deset uzastopnih sezona u Autonomnoj Pokrajini Vojvodini (APV), Srbija, uključujući uzrasno-specifičnu i nedeljnu distribuciju i karakteristike smrtnih ishoda povezanih sa gripom. Osim toga, analizirani su i trendovi vakcinacije protiv gripa u APV tokom poslednjih deset sezona. **Metode**. Sprovedena je retrospektivna studija korišćenjem podataka iz sentinelnog nadzora nad oboljenjima sličnim gripu (*influenza-like illness* – ILI) i akutnim respiratornim infekcijama (*acute respiratory infections* – ARI) kod ambulantnih bolesnika, odnosno nad

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teškom akutnom respiratornom bolešću (*severe acute respiratory infection* – SARI) i akutnim respiratornim distres sindromom (*acute respiratory distress syndrome* – ARDS) kod hospitalizovanih bolesnika sa smrtnim ishodom, kao i podataka o broju osoba vakcinisanih protiv gripa u APV. Laboratorijska potvrda virusa gripa kod bolesnika sa smrtnim ishodom dobijena je u Centru za virusologiju Instituta za javno zdravlje Vojvodine, Novi Sad, Srbija. **Rezultati**. Stope incidencije ILI i ARI varirale su u zavisnosti od sezone, sa pikovima uglavnom između 5. i 10. nedelje nadzora. Sezona 2020/21 imala je značajno nisku aktivnost virusa gripa, dok su sezone 2021/22–2024/25 imale obrasce tipične za sezonu gripa. Tokom perioda nadzora, deca uzrasta 0–4 i 5–14 godina imala su najviše stope incidencije. Tokom deset sezona registrovana su 202 smrtna ishoda povezana sa laboratorijski potvrđenim gripom, uglavnom kod muškaraca uzrasta 24–64 godine, koja su se dogodila pretežno tokom zimskih meseci. Nijedan bolesnik nije bio vakcinisan. Najzastupljeniji podtip virusa gripa tipa A bio je A (H1N1)pdm09 (59,4%). Smrtni slučajevi bili su gotovo ravnomerno raspoređeni između dijagnoza SARI (49,0%) i ARDS (51,0%), pri čemu je ARDS bio češći kod odraslih starosti 24–64 godine. Nije uočena statistički značajna razlika u raspodeli smrtnih ishoda između perioda pre i posle pandemije izazvane koronavirusom 2019 (p = 0,6870). Obuhvat vakcinacijom protiv gripa visokorizične populacije ostao je nizak, dostižući maksimum od 17,5% kod osoba ≥ 65 godina u sezoni 2020/21. Među zdravstvenim radnicima, obuhvat je dostigao vrednost od preko 40,0% u sezoni 2020/21, ali je u narednim sezonama opao na 20,0–25,0%. **Zaključak**. Integrisani sentinelni i bolnički nadzor obuhvata nadzor nad ambulantnim, teškim slučajevima i smrtnim ishodima povezanim sa gripom. Značajno povećanje obuhvata imunizacijom protiv gripa u populacijama sa povećanim rizikom od obolevanja od gripa moglo bi smanjiti stope hospitalizacije, učestalost komplikacija i smrtnost povezanu sa virusom gripa.

Ključne reči:
covid-19; epidemiologija; grip; respiratorni trakt, infekcije; srbija; vakcinacija; virologija.

Introduction

Influenza remains a major global public health concern, contributing significantly to morbidity and mortality, particularly among young children, older adults, and individuals with underlying medical conditions [1, 2]. Seasonal influenza epidemics cause substantial strain on healthcare systems, with considerable year-to-year and regional variation in their intensity, timing, and age distribution [3]. Continuous influenza surveillance is crucial for understanding seasonal dynamics, guiding immunization policies, and evaluating the disease burden, particularly in the context of emerging respiratory threats [4–8].

The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in early 2020 profoundly disrupted the epidemiology of other respiratory viruses, including influenza [9]. Non-pharmaceutical interventions such as school closures, mask mandates, physical distancing, remote working, and international travel restrictions resulted in historically low influenza activity during the 2020/21 season in many countries, including those in the European region [10–13]. In the aftermath of the pandemic, influenza activity gradually rebounded, although with atypical timing and intensity in some seasons [14].

The Autonomous Province of Vojvodina (APV), northern Serbia, has established an integrated system that combines sentinel surveillance of influenza-like illness (ILI) and acute respiratory infection (ARI) in outpatient settings, hospital-based surveillance of severe acute respiratory infection (SARI) and acute respiratory distress syndrome (ARDS), and virological testing of respiratory samples collected from influenza-suspected cases across the province. These surveillance systems enable comprehensive monitoring of influenza activity and disease burden and have been described in detail previously [15, 16].

In Serbia, influenza vaccination is mandatory for individuals older than 6 months with chronic conditions, pregnant women, adults aged over 65 years, family members of high-risk individuals, residents and staff of gerontological and social-health institutions, as well as for health-care workers (HCWs) with chronic illnesses, pregnant HCWs, and HCWs providing medical services to pregnant women or patients at high risk of complications [17–19].

The aim of this study was to analyze the weekly and age-specific ILI and ARI incidence, compare clinical and epidemiological features of influenza-related deaths in adults (24–64 vs. ≥ 65 years), and to describe the distribution of influenza vaccination over ten seasons (2015/16–2024/25) in APV.

Methods

This observational study was based on data collected through sentinel surveillance of ILI and ARI among outpatients, surveillance of hospitalized influenza patients with SARI and/or ARDS, and virological surveillance in APV, over ten consecutive influenza seasons (from calendar week 40 to week 20 of the following year, i.e., from the first week of October to the fourth week of May). During the study period (2015/16 to 2024/25), we analyzed weekly seasonal and age-specific trends of ILI and ARI, and compared the characteristics of fatal influenza cases between individuals aged 24–64 years and those aged ≥ 65 years. We applied the same methodology for sentinel and hospital-based surveillance of ILI, ARI, SARI, and ARDS as previously reported [15, 16]. Sentinel surveillance, in line with World Health Organization (WHO) and national recommendations, was conducted in all primary health care centers of APV and covered five age groups (0–4, 5–14, 15–29, 30–64, and ≥ 65 years). Outpatient cases (ILI and ARI) were reported weekly by sentinel general practitioners and

pediatricians. Hospital surveillance involved daily reporting of all SARI and ARDS cases from acute care hospitals, applying WHO case definitions. Laboratory confirmation of influenza was performed using real-time polymerase chain reaction–according to the protocols of the United States Centers for Disease Control and Prevention at the Center for Virology, Institute of Public Health of Vojvodina (IPHV), Novi Sad, Serbia [15, 16, 20].

The number of persons vaccinated against influenza, stratified by age groups within high-risk populations and by HCW status, was collected annually through collaboration between IPHV and the six district Institutes of Public Health located in Subotica, Sombor, Pančevo, Sremska Mitrovica, Kikinda, and Zrenjanin, Serbia.

Statistical analysis

Weekly incidence rates of ILI and ARI were calculated *per* 100,000 inhabitants, and age-specific weekly rates were estimated for the five previously defined age groups. The epidemic threshold (baseline) of 246.3 cases *per* 100,000 inhabitants, representing the medium level of influenza activity, was defined based on weekly ILI incidence rates from five pre-pandemic (2004/05–2008/09) sentinel seasons in APV [20].

Comparisons between the 24–64-year and ≥ 65-year age groups were performed by gender, place of residence, month of death notification, number of comorbidities, influenza virus type, clinical diagnosis (SARI or ARDS), and observation period (seasons 2015/16–2019/20 vs. 2020/21–2024/25), using the Chi-squared or Fisher's exact test, as appropriate. For continuous variables–specifically, the number of days from symptom onset to laboratory confirmation, and from symptom onset to death–Student's *t*-test was applied. A p-value < 0.05 was considered statistically significant. All statistical analyses were conducted using SPSS software, version 21 (IBM Corp., Armonk, NY, USA).

Ethical considerations

Data from this retrospective study were obtained from surveillance conducted at the Center for Disease Control and Prevention of the IPHV, Novi Sad, which coordinates influenza surveillance across the entire territory of APV. As this is part of routine surveillance practice established for many years [15, 16, 20], approval from the Ethics Committee was not required in Serbia. All patient data were anonymized and de-identified in accordance with ethical standards.

Results

Influenza-like illness

Over ten consecutive influenza seasons (2015/16 to 2024/25), weekly incidence rates of ILI in APV displayed substantial seasonal variability, with epidemic activity usually occurring between weeks 5 and 10. The epidemic threshold (baseline: 246.3 cases *per* 100,000 inhabitants) was surpassed in all pre-coronavirus disease 2019 (COVID-19) seasons (2015/16–2019/20) and in the recent three influenza seasons (2022/23–2024/25). Before the COVID-19 pandemic, seasonal peaks were typically observed between weeks 5 and 7, with exceptionally high activity in 2019/20 (peak 881.1 *per* 100,000 in week 6) and other intense waves in 2017/18 (500.5 *per* 100,000, week 5) and 2018/19 (497.1 *per* 100,000, week 7). In contrast, ILI incidence remained far below the epidemic threshold throughout 2020/21, coinciding with the onset of the COVID-19 pandemic. The 2021/22 season showed a delayed and more gradual pattern compared with pre-pandemic years, with incidence peaking later than usual in weeks 11–13 (maximum 249.1 *per* 100,000) and only approaching but not exceeding the epidemic threshold. In subsequent seasons, influenza activity rebounded. In 2022/23, incidence peaked at 343.6 *per* 100,000 (week 6), while in 2023/24 a higher peak of 422.0 *per* 100,000 was recorded (week 7). Preliminary 2024/25 data indicate sustained transmission, with the epidemic threshold exceeded from week 6 and a peak of 273.6 *per* 100,000 in week 8 (Figure 1).

Across all seasons, the highest incidence rates of ILI were consistently observed in children aged 0–4 and 5–14 years, with peak weekly rates exceeding 700–1,000 *per* 100,000 inhabitants during several weeks (e.g., weeks 6–8 in 2015/16, 2017/18, 2018/19, and 2019/20). Adolescents and young adults (15–29 years) exhibited intermediate rates, while the lowest incidence was observed among individuals aged ≥ 65 years, although substantial increases were noted during high-intensity seasons (e.g., 2022/23 and 2023/24). The timing and magnitude of seasonal peaks varied between seasons. Several seasons (e.g., 2017/18, 2018/19, and 2019/20) showed sharp, early peaks dominated by pediatric age groups, whereas others, such as 2021/22, had later peaks extending into weeks 12–15, coinciding with broader age distribution of cases. The 2020/21 season showed markedly attenuated ILI activity across all age groups (Figure 2).

Acute respiratory infections

From the 2015/16 through the ongoing 2024/25 respiratory seasons, weekly incidence rates of ARI in APV fluctuated substantially, with clear seasonal peaks each year, typically between epidemiological weeks 4 and 10. Incidence rates ranged from below 200 *per* 100,000 inhabitants during inter-epidemic weeks to over 2,500 *per* 100,000 inhabitants during the peak of ARI activity. The highest peak across the surveillance period was observed in the 2022/23 season (week 6), reaching 2,703 *per* 100,000 inhabitants. Other notable high-intensity seasons included 2017/18 (peak 2,465 *per* 100,000 in week 5), 2018/19 (2,060 *per* 100,000 in week 5), and 2019/20 (2,183 *per* 100,000 in week 5). By contrast, the 2020/21 season (coinciding with the start of the COVID-19 pandemic) showed markedly lower activity, with weekly rates remaining below 1,100 *per* 100,000 inhabitants. Seasonal peaks tended to occur in February (weeks 5–7) in most pre-pandemic seasons, whereas post-2020 fluctuations

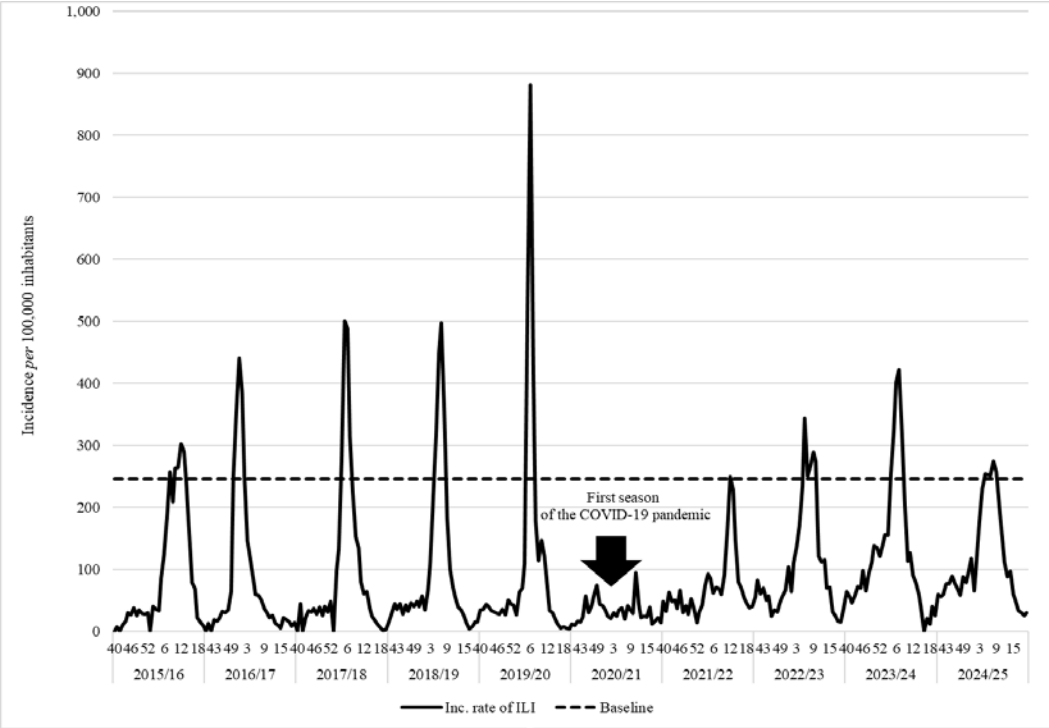

Fig. 1 – Weekly (October–May) incidence of influenza-like illness (ILI) *per* 100,000 inhabitants in the Autonomous Province of Vojvodina, Serbia, 2015/16–2024/25 seasons. COVID-19 – coronavirus disease 2019; Inc. – incidence.

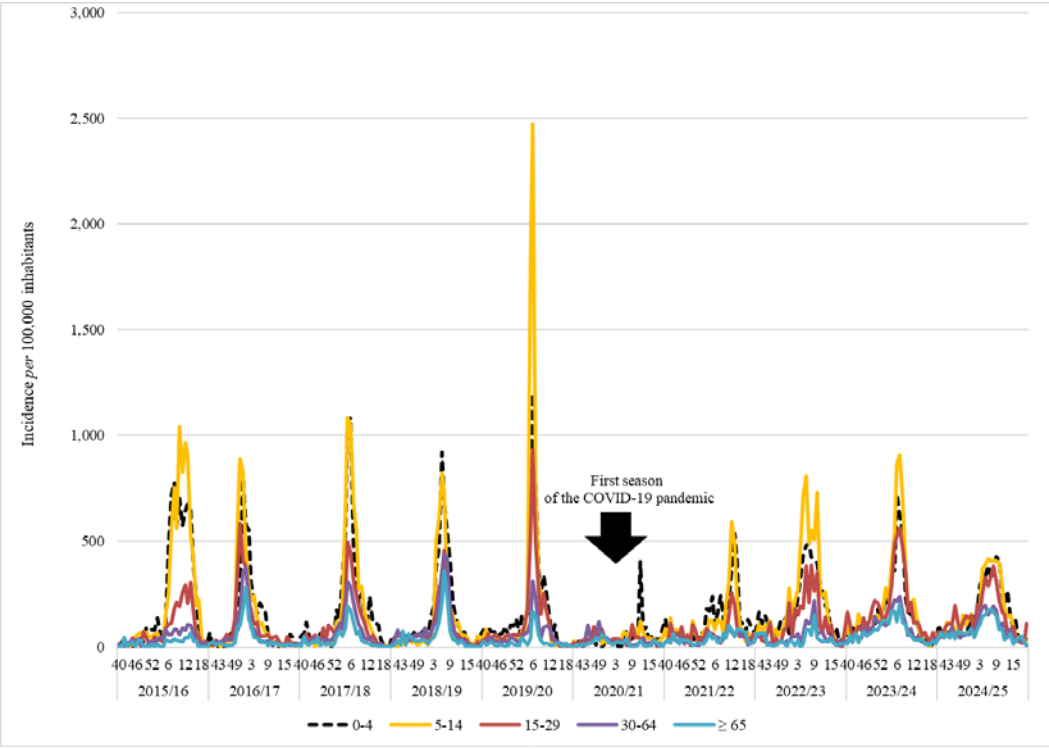

Fig. 2 – Weekly (October–May) age-specific incidence of influenza-like illness *per* 100,000 inhabitants in the Autonomous Province of Vojvodina, Serbia, 2015/16–2024/25 seasons. COVID-19 – coronavirus disease 2019.

demonstrated more variable patterns. The 2021/22 season exhibited a prolonged late peak, extending from weeks 10 to 13 with weekly rates above 1,800 *per* 100,000 inhabitants, while 2023/24 had an earlier and sharper peak in weeks 4–6 (maximum 2,396 *per* 100,000 in week 5). The current 2024/25 season shows a moderate trajectory, with incidence peaking thus far at 1,655 *per* 100,000 inhabitants (week 6) (Figure 3).

Weekly age-specific incidence rates of ARI *per* 100,000 inhabitants in APV showed that children aged 0–4 years and school-aged children (5–14 years) consistently exhibited the highest weekly ARI incidence, frequently exceeding 4,000–6,000 cases *per* 100,000 inhabitants during seasonal peaks. Adults aged 15–29 years showed intermediate incidence rates, whereas adults aged 30–64 years and those ≥ 65 years had substantially lower rates, typically below 1,000 cases *per* 100,000 inhabitants except during major peaks. Marked inter-seasonal variability was observed. Peak activity commonly occurred between weeks 5 and 10. However, the 2021/22 season demonstrated a delayed peak compared with pre-COVID-19 seasons, while the 2020/21 season recorded notably lower incidence across all age groups (Figure 4).

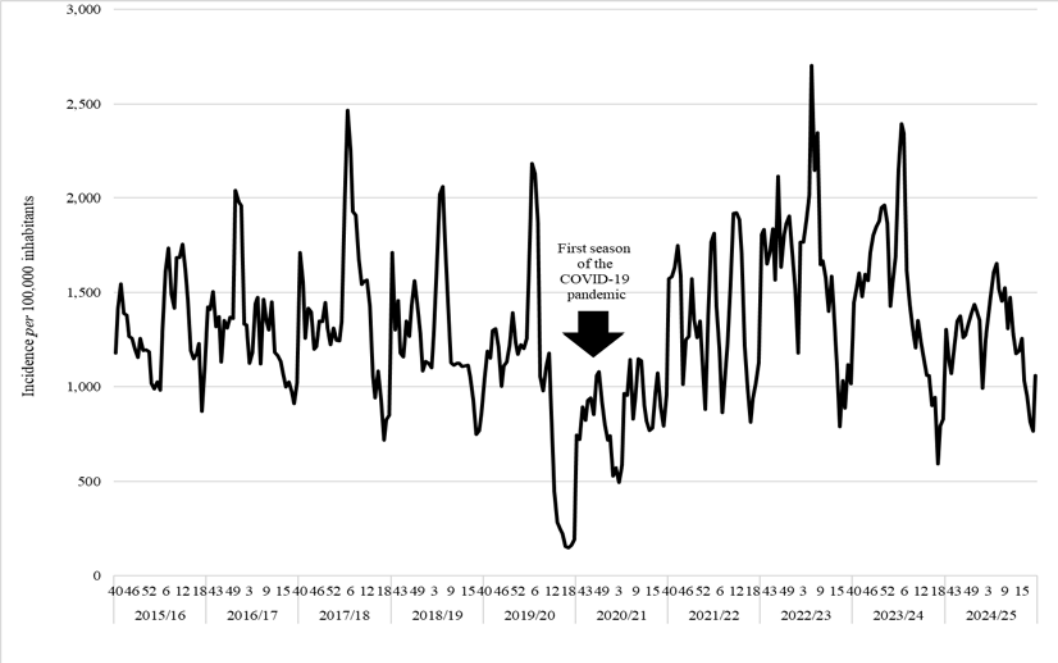

Fig. 3 – Weekly (October–May) incidence of acute respiratory infections *per* 100,000 inhabitants in the Autonomous Province of Vojvodina, Serbia, 2015/16–2024/25 seasons.
COVID-19 – coronavirus disease 2019.

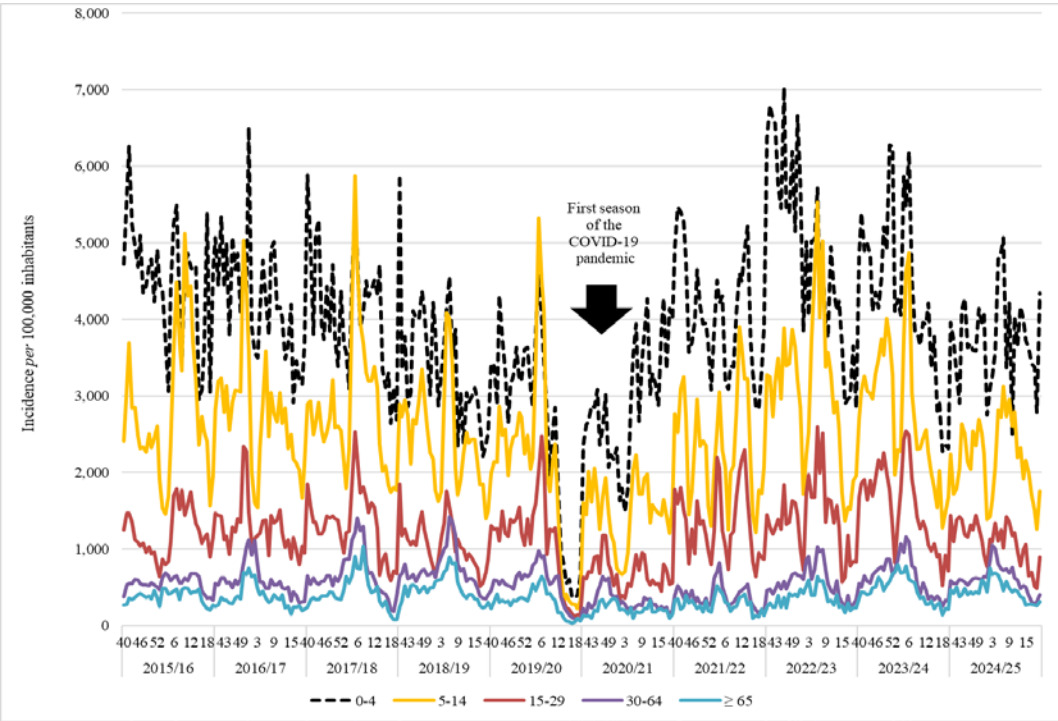

Fig. 4 – Weekly (October–May) age-specific incidence of acute respiratory infections *per* 100,000 inhabitants in the Autonomous Province of Vojvodina, Serbia, 2015/16–2024/25 seasons.
COVID-19 – coronavirus disease 2019.

Laboratory-confirmed influenza-associated deaths

Between the 2015/16 and 2024/25 influenza seasons, a total of 202 laboratory-confirmed influenza-associated deaths were reported in APV. The highest number of fatal cases was recorded during the 2018/19 season, with 57 (28.2% of all reported fatalities) deaths, followed by 37 (18.3%) deaths in the 2017/18 season, and 35 (17.3%) deaths in the 2019/20 season. In contrast, no influenza-related deaths were recorded during the 2020/21 season, which coincided with the first year of the COVID-19 pandemic (Figure 5).

None of the patients with fatal outcomes had received vaccination prior to influenza. Among the 202 laboratory-confirmed fatal influenza cases, 56.4% occurred in males. The proportion of male deaths was significantly higher among individuals aged 24–64 years (65.1%) compared to those aged ≥ 65 years (50.0%) (p = 0.0326). The majority of cases resided in urban areas (54.5%), with no significant difference in place of residence between the two age groups. The mean time from symptom onset to laboratory confirmation of influenza was similar across age groups (overall mean: 3.0 ± 2.9 days; p = 0.9145). Likewise, the average duration from illness onset to death did not differ significantly across age groups (mean: 12.5 ± 10.2 days; p = 0.7277). Most deaths occurred during the winter months, with the highest number reported in February (46.5%), followed by January (25.2%) and March (19.4%), but the monthly distribution of deaths did not differ significantly between the two age groups (p = 0.7199). Regarding comorbidities, individuals aged ≥ 65 years were more likely to have multiple underlying conditions, with only 0.9% having no comorbidities, compared to 7.0% in the 24–64 age group (p = 0.0292). Over ten consecutive influenza seasons, influenza A(H1N1)pdm09 was the most frequently identified virus type (59.4%), with a similar distribution across two age groups (p = 0.7056). Other detected types included A(H3N2) (19.4%), influenza B (15.3%) and unsubtyped influenza A (5.9%). Regarding clinical presentation, 51.0% of patients were diagnosed with ARDS, which was more frequent in the 24–64 age group than in older adults (58.1% vs. 45.7%), although the difference was not statistically significant (p = 0.0808). When stratified by age and surveillance period, 84.9% of fatal cases aged 24–64 years and 82.8% of those aged ≥ 65 years occurred in the earlier period (2015/16–2019/20), with no statistically significant difference in the distribution of fatal cases between the two periods (p = 0.6870) (Table 1).

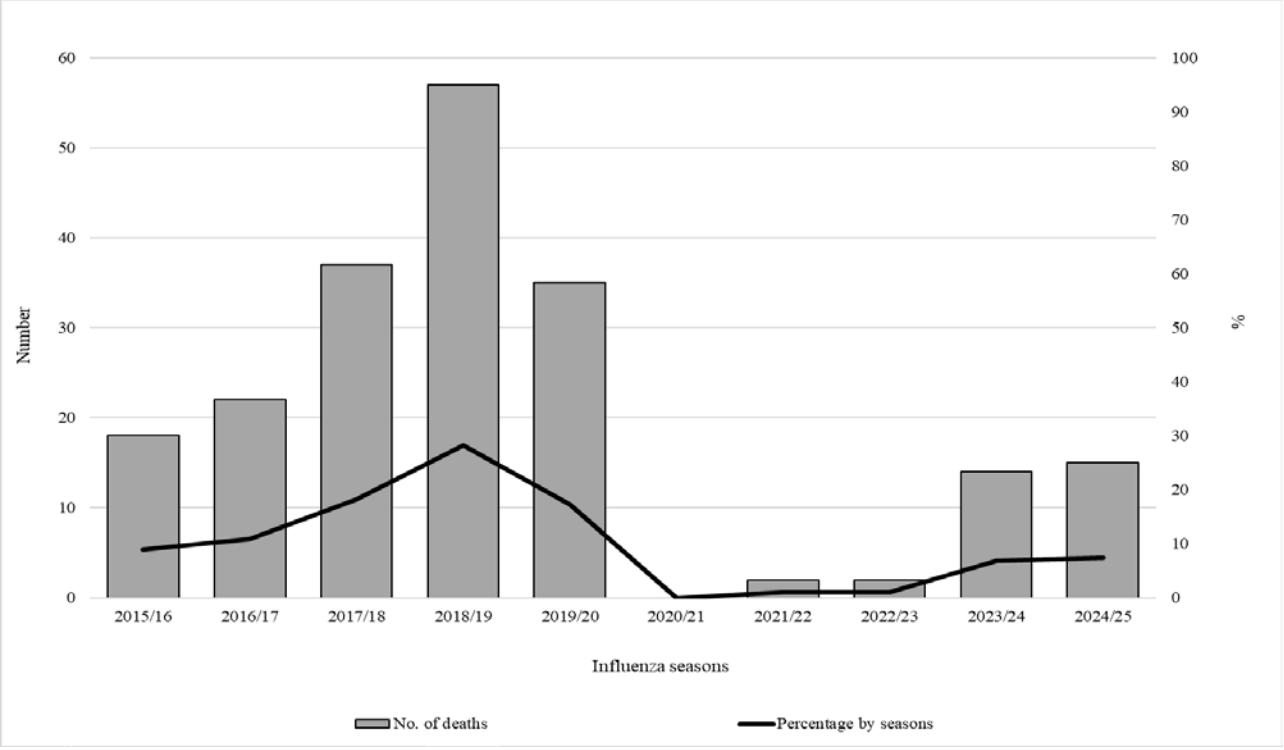

Fig. 5 – Influenza-associated deaths by years in the Autonomous Province of Vojvodina, Serbia, 2015/16–2024/25 seasons.
No. – number.

Table 1

Comparison of fatal influenza cases aged 24–64 and ≥ 65 years in the Autonomous Province of Vojvodina, Serbia, 2015/16–2024/25 seasons

Parameters	Fatal influenza cases			*p*-value
	total (n = 202)	24–64 years (n = 86)	≥ 65 years (n = 116)	
Gender				
male	114 (56.4)	56 (65.1)	58 (50.0)	**0.0326**
female	88 (43.6)	30 (34.9)	58 (50.0)	
Place of residence				
rural	92 (45.5)	41 (47.7)	51 (44.0)	0.6016
urban	110 (54.5)	45 (52.3)	65 (56.0)	
Days between onset and laboratory confirmation	3.0446 ± 2.8622	3.0698 ± 2.6112	3.0259 ± 3.0459	0.9145
Days between onset of illness and death	12.5347 ± 10.1722	12.2442 ± 9.2022	12.7500 ± 10.8699	0.7277
Month of death				
December	4 (2.0)	2 (2.3)	2 (1.7)	0.7199
January	51 (25.2)	24 (27.9)	27 (23.3)	
February	94 (46.5)	34 (39.5)	60 (51.7)	
March	39 (19.4)	19 (22.2)	20 (17.3)	
April	14 (6.9)	7 (8.1)	7 (6.0)	
Number of comorbidities*				
0	7 (3.5)	6 (7.0)	1 (0.9)	**0.0292**
1	88 (43.5)	36 (41.9)	52 (44.8)	
2	67 (33.2)	33 (38.3)	34 (29.3)	
3	32 (15.8)	8 (9.3)	24 (20.7)	
> 3	8 (4.0)	3 (3.5)	5 (4.3)	
Type of influenza				
A non-subtyped	12 (5.9)	4 (4.7)	8 (6.9)	0.7056
A (H1N1)pdm09	120 (59.4)	55 (64.1)	65 (56.0)	
A (H3N2)	39 (19.4)	15 (17.4)	24 (20.7)	
B	31 (15.3)	12 (14.0)	19 (16.4)	
Diagnosis				
SARI	99 (49.0)	36 (41.9)	63 (54.3)	0.0808
ARDS	103 (51.0)	50 (58.1)	53 (45.7)	
Period				
2015/16–2019/20	169 (83.7)	73 (84.9)	96 (82.8)	0.6870
2020/21–2024/25	33 (16.3)	13 (15.1)	20 (17.2)	

SARI – severe acute respiratory infection; ARDS – acute respiratory distress syndrome; n – number.
All values are given as numbers (percentages) or mean ± standard deviation.
Values that differ significantly ($p < 0.05$) are marked in bold.
***Note*: * – included chronic cardiovascular, cerebrovascular, pulmonary, renal, and liver diseases, immunodeficiency (including malignancy and human immunodeficiency virus), diabetes, and obesity, as well as long-term smoking and alcohol consumption.**

Immunization against influenza

Between the 2015/16 and 2024/25 seasons, the number of persons vaccinated against influenza in APV ranged from 57,570 in 2015/16 to a peak of 108,374 in 2020/21, averaging 77,842 *per* season (Figure 6).

The majority of vaccinated persons were aged ≥ 65 years (ranging from 36,989 to 66,337 annually), followed by adults aged 20–64 years (19,001–40,814). The number of vaccinated children at high risk of complications was low: 5–19 years (ranging from 306 to 1,186) and 6 months–4 years (fewer than 180 in most seasons). A sharp increase occurred in 2020/21 during the COVID-19 pandemic, after which numbers declined but stayed above pre-2019/20 levels. More precisely, the highest vaccination uptake was observed among those aged ≥ 65 years (rising from 9.8% in 2015/16 to 17.5% in 2020/21, and later stabilizing at 14.0–16.0%). Adults aged 20–64 years had modest coverage (1.9–4.0%), while adolescents (5–19 years) and young children (6 months–4 years) remained below 0.5% (Figure 7).

Between 2015/16 and 2018/19, 4,000–5,000 HCWs were vaccinated annually, corresponding to 16.0–21.0% coverage. A marked increase was observed in 2019/20 (≈ 8,900 vaccinated; 32.1% coverage), with a peak in 2020/21 when over 11,000 HCWs received the vaccine, reaching more than 40.0% coverage. After this peak, uptake declined sharply to about 20.0% in the 2021/22 season, and then fluctuated between 21.0% and 25.0% (5,400–6,800 vaccinated annually) during the 2022/23–2024/25 seasons (Figure 8).

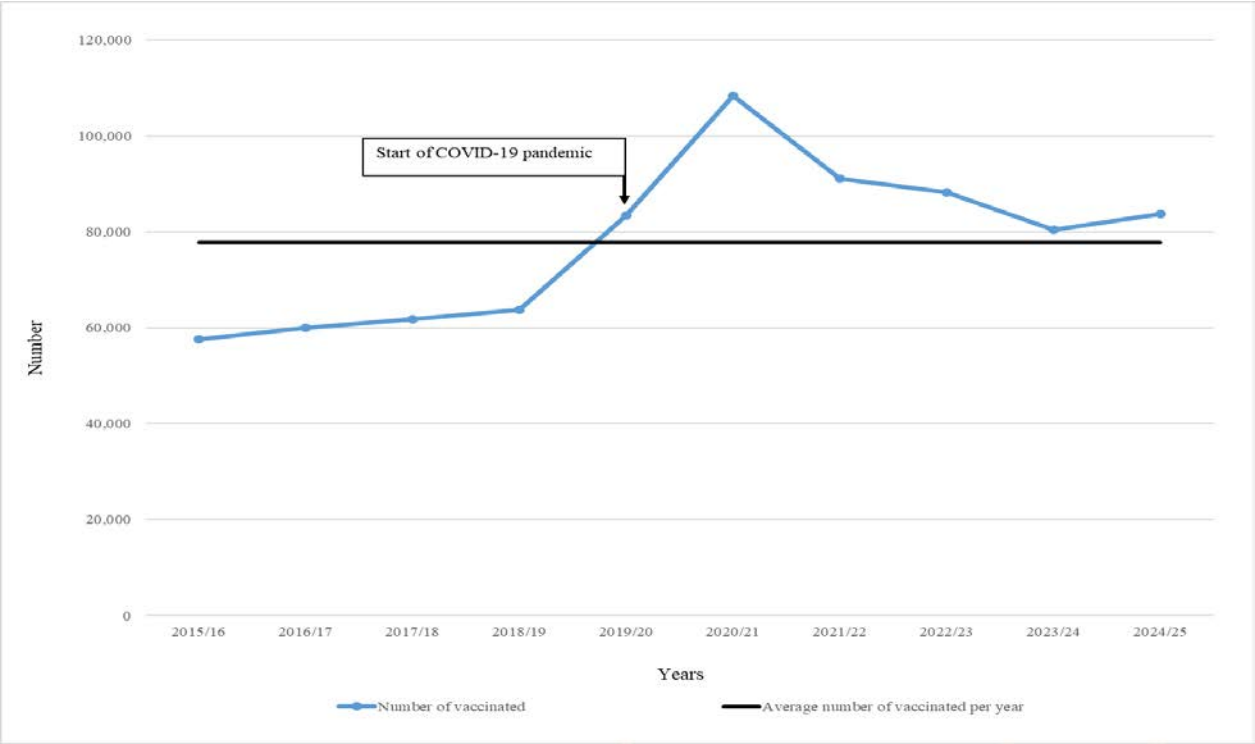

Fig. 6 – Number of high-risk individuals vaccinated against influenza in the Autonomous Province of Vojvodina, Serbia, 2015/16–2024/25 seasons.
COVID-19 – coronavirus disease 2019.

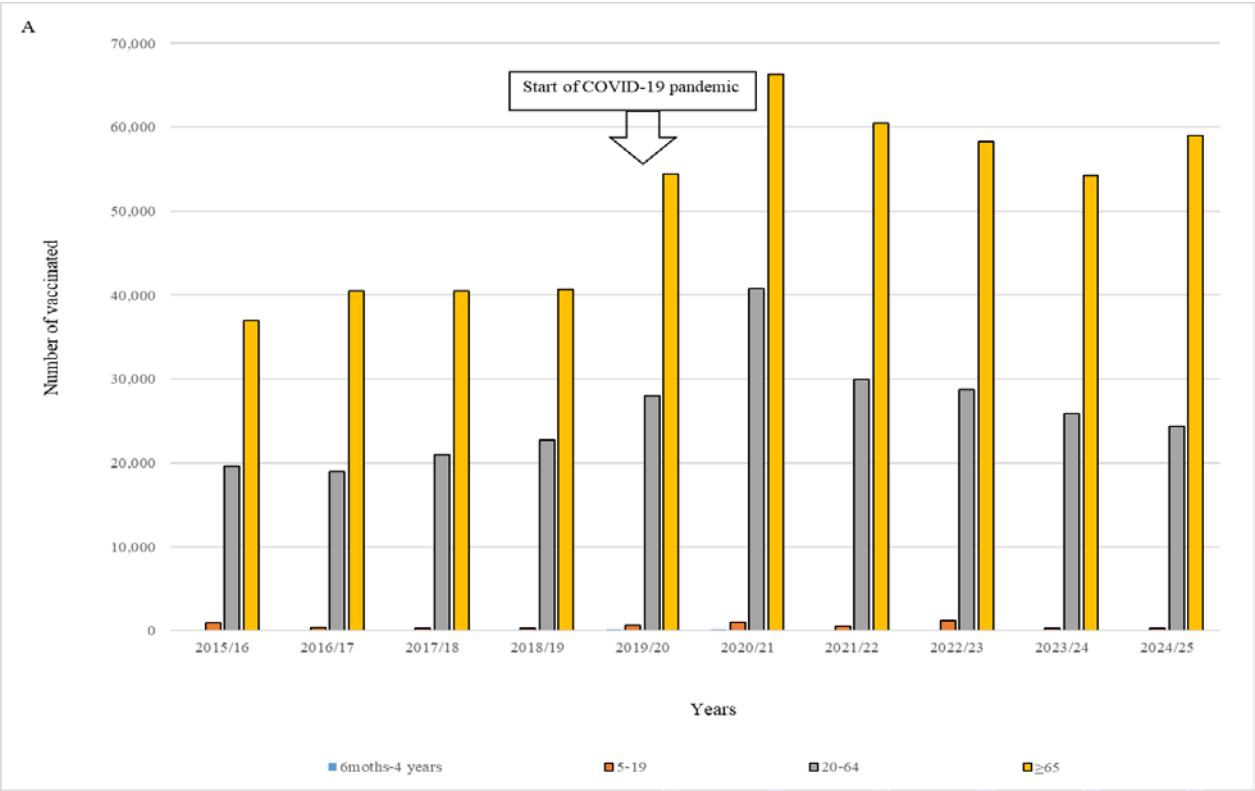

Fig. 7 – Number (A) and percentage (B) of high-risk individuals vaccinated against influenza by age groups in the Autonomous Province of Vojvodina, Serbia, 2015/16–2024/25 seasons.
COVID-19 – coronavirus disease 2019.

***Note*: Fig. 7 continued on next page.**

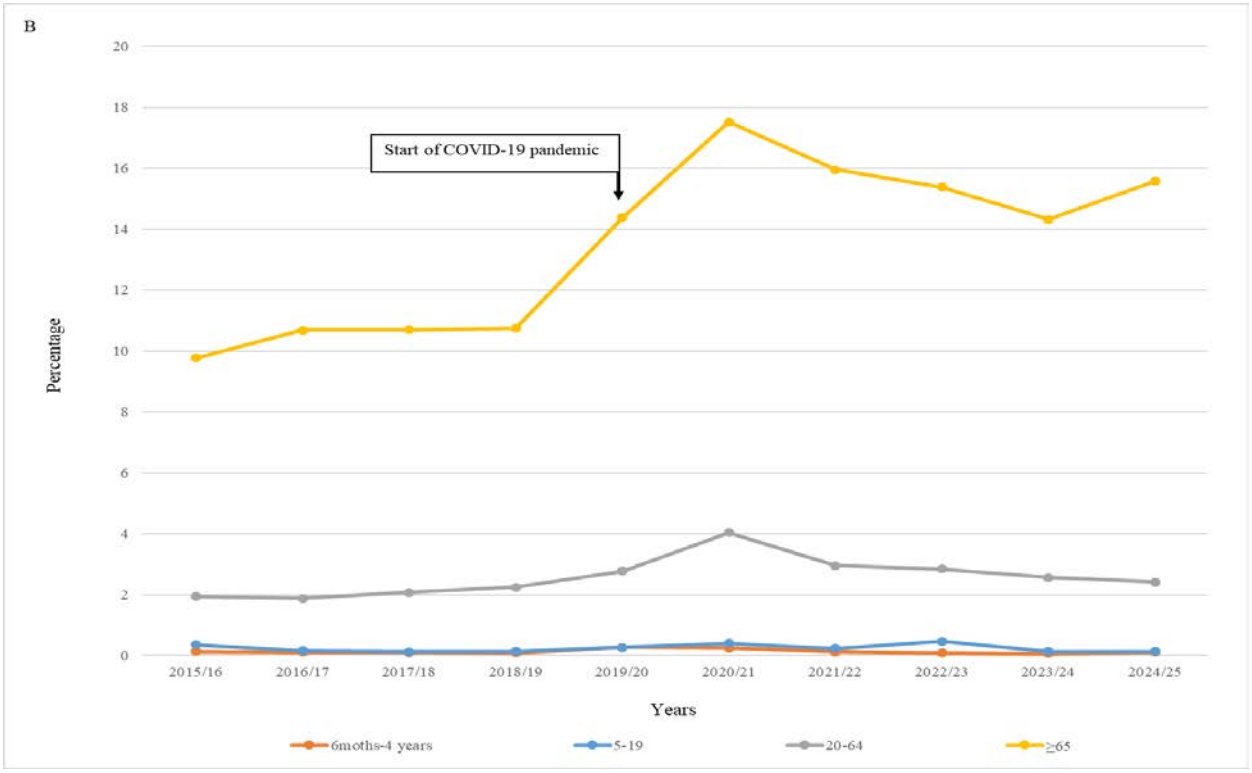

Fig. 7 (Continued) – Number (A) and percentage (B) of high-risk individuals vaccinated against influenza by age groups in the Autonomous Province of Vojvodina, Serbia, 2015/16–2024/25 seasons. COVID-19 – coronavirus disease 2019.

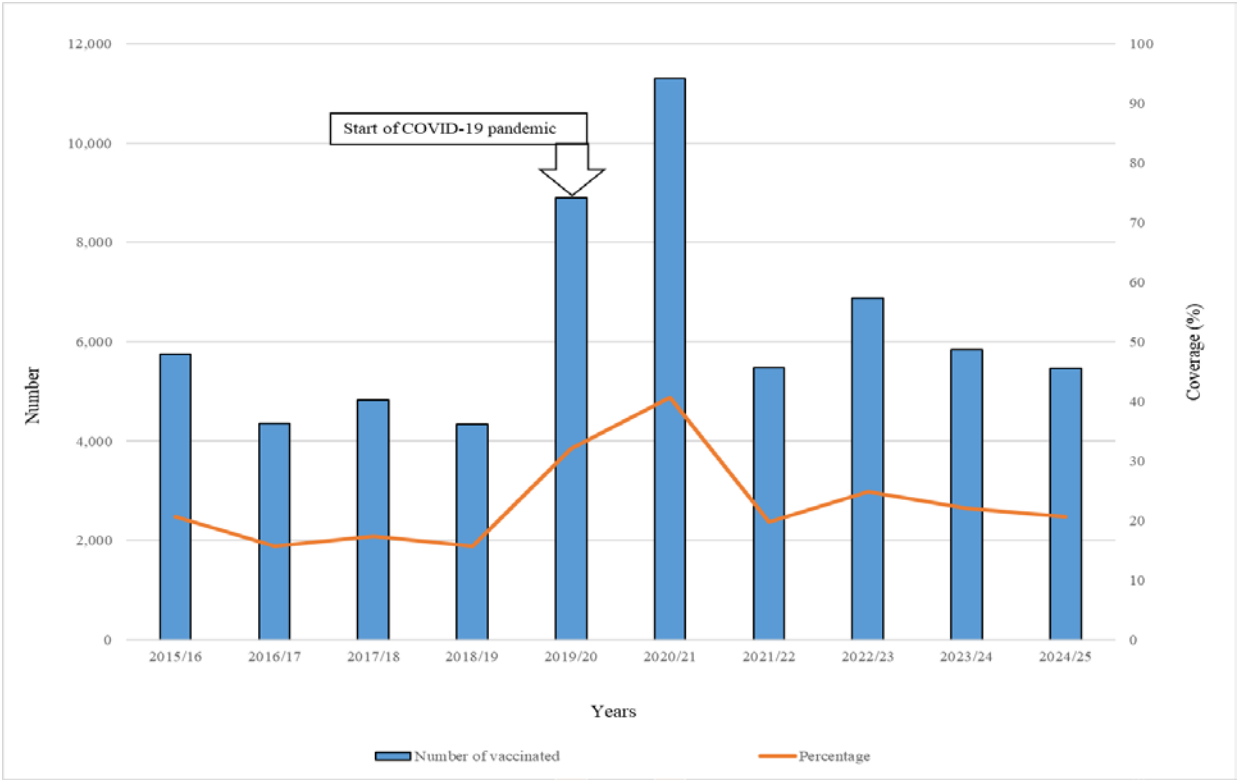

Fig. 8 – Coverage of immunization against influenza among healthcare workers in the Autonomous Province of Vojvodina, Serbia, 2015/16–2024/25 seasons. COVID-19 – coronavirus disease 2019.

Discussion

Building on previously published results of influenza surveillance in APV over five seasons (2010/11–2014/15) [15, 16], this study provides a comprehensive overview of influenza activity in the province over the subsequent ten consecutive seasons (2015/16–2024/25), encompassing both the pre- and post-COVID-19 pandemic periods.

As observed across European countries and globally, influenza activity was virtually absent during the 2020/21 season [9–12]. For instance, in the WHO European Region, the epidemic threshold of 10% positivity among tested samples was not reached during the 2020/21 influenza season, and detections of seasonal influenza, as well as ILI and ARI cases, were lower than those typically observed even during regular summer months [21]. The widespread implementation of non-pharmaceutical interventions against SARS-CoV-2, along with virus competition and the lower R_0 of influenza compared to SARS-CoV-2, likely contributed to the pronounced reduction in influenza circulation [22].

Sentinel surveillance is considered the gold standard for monitoring influenza activity, providing high-quality data from a defined outpatient population (ILI or ARI), together with virus characterization analyses [21]. Based on our sentinel surveillance results, we found that, for reasons yet unexplained, the highest weekly ILI incidence rates in APV over ten consecutive seasons were recorded in the season preceding the COVID-19 pandemic. Our results also showed that, across ten consecutive seasons, the highest weekly peak incidence was usually recorded between weeks 5 and 10. Excluding the 2020/21 influenza season (the beginning of the COVID-19 pandemic), atypical influenza dynamics persisted into 2021/22, characterized by delayed and attenuated epidemic waves, with incidence peaking later than usual, between weeks 11 and 13–a pattern also observed in other countries, and explained by the withdrawal of SARS-CoV-2 from circulation, after which influenza virus re-emerged [23, 24]. However, from 2022/23 onwards, seasonal influenza activity began returning to pre-pandemic levels in both timing and intensity, although some variation in peak magnitude and age distribution remained [25].

The highest incidence of ILI and ARI consistently occurred in younger populations, particularly among those aged 0–4 and 5–14 years, underscoring their role as key drivers of influenza virus transmission. These findings are consistent with reports from other European surveillance networks and are widely recognized in the literature [25–27]. In contrast, individuals aged ≥ 65 years showed lower incidence rates of both ILI and ARI but accounted for a substantial proportion of severe and fatal outcomes, particularly during high-intensity seasons.

Our results also showed that ARI incidence rates were highest in the 2017/18 season, but substantially higher in the post-COVID-19 period, specifically during the 2022/23 and 2023/24 seasons. The increased frequency of ARI in the post-COVID period may be attributed to the resurgence of respiratory viruses, not only influenza, which had been suppressed during the 2020–2022 period. Unlike rhinoviruses and enteroviruses, which persisted despite interventions such as masking and school closures–likely due to their greater surface stability and alternative transmission routes–many other respiratory viruses notably increased following the mitigation of the COVID-19 pandemic [28–32].

During 2020/21, ILI and ARI incidence declined in all age groups, but the highest rates were recorded in children aged 0–4 years, probably reflecting continued kindergarten attendance during the COVID-19 pandemic. The predominance of ILI in school-aged children (5–14 years)–a group considered the main reservoir for seasonal influenza transmission [30] compared with preschool-aged children (0–4 years)–was first observed at the end of the 2021/22 influenza season, coinciding with the re-emergence of seasonal influenza, and persisted until the end of the study period (2024/25 season).

Over the ten-season period, 202 laboratory-confirmed influenza-associated deaths were recorded. The highest number of fatal cases occurred over three consecutive seasons (2017/18–2019/20), during which 64% of all fatal cases were recorded, coinciding with the highest ILI incidence observed across all seasons in APV. During the 2020/21 season, when ILI incidence was below baseline, no deaths attributable to influenza were reported.

Our analysis revealed a higher proportion of male deaths among adults aged 24–64 years compared to those aged ≥ 65 years. Although the reasons for these gender differences are not yet fully understood [33, 34], there is some evidence that estradiol is a potent anti-inflammatory hormone that reduces the severity of influenza A virus infection in females [35]. This may explain the pattern observed in our cohort aged 24–64 years, but not in those aged ≥ 65 years. The equal distribution of influenza-related fatal cases among individuals aged ≥ 65 years, regardless of gender, may be explained by immunosenescence associated with increasing age and reduced physiological resilience, which together impair the ability to mount an effective immune response and increase the risk of complicated clinical forms of influenza [1–3, 8, 14, 25, 26].

Although not statistically significant, we observed a slight predominance of urban residence among fatal cases compared to rural areas, which aligns with known patterns of influenza transmission in densely populated regions [36].

We found that time intervals from symptom onset of influenza to laboratory confirmation and to death were similar across the 24–64 and ≥ 65-year age groups, suggesting comparable disease progression once severe illness developed. Although data on outcome severity and length of hospital stay are available [37–39], no evidence was found to support uniform disease progression across these two age groups, suggesting this warrants further investigation. The reasons for this are likely related to the nearly even distribution of comorbidities across both age groups studied, except for the presence of three concurrent comorbidities, which were significantly more frequent among influenza-hospitalized patients aged ≥ 65 years. The impact of comorbidities on influenza outcomes has been well described previously [40–43].

During the study period, almost 60% of all fatal cases were attributed to influenza A(H1N1)pdm09. The predominance of A(H1N1)pdm09 among fatal cases, with a similar distribution across age groups, reflects circulating strain patterns reported across Europe and globally in recent seasons [5, 44]. We also found that clinical presentations (SARI or ARDS) in fatal cases were nearly evenly distributed, with no significant trend toward more frequent ARDS diagnoses among younger adults, a finding consistent with previous studies [45, 46]. Due to the absence of notable influenza activity during the COVID-19 period, most fatal cases (84%) occurred in earlier observed seasons (2015/16–2019/20). However, no significant shifts in the distribution of fatal cases between the two age groups were observed over time, indicating stable fatality patterns despite fluctuations in influenza activity and public health interventions.

Public interest in influenza vaccination rose markedly immediately before and during the onset of the COVID-19 pandemic. In APV, during the October–November 2020 period, all influenza vaccine doses from the first distribution tranche in primary care were rapidly administered, reflecting heightened demand. However, additional vaccine supplies were delayed until late 2020, by which time interest had already started to decline [47].

The Spanish nationwide ENE-COVID study, involving nearly 29,000 adults from established risk groups, showed a marked increase in influenza vaccination coverage during the first year of the COVID-19 pandemic – from 31% in 2019 to almost 47% in 2020. The largest gains were among older adults (from 58% to 75%) and HCWs (over 20 percentage points). Only the ≥ 65-year group reached the national target, highlighting persistent gaps in other priority groups. These findings indicate that the COVID-19 pandemic boosted influenza vaccination, but structural and behavioral barriers remain, emphasizing the need for targeted strategies to further improve uptake [48].

A recent multi-country analysis from France, Italy, Spain, and the United Kingdom showed that current influenza vaccination programs, though below the WHO 75% target, prevent substantial morbidity, mortality, and healthcare costs, particularly among older adults and those with chronic conditions. In 2021/22, coverage ranged from 44% to 65%, averting an estimated 1.9 million cases and 38,000 deaths. Achieving the target could prevent nearly one million additional cases, highlighting both the value of vaccination and the need for reinforced strategies to close persistent gaps in high-risk groups [49].

Despite being mandatory, influenza immunization coverage among HCWs in APV remained suboptimal throughout the study period. Coverage rose substantially during the first pandemic year, peaking at 40.7% in 2020/21– the highest observed in the study period. This surge likely reflects a combination of pandemic-related risk perception and prior educational initiatives on the importance of influenza vaccination, conducted in 2019 across several tertiary healthcare institutions in APV, coinciding with the introduction of the quadrivalent influenza vaccine in Serbia. However, the absence of high-intensity influenza virus circulation during 2019–2021 likely reduced perceived risk, and vaccination uptake subsequently reverted to pre-pandemic levels in APV, despite influenza re-emerging from 2021/22 onwards at intensities typical of the pre-COVID era. This pattern underscores the challenge of sustaining increased uptake once the acute sense of pandemic threat dissipates.

Comparable observations have been described in other regions. A three-year evaluation of influenza vaccination among healthcare workers at a large university hospital in Pisa showed a surge from persistently low coverage, 10–12% in 2018/19–2019/20, to 39.3% in 2020/21, reflecting the COVID-19 pandemic's role as a catalyst. A survey indicated that 71% of respondents considered influenza vaccination more important in the pandemic context [50]. Similarly, a European review of 14 studies reported a 17–38% increase in influenza vaccine uptake among HCWs in 2020/21 compared with 2019/20, driven by COVID-19-related factors such as greater vaccine willingness, fear of infection, and symptom confusion. Despite these gains, hesitancy persists, suggesting that only mandatory vaccination policies may sustain high coverage in Europe [51].

Public health relevance

Our findings offer important insights into temporal trends and age-specific patterns of influenza activity before, during, and after the COVID-19 pandemic in a southeastern European context.

Similar to previously published experiences in influenza surveillance in APV [15, 16, 20, 30, 52–57], our results continually underscore the importance of maintaining resilient, comprehensive, multi-component surveillance systems that can detect atypical influenza seasons and inform timely public health responses. The consistent observation of high incidence of both ILI and ARI among children, alongside severe outcomes in older adults, supports the prioritization of these groups in seasonal vaccination programs and other preventive measures. In a typical influenza season, particularly school-aged children are most frequently affected and act as the main reservoirs for influenza transmission, while severe cases and deaths occur predominantly among older adults and individuals with comorbidities. This pattern becomes even more pronounced during seasons of higher ILI intensity. Furthermore, the disruption of typical seasonal dynamics during the pandemic underscores the need to better understand long-term shifts in population immunity and virus circulation. These findings may inform European-level strategies on pandemic preparedness, influenza vaccination, and the integration of respiratory virus surveillance.

Considering the number of individuals vaccinated against influenza in high-risk populations and among HCWs, our findings highlight the need for urgent interventions to increase influenza vaccine coverage.

Strengths and limitations of the study

This study has several notable strengths. Our study was based on integrated and continuous surveillance of ILI, ARI,

and severe outcomes (SARI/ARDS) over a ten-season period in a defined geographical region. The use of standardized case definitions, consistent surveillance methodology, and age-specific incidence analysis allowed for robust monitoring of seasonal patterns and the impact of the COVID-19 pandemic. Additionally, the inclusion of virological data and fatal outcomes offers a comprehensive view of the influenza burden, going beyond outpatient trends.

However, several limitations should be acknowledged. First, although sentinel surveillance provides valuable trend data, it may underestimate the absolute number of influenza cases, particularly in populations with limited healthcare-seeking behavior. Second, hospital-based surveillance may miss severe cases who were not admitted or tested, especially during the peak of the COVID-19 pandemic when healthcare systems were under strain. Third, virological testing was not performed for all suspected cases, particularly in outpatient settings, and subtype identification was not always possible. Fourth, the lack of classification of patients by community- vs. healthcare-associated influenza may have introduced bias in the variable "Days between onset and laboratory confirmation". Fifth, the lack of information on the number of days spent on mechanical ventilation for patients with ARDS may have influenced the calculated mean value of the variable "Days between onset of illness and death". Lastly, except for the ≥ 65-year-old age group, we did not have access to the number (denominator) of patients with comorbidities for whom influenza vaccination is mandatory. However, given the exceptionally low percentages observed across age groups, it can be assumed that coverage in these risk groups was also extremely low. Although insufficient, influenza vaccination coverage was measured for all HCWs in institutions in APV. Therefore, it can be assumed that coverage is higher among HCWs working in departments with increased risk.

Despite the numerous limitations of this study, we believe they did not substantially affect the main findings of our research.

Conclusion

Our findings emphasize the need for preparedness for atypical influenza seasons in the post-COVID-19 era and targeted prevention strategies, including vaccination of high-risk groups. Children consistently experience the highest incidence, while most influenza-related deaths occur in seasons of peak influenza-like illness activity, with middle-aged men overrepresented among fatalities. Notably, none of the fatal cases were vaccinated, highlighting the urgent need to improve vaccine uptake through physician awareness and public health education. Persistently low vaccination coverage among high-risk individuals, contrasted with higher but unstable uptake among health-care workers, underscores the need for strengthened and sustainable immunization strategies. Temporary increases in coverage during the COVID-19 pandemic suggest that public awareness can improve uptake, but sustained efforts are essential.

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Validity and reliability of the A-test in assessing the functional capacity of patients with heart failure

Validnost i pouzdanost A-testa u proceni funkcionalnog kapaciteta obolelih od srčane insuficijencije

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Abstract

Background/Aim. Before initiating early rehabilitation, it is essential to assess the functional status of patients with heart failure (HF). Although the Barthel Index (BI) is well-established, the A-test, originally developed for traumatology and orthopedic patients, has recently been introduced into broader clinical practice. The aim of this study was to evaluate the validity and reliability of the A-test in assessing functional status in patients with HF. **Methods.** The study included patients of both sexes with HF, regardless of age or left ventricular ejection fraction, classified according to the New York Heart Association (NYHA) I–IV class, with Mini-Mental State Examination (MMSE) scores ≥ 24, who were tested daily at the same time. Two experienced senior physiotherapists performed the testing, and they completed the A-test and BI separately. **Results.** A total of 77 patients were enrolled, two-thirds of whom were male, with a mean age of 72 years and a mean ejection fraction of 41.83% [standard deviation (SD) = 11.46]. The average NYHA class at admission, 2.75 (SD = 1.05), and discharge, 2.42 (SD = 1.12), showed a statistically significant improvement (Z = -4.914, $p < 0.001$). Content validity was supported by low floor (minimal score = 2.0%) and ceiling (maximal score = 10.8%) effects, a wide range of scores (0–50), and appropriate score distribution. Concurrent validity was confirmed by a strong correlation between the A-test and BI (ϱ = 0.991, $p < 0.001$). Predictive validity was demonstrated, as the A-test score on the first day significantly predicted BI at discharge (β = 0.880, $p < 0.001$, 95% confidence interval: 1.087–1.562). Construct validity was confirmed through four hypotheses. Patients in NYHA class I–II, with higher MMSE scores and higher left ventricular ejection fractions, scored significantly better on the A-test. Age had a significant effect on the results. The A-test demonstrated excellent internal consistency (α = 0.971) and high inter-rater reliability ($\varkappa$ = 0.842). **Conclusion.** The A-test is a valid and reliable instrument for evaluating the functional capacity of patients with HF.

Key words:
heart failure; physical and rehabilitation medicine; predictive value of tests; rehabilitation.

Apstrakt

Uvod/Cilj. Pre početka rane rehabilitacije, potrebno je obolelima od srčane insuficijencije (*heart failure* – HF) uraditi procenu funkcionalnog statusa. Iako je Bartelov indeks (BI) dobro poznat, nedavno je u širu kliničku praksu uveden A-test, osmišljen primarno za pacijente iz oblasti traumatologije i ortopedije. Cilj studije bio je da se ispita validnost i pouzdanost A-testa za procenu funkcionalnog stanja obolelih od HF. **Metode.** U studiju su bili uključeni oboleli od HF, pripadnici oba pola, bez obzira na starost ili ejakcionu frakciju leve komore, klasifikovani prema *New York Heart Association* (NYHA) I–IV klase, sa rezultatima ≥ 24 na mini-testu mentalnog stanja (*Mini-Mental State Examination* – MMSE), koji su testirani svakog dana u isto vreme. Dva iskusna viša fizioterapeuta su vršila testiranje i odvojeno popunjavala A-test i BI. **Rezultati.** Studijom je obuhvaćeno ukupno 77 bolesnika, dve trećine muškog pola, prosečne starosti 72 godine i prosečne ejekcione frakcije 41,83% [standardna devijacija (SD) = 11,46]. Prosečna NYHA klasa na prijemu iznosila je 2,75 (SD = 1,05) a na otpustu 2,42 (SD = 1,12), što je predstavljalo statistički značajno poboljšanje (Z = -4,914, $p < 0,001$). Validnost sadržaja potvrđena je niskim efektima poda (minimalni skor = 2,0%) i plafona (maksimalni skor = 10,8%), širokim opsegom vrednosti skorova (0–50) i odgovarajućom distribucijom skorova. Konkurentna validnost je potvrđena jakom korelacijom između A-testa i BI (ϱ = 0,991, $p < 0,001$). Prediktivna validnost je dokazana jer je skor A-testa prvog dana značajan prediktor za BI na otpustu

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(β = 0,880, p < 0,001, 95% *confidence interval*: 1,087–1,562). Konstruktivna validnost potvrđena je kroz četiri hipoteze. Bolesnici NYHA I i II klase, sa višim rezultatima na MMSE testu i višim ejekcionim frakcijama leve komore, pokazali su značajno bolje rezultate na A-testu. Godine starosti imale su značajan uticaj na rezultate. A-test je pokazao izuzetnu internu konzistentnost (α = 0,971) i visoku pouzdanost između ocenjivača ($\varkappa$ = 0,842). **Zaključak.** A-test je validan i pouzdan instrument za procenu funkcionalne sposobnosti obolelih od HF.

Ključne reči:
srce, insuficijencija; medicina, fizikalna i rehabilitacija; testovi, prognostička vrednost; rehabilitacija.

Introduction

Heart failure (HF) is the inability of the heart to pump sufficient amounts of blood to peripheral tissues and is the leading cause of death in the developed world [1–3]. More than 64 million people suffer from HF, half of whom have reduced left ventricular ejection fraction (EF) – LVEF [1, 2, 4]. The five-year mortality rate is 50–75% [2]. Almost all heart diseases lead to HF, most commonly arterial hypertension [5], atrial fibrillation [6], anemia [7], as well as endocrine diseases, primarily diabetes [8, 9]. Symptoms of HF include fatigue, shortness of breath, and inability to sleep on a flat surface. Cases of HF with preserved EF, intermediate range, and reduced LVEF have been described. Preserved LVEF, ≥ 50%, is indicative of diastolic HF, moderately preserved LVEF represents a "gray zone" for patients with values between 40–49%, whereas those with LVEF < 40% are classified as having reduced LVEF [10].

According to the New York Heart Association (NYHA), the NYHA classification is based on the severity of symptoms and physical activity, dividing patients into four groups: Class I – the patient has no limitations in physical activity; Class II – usual activities cause fatigue, shortness of breath, or palpitations; Class III – the patient has significant limitations in physical activity; Class IV – symptoms of HF are present at rest and worsen with minimal physical exertion [10].

According to our study results, cardiac rehabilitation and acupuncture, in addition to drug therapy, may be beneficial in improving the functional ability of HF patients as measured by the A-test [11]. Exercise is the gold standard in the rehabilitation of cardiac patients. Before starting rehabilitation, it is necessary to assess the functional status of patients and continue to monitor their recovery.

The Barthel Index (BI) is commonly used in the rehabilitation of cardiac patients with HF [12–14]. BI assesses the functional independence of patients (feeding, dressing, toileting, personal hygiene, transfers, walking, bowel and bladder control), mainly in the self-care and walking and moving domain according to the International Classification of Functioning, Disability, and Health (ICF) established by the World Health Organization in 2001. A BI score < 85 at discharge indicates increased mortality [15]. BI has a maximum value of 100 when the patient is independent, and a minimum value of 0, indicating complete dependence.

The A-test, created in the early 2000s for evaluating functional recovery, was primarily used to assess patients during the early stages of rehabilitation in orthopedic wards. Today, however, it serves as a useful assessment tool in the early rehabilitation of patients who have undergone cardiac, thoracic, vascular, or neurosurgical procedures [16–18]. Similar to the BI, it tests ten patient activities, mainly within the domain of changing basic body positions and walking and moving according to the ICF, using a 6-level ordinal scale (0–5), where 0 represents the minimum and 50 the maximum score. The A-test is a reliable instrument for everyday evaluation of functional recovery during early rehabilitation of patients surgically treated in an orthopedic ward [16]. The A-test can identify which patients are progressing and which are lagging behind in functional recovery on each day of rehabilitation [17]. Patients should not be assessed according to diagnosis, but rather according to the type and severity of functional deficit [18–20].

The aim of this study was to evaluate the validity and reliability of the A-test in patients with HF. Validity refers to the extent to which an instrument measures what it is intended to measure [18, 21]. Reliability refers to the ability of an instrument to produce consistent results when applied two or more times [21].

Methods

This was a prospective, clinically controlled study, with one experimental group of 77 patients tested using two functional tests by two examiners. The research was conducted at the Clinic for Cardiology of the Military Medical Academy (MMA), Belgrade, Serbia. The research team consisted of a cardiologist, a physiatrist, a speech therapist trained in psychological testing, and two senior physiotherapists. The study was approved by the Ethics Committee of the MMA (No. 56/2022, from December 1, 2022) and the Ethics Committee of the Faculty of Medicine of the MMA (No. 1/4/2023, from February 21, 2023) within the framework of the doctoral dissertation decision of the Senate of the University of Defence (No. 27-272, from July 25, 2023). All patients provided written informed consent to participate in the study.

Patient selection

Patients diagnosed with HF were selected based on their clinical presentation, medical history, and echocardiographic findings. As a prerequisite for participation in the clinical study, the patient's cognitive status was determined using the Mini-Mental State Examination (MMSE), and patients with a score of 24 or above were included in the study. Patients of both sexes, without restrictions regarding age or LVEF values, classified as NYHA class I–IV, as determined by a physician at the beginning and end of hospitalization, were included.

Exclusion criteria

Immobile patients (those with quadriplegia, paraplegia, hemiplegia, or severe degenerative and inflammatory rheumatism), as well as patients with anemia (hemoglobin < 90 g/L) or other severe comorbidities that prevent functional testing, were excluded from the study. Exclusion criteria also included instability of vital parameters: arterial blood pressure > 180/120 mmHg or < 90/60 mmHg, heart rate ≥ 130 beats *per* min, malignant cardiac arrhythmias, febrile conditions, or instability of vital signs within the previous 24 hrs.

A-test and Barthel Index

Two experienced senior physiotherapists tested each patient together at the same time every day during hospitalization, and filled out the tests separately. The A-test evaluates ten activities: changing position in bed (from supine to side-lying and from supine to sitting on the edge of the bed), getting in and out of bed, standing, walking, sitting in a chair, and using the toilet. The last two activities are walking up and down the stairs and walking endurance. The A-test uses a 6-level ordinal scale ranging from 0 to 5, where a score of 0 indicates that the patient is unable to perform the activity, and a score of 5 represents independent and safe performance (Appendix 1). Walking endurance is scored separately: 0 for inability to walk; 1 for walking up to 5 m (which is room-level walking); 2 for walking up to 15 m; 3 for walking up to 50 m; 4 for walking up to 100 m; and 5 for walking over 100 m. The minimum A-test score is 0, while the maximum is 50 [16]. The well-known BI also assesses ten activities, using different scales (from a 2-level ordinal scale for bathing to a 4-level ordinal scale for mobility on a level surface), with 0 as the minimum and 100 as the maximum score.

Feeding, personal toileting, bathing, dressing, transfers when using the toilet and bed-chair, walking and stair climbing, and sphincter control were tested [22, 23].

Sample size and study power

The sample size required to test the validity and reliability of the A-test in patients with HF across all four NYHA classes was a minimum of 60 subjects. The sample size of 60 subjects was determined according to the standard formula for repeated measures analysis of variance, assuming an alpha (α) error of 0.05, a study power of 80% (β = 0.20), and an internal measurement correlation of ρ = 0.50. The sample size (N) was calculated according to the formula: $N = [2 \times (Z_{1-\alpha/2} + Z_{1-\beta})^2 \times (1 - \rho)] / (m \times f^2)$, i.e., $N = [2 \times (1.96 + 0.84)^2 \times (1–0.50)] / (m \times f^2) = 60$.

Statistical analysis

The validity of the A-test was assessed through three complementary aspects of validity: content, criterion validity, and construct validity. Content validity was determined by analyzing the distribution of results, floor and ceiling effects, and the width of the range of results. The following statistical indicators were used: the percentage of minimum and maximum results by day (floor/ceiling effect), skewness (Sk) values, and the theoretical range of the scale (0–50). Criterion validity was assessed in two ways: concurrent validity was examined using Spearman's correlation between A-test results and BI; predictive validity was examined using univariate linear regression analysis, which found that A-test results on day 1 significantly predicted functional outcome (BI on day 6). Construct validity was assessed by testing hypothetically expected differences in test results in accordance with theoretical assumptions. This validity was measured by the Mann-Whitney (*U*) test and Spearman's product-moment correlation coefficient. Reliability of the A-test was assessed in two ways: internal consistency and inter-rater reliability. Cronbach's alpha coefficient was used to assess internal reliability. The agreement between two independent raters was examined using the kappa (κ) coefficient, which measures inter-rater reliability. Intraclass Correlation Coefficient (ICC) was used to evaluate the A-test's stability across time points. The ICC was calculated using a two-way random effects model with absolute agreement and average measures – ICC (2, k). The normality of distribution for continuous variables was assessed using the Shapiro-Wilk test. Categorical variables were summarized using frequencies and percentages. For continuous variables, those following a normal distribution were presented as means with standard deviations (SD), while non-normally distributed variables were described using medians (Me). Statistical analysis was performed using the IBM SPSS Statistics for Windows, version 25.0 (IBM Corp., Armonk, NY, USA).

Results

The study sample consisted of a total of 77 patients, the majority of whom were male, i.e., 66.2%, while 33.8% were female. The majority of participants were older than 65 years (80.5%), with a mean age of 72.04 years (SD = 11.12), a median of 72 years, and a range of 38 to 99 years. Cognitive functioning, assessed by MMSE, showed that the subjects scored an average of 27.42 points (SD = 2.14), indicating preserved cognition. The median MMSE score was 28 points, with a range of 24 to 30. Regarding cardiac function, the mean EF was 41.83% (SD = 11.46), with a median of 44% and values ranging from 20% to 66%. Functional status was also assessed using the NYHA classification. At admission, the mean NYHA class was 2.75 (SD = 1.05), while a slight improvement was noted at discharge with a mean value of 2.42 (SD = 1.12). The range of the NYHA class ranged from I to IV in both measurements. The Wilcoxon test indicates a significant difference in NYHA scores between admission and discharge ($Z = -4.914$, $p < 0.001$). At admission, 15.6% of patients were classified as NYHA class I, 23.4% as NYHA class II, 31.2% as NYHA class III, while 29.9% of patients were in the NYHA class IV. At discharge, 28.6% of patients were in the NYHA class I, 22.1% in NYHA class II, 28.6% in NYHA class III, while 20.8% of patients remained in the NYHA class IV (Table 1). The sum of the total num-

ber of measurements *per* therapist was 444, which means the total number of measurements taken by two therapists was 888. The smallest number of measurements *per* patient was 3, while the largest was 13 for each therapist.

Descriptive statistics for the results of the A-test and BI during the 13 days of hospitalization are presented. Based on the values, a gradual increase in the average results of both tests can be observed during the first ten days, which indicates progress in the functional recovery of the patients. After the tenth day, stable values are recorded, with reduced variability, which may be a consequence of the reduced number of subjects. The total median values for the entire period were Me = 36.36 for the A-test and Me = 73.64 for BI (Table 2). The A-test was validated based on three aspects of validity: content validity, criterion validity, and construct validity.

Table 1

General data about patients

Parameter	Values
Gender	
male	51 (66.2)
female	26 (33.8)
Age, years	
≤ 65	15 (19.5)
> 65	62 (80.5)
mean ± SD; median (range)	72.04 ± 11.12; 72 (38–99)
MMSE, mean ± SD; median (range)	27.42 ± 2.14; 28 (24–30)
Ejection fraction, mean ± SD; median (range)	41.83 ± 11.46; 44 (20–66)
NYHA categories at admission	
I	12 (15.6)
II	18 (23.4)
III	24 (31.2)
IV	23 (29.9)
NYHA at admission*, mean ± SD; median (range)	2.75 ± 1.05; 3 (1–4)
NYHA at discharge*, mean ± SD; median (range)	2.42 ± 1.12; 2 (1–4)
NYHA categories at discharge	
I	22 (28.6)
II	17 (22.1)
III	22 (28.6)
IV	16 (20.8)

MMSE – Mini-Mental State Examination; SD – standard deviation; NYHA – New York Heart Association.
Data are shown as numbers (percentages), or mean ± SD; median (range), where indicated.
***Note*. *The Wilcoxon test showed a statistically significant difference in NYHA scores between admission and discharge ($p < 0.001$).**

Table 2

Minimum, maximum, and median values of the A-test and Barthel Index

Parameter	Minimum	Maximum	Median
A-test			
day 1	0	50	36
day 2	0	50	36
day 3	0	50	37
day 4	0	50	37
day 5	0	50	38
day 6	3	50	41
day 7	0	50	40
day 8	0	45	39.5
day 9	5.5	43	36
day 10	29	43	40.5
day 11	33	42	39
day 12	33	33	33
day 13	33	33	33
overall	1	50	36.36
Barthel Index			
day 1	0	100	65
day 2	0	100	70
day 3	0	100	70
day 4	5	100	75

Table 2 (continued)

Parameter	Minimum	Maximum	Median
day 5	5	100	75
day 6	10	100	80
day 7	10	100	80
day 8	5	70	61
day 9	20	90	67.5
day 10	55	90	87.5
day 11	55	90	85
day 12	55	55	55
day 13	55	55	55
overall	7.5	100	73.64

Table 3

Floor and ceiling effect for the A-test

Score (%)	Days of rehabilitation												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Minimal	3.9	3.9	2.6	1.5	1.9	0	4.0	8.3	0	0	0	0	0
Maximal	18.2	18.2	19.5	16.9	18.9	28.2	20.0	0	0	0	0	0	0

Table 4

Distribution characteristics of the A-test by day and overall

Parameter	Day													Overall
	1	2	3	4	5	6	7	8	9	10	11	12	13	
n	77	77	77	65	53	39	25	12	8	6	3	1	1	77
Sk	-0.322	-0.457	-0.740	-0.883	-0.961	-1.373	-1.386	-1.789	-1.570	-0.923	-0.935	/	/	-0.790
Ku	-1.308	-1.080	-0.526	-0.098	-0.009	1.583	1.218	2.083	2.623	-0.930	/	/	/	-0.324

n – number of patients; Sk – skewness; Ku – kurtosis.

***Note*: The symbol "/" indicates that the correlation for days 11, 12, and 13 of follow-up was not tested due to the small number of subjects.**

Content validity

We assumed that the A-test would demonstrate a satisfactory level of content validity if the scores obtained during the 13 days of hospitalization covered the full range from 0 to 50, if the skewness value of the distribution was below 1.00, and if fewer than 15% of the scores represented the lowest or highest possible value on the scale. To determine content validity, we used the floor and ceiling effect, measures of asymmetry, and the range of values.

The floor and ceiling effects were analyzed throughout the 13 days of early rehabilitation. Content validity, which assesses the extent to which the instrument covers key aspects of functional capacity, was confirmed by the wide score range, low floor (minimal score 2.0%) and ceiling (maximal score 10.8%) effects, and symmetrical distribution (Table 3).

The distribution shape parameters (skewness and kurtosis) were analyzed across all 13 days of early rehabilitation, with the number of respondents gradually decreasing, particularly affecting the reliability of estimates after day 8 ($N < 10$). From day 1 to day 10, all scores showed negative skewness, suggesting that the majority of participants achieved higher functional scores. Notably, from day 6 to 9, skewness values exceeded ±1, indicating a significant deviation from symmetry (Table 4).

Kurtosis values were negative from day 1 to 5, reflecting flatter-than-normal distributions, while a shift to positive values occurred from day 6 to day 9, suggesting increased score concentration around the mean and reflecting a more peaked (leptokurtic) distribution during this later phase of rehabilitation (e.g., day 6 = 1.583; day 9 = 2.623) (Figure 1).

When considering the total A-test scores across all 13 days, the average skewness (Sk = -0.790) and kurtosis (Ku = -0.324) remained within acceptable limits (±1), indicating an approximately symmetric and moderately flattened distribution. These findings suggest that the test was capable of capturing variations in functional capacity across time without substantial ceiling or floor effects, fulfilling the second criterion of content validity (Figure 1).

The third criterion of content validity, which relates to the range of values, was also met, given that the range of values ranged from 0 to 50, which is both the theoretical minimum and maximum.

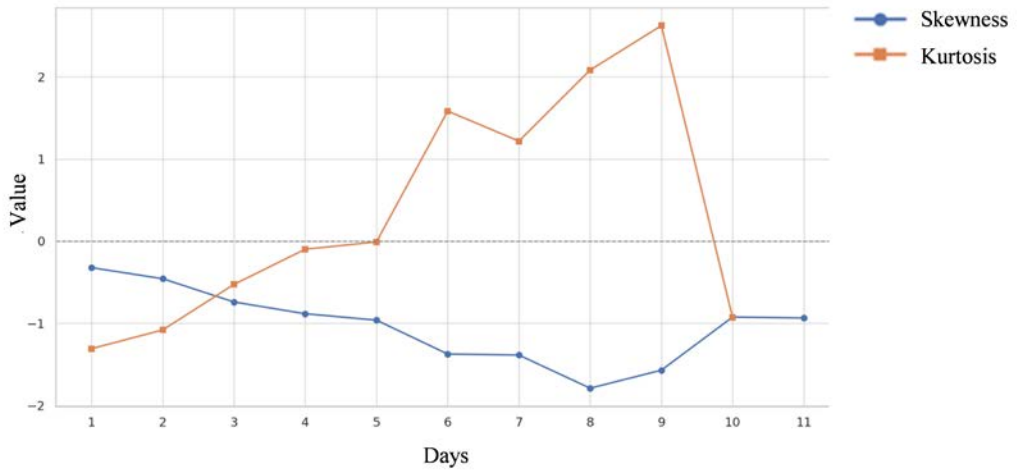

Fig. 1 – Skewness and kurtosis of A-test scores by day.

Table 5

Spearman's correlation between the A-test composite scores and the Barthel Index

Parameter	Value
ρ	0.991
p-value	< 0.001
n	77

ρ – Spearman's correlation coefficient; *p* – statistical significance; n – number of patients.

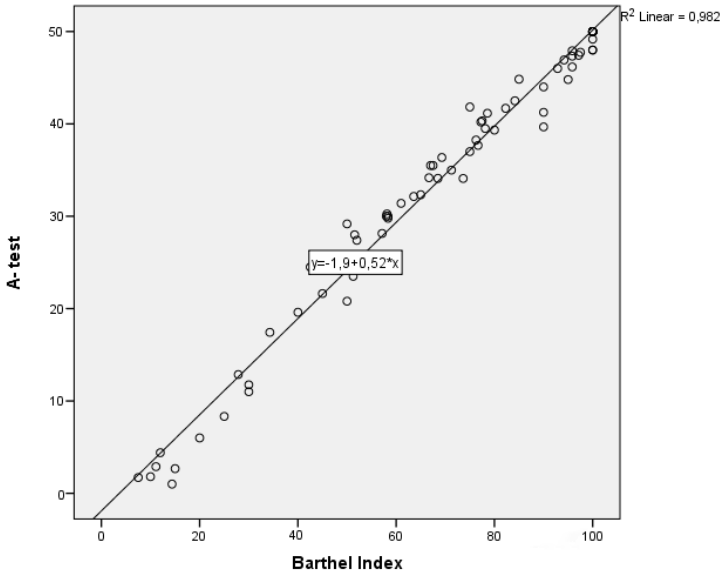

Fig. 2 – Correlation between A-test composite scores and Barthel Index.

Criterion validity

Criterion validity refers to the assessment of whether a test adequately predicts or correlates with external criteria. In this context, we examined two types of validity: concurrent validity and predictive validity. Since the A-test aims to assess the functional status of patients, the results of BI were used for the criterion assessment of concurrent validity. Spearman's correlation coefficient (rho) was used to test association.

Using Spearman's correlation coefficient, we examined whether the results of the A-test were statistically significantly related to BI. The results show a very high correlation ($\rho = 0.991$, $p < 0.001$) (Table 5). Such a high correlation indicates almost identical results of the two tests (Figure 2).

For testing the correlation, the averages of both researchers and the average for all testing days were taken.

The correlation between the results of the A-test and BI over a ten-day period was determined, using Spearman's product-moment correlation coefficient. The results show a very strong positive and statistically significant correlation between the results of these two tests over all ten days. The correlations range from ρ = 0.929 (day 9) to ρ = 0.989 (day 2) (Table 6).

The predictive validity of the A-test was examined through the ability of the test to predict the later functional outcome of patients. For this purpose, univariate linear regression analysis was used to examine whether the results of the A-test on day 1 were a statistically significant predictor of BI at discharge. In order to ensure a sufficient number of subjects, day 6 was taken as the date of discharge.

The analysis of the prediction of BI at discharge using the initial A-test score as a predictor is presented. The data indicate that the A-test measured on the first day is a significant predictor of BI at discharge [β = 0.880, $p < 0.001$, 95% confidence interval (CI): 1.087–1.562]. The coefficient of determination is very high, and the value $R^2 = 0.769$ means that the model explains 76.9% of the variance of BI at discharge, which indicates a very good predictive power of this model (Table 7).

Construct validity

The results of the A-test are presented according to the age of the respondents. The average results of the A-test were better in respondents younger than 65 years (Me = 48.00) compared to those older than 65 years (Me = 32.24). This difference was statistically significant ($U = 135.00$, $p < 0.001$) (Table 8). The first hypothesis, that the results of the A-test would be statistically significantly better in those younger than 65 years compared to those older than 65 years, was confirmed.

Table 9 shows the results of the A-test according to the NYHA class at admission and at discharge. At admission, patients in the NYHA class I had a median A-test result of Me = 50.00, in the NYHA class II, Me = 46.09, in class III, Me = 34.59, while in the NYHA class IV, the lowest median value was recorded, Me = 17.43. The difference in A-test results between categories at admission is statistically significant ($U = 62.05$, $p = 0.000$). At discharge, the median A-test for the NYHA class I was Me = 50.00, for NYHA class II Me = 40.20, for NYHA class III Me = 30.13, while in the NYHA class IV, the lowest value was recorded, Me = 9.67. The difference in the A-test scores in relation to the NYHA class at discharge is also statistically significant ($U = 65.77$, $p = 0.000$). These findings confirm that a higher NYHA

Table 6

Correlation between the A-test and the Barthel Index by measurement days

Assessment day	ρ	*p*-value
Day 1	0.989	< 0.001
Day 2	0.989	< 0.001
Day 3	0.984	< 0.001
Day 4	0.973	< 0.001
Day 5	0.953	< 0.001
Day 6	0.976	< 0.001
Day 7	0.949	< 0.001
Day 8	0.935	< 0.001
Day 9	0.929	0.001
Day 10	0.985	< 0.001

ρ – Spearman's correlation coefficient; *p* – statistical significance.

Table 7

Prediction of the Barthel Index at discharge based on day 1 A-test score

β	*t*-value	*p*-value	95% CI limit (lower–upper)	R^2
0.880	11.298	< 0.001	1.087–1.562	0.769

CI – confidence interval.
***Note*. Univariate linear regression analysis was performed.**

Table 8

Results of the A-test according to the patient's age

Statistic	Age (years)		All patients
	≤ 65	> 65	
Minimum	34.1	1	1
Maximum	50	50	50
Median	48	32.24	36.36
U			135
p-value			< 0.001

***U* – Mann-Whitney test; *p* – statistical significance.**

class, indicating a more severe functional class, consistently accompanies lower A-test scores, both at admission and at discharge. This was our second hypothesis, which was therefore confirmed.

The correlation between the A-test results and the MMSE scale was examined. The analysis revealed a statistically significant positive correlation of medium intensity ($\rho = 0.596$, $p < 0.001$), indicating that better cognitive status, measured by the MMSE scale, was associated with better A-test results. A total of 77 subjects were included in the analysis (Table 10). Hypothesis 3, that patients with higher MMSE scores would achieve better A-test results, was thus confirmed.

The relationship between the A-test results and EF was also examined. A statistically significant positive correlation of weaker intensity was obtained ($\rho = 0.269$, $p = 0.018$), indicating that higher values of EF are associated with better results on the initial measurement of the A-test. A total of 77 subjects were included in the analysis (Table 11). Hypothesis 4, that patients with a higher EF would have better A-test results at the initial measurement, was confirmed.

Reliability of the A-test

The reliability of the A-test was measured through internal consistency and reproducibility.

Internal consistency is a form of reliability that refers to the degree to which the items of an instrument are interconnected, that is, to what extent they jointly measure the same construct. In this study, Cronbach's alpha was used as the main indicator of the internal reliability of the A-test.

The A-test demonstrated excellent internal consistency during the first 11 days of rehabilitation, with Cronbach's alpha values consistently high ($\alpha = 0.970–0.972$). The overall reliability ($\alpha = 0.971$) confirms that the test items reliably assess the same construct, functional status, throughout the rehabilitation process. These findings support the use of the A-test as a stable and consistent tool for monitoring patient progress in clinical settings (Table 12).

Reproducibility of the A-test was measured by inter-rater reliability. The A-test showed very high inter-rater reliability throughout the rehabilitation period, with κ coefficients ranging from 0.860 to 1.000. According to the Landis

Table 9

The A-test results according to the NYHA at admission and discharge

Statistic	NYHA at admission				NYHA at discharge			
	I	II	III	IV	I	II	III	IV
Minimum	46.92	35.5	20.8	1	41.15	30	20.8	1
Maximum	50	50	42.5	34.1	50	48	41.83	29.78
Median	50	46.09	34.59	17.43	50	40.2	30.13	9.67
U	62.05				65.77			
p-value	< 0.001				< 0.001			

NYHA – New York Heart Association; *U* – Mann-Whitney test; *p* – statistical significance.

Table 10

Spearman's correlation between the A-test and MMSE test scores

Parameter	Value
ρ	0.596
p-value	< 0.001
n	77

MMSE – Mini-Mental State Examination; ρ – Spearman's correlation coefficient; *p* – statistical significance; n – number of patients.

Table 11

Spearman's correlation between the A-test results at initial measurement and the ejection fraction

Parameter	Value
ρ	0.269
p-value	0.018
n	77

ρ – Spearman's correlation coefficient; *p* – statistical significance; n – number of patients.

Table 12
Reliability of the A-test

A-test	α
Day 1	0.972
Day 2	0.969
Day 3	0.970
Day 4	0.967
Day 5	0.967
Day 6	0.968
Day 7	0.968
Day 8	0.968
Day 9	0.970
Day 10	0.970
Day 11	0.970
Overall	0.971

α – Cronbach alpha coefficient.

Table 13
Inter-examiner agreement of results on the A-test

A-test	κ
Day 1	0.931
Day 2	0.945
Day 3	0.930
Day 4	0.951
Day 5	0.980
Day 6	1.000
Day 7	0.955
Day 8	0.908
Day 9	0.860
Day 10	1.000
Day 11	1.000
Day 12	n/a
Day 13	n/a
Overall	0.842

n/a – not applicable.

and Koch classification [24], all values reflect very good agreement ($\kappa \geq 0.81$), even at the lowest point ($\kappa = 0.860$ on day 9). The overall inter-rater reliability ($\kappa = 0.842$) confirms that the A-test yields consistent results across different examiners, supporting its applicability in clinical practice for reliable patient assessment (Table 13).

Discussion

The mean age of the subjects and the distribution by gender are similar to our previous studies in this area, as well as to the works we cited [11]. A statistically significant decrease in NYHA class at discharge, compared to admission, is an indicator of good, modern drug therapy for HF [25]. The A-test is designed to assess the basic functional abilities that the patient should regain during the initial phase of recovery.

Content validity refers to the extent to which an instrument covers items relevant and essential to the concept being measured. When an instrument covers all important aspects of the target area and is tailored to the purpose for which it is used, it is considered to have good content validity [26]. The A-test demonstrated good content validity, with a wide range of scores, minimal asymmetry, and a low percentage of extreme scores – indicating a low floor and ceiling effect.

Concurrent validity refers to the degree to which the A-test scores correlate with an existing BI used for the same or similar construct. If a new test shows a high correlation with existing measures, it is considered to have good concurrent validity. This type of validity is particularly important for determining whether the A-test truly measures the same thing as other validated instruments. The results indicate a stable and strong relationship between A-test scores and functional independence measured by BI in subjects across all follow-up days, with the exception that correlations for days 11, 12, and 13 were not tested due to the small number of participants.

Predictive validity refers to the ability of the A-test to predict future behavior or outcomes based on measurements. High predictive validity indicates that the test has the ability to forecast future events based on current results successfully. The A-test score on the first day is a significant predictor of BI at discharge, according to the values of the coefficient of determination.

The construct validity of the A-test has been confirmed with several measurements, where the value of the A-test depends on the patient's functional ability and age.

Reliability is a basic psychometric property of the instrument and refers to the degree to which the measurement is consistent, i.e., in which the instrument provides stable and reproducible results [21]. Internal consistency is an indicator of the homogeneity of the scale and is important for assessing whether all items of the instrument can be considered parts of the same measurement. The most commonly used indicator of internal consistency is Cronbach's alpha, which is calculated based on the average correlation between items. The Cronbach's alpha value ranges from 0 to 1, with higher values indicating greater internal consistency. It is common for $\alpha \geq 0.70$ to be considered an acceptable level of reliability for research purposes, while values above 0.80 are interpreted as good, and above 0.90 as a very high level of consistency. Internal consistency can also be used to analyze what happens to alpha if individual items are removed from the scale. This analysis helps identify items that may be disrupting the internal structure of the instrument. In our study, the A-test showed a very high level of consistency.

Inter-rater reliability refers to the degree of agreement between different raters who apply the same instrument under the same conditions. A high level of inter-rater reliability indicates that the results do not depend on who conducts the measurement, but that the instrument itself consistently measures what is being assessed. Assessments were independently recorded by two raters on the A-test for each respondent at all measurement intervals. We measured the agreement between their assessments using the κ coefficient. The usual classification of κ coefficient of agreement values is as follows: $\kappa < 0.00$ – poor agreement; $\kappa = 0.00–0.20$ – slight agreement; $\kappa = 0.21–0.40$ – fair agreement; $\kappa = 0.41–0.60$ – moderate agreement; $\kappa = 0.61–0.80$ – substantial agreement; and $\kappa = 0.81–1.00$ – almost perfect agreement. In our study, the two senior physiotherapists showed very good agreement.

The results obtained in this study show that the A-test has extremely good psychometric characteristics in terms of reliability. Both internal consistency and inter-rater reliability show very high values. Cronbach's alpha coefficients over the eleven days of measurement, as well as for the total score, are consistently above 0.97, indicating exceptional test homogeneity and consistent measurement of the same construct – the functional status of patients. At the same time, κ coefficient values, ranging from 0.860 to 1.000, indicate almost perfect agreement between the two raters. These findings confirm that the A-test is a reliable instrument for assessing the functional recovery of patients during early rehabilitation, regardless of who performs the assessment.

ICC was calculated to evaluate the temporal stability of the A-test scores across repeated measurements. The obtained ICC (2, k) value was 0.971, indicating excellent reliability and minimal within-subject variability over time.

Conclusion

The A-test demonstrated strong validity and reliability metrics, confirming its clinical utility in assessing functional capacity in patients with heart failure. Its structured scoring system allows for precise monitoring of early rehabilitation progress. The A-test is easily applied in clinical practice and monitors all patient activities from lying in bed to walking. Future studies may further explore its responsiveness and predictive power in larger and more diverse cardiac populations.

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Appendix 1.

The A-test – tabular presentation of the list used by physiotherapists [16,17].

A-test	Patient name					
	Diagnosis:					
	Day of rehabilitation					
1.	Bed mobility (from supine to side-lying)					
2.	From supine to sitting on the bed edge					
3.	Getting out of bed					
4.	Standing					
5.	Going back to bed					
6.	Walking with aids					
7.	Use of the toilet					
8.	Sitting on the chair					
9.	Walking up and down the stairs					
10.	Walking endurance					
	Summary					

Score: 0 – activity is not achieved; 1 – needs full assistance of a physiotherapist; 2 – requires adherence by a physiotherapist; 3 – activity performed with verbal suggestions of therapists; 4 – completely independent but insecure; 5 – fully independent and secure.

Score for walking endurance: 0 – unable to walk; 1 – walks up to 5 m (room-level walk); 2 – walks up to 15 m; 3 – walks up to 50 m; 4 – walks up to 100 m; 5 – walks over 100 m.

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Inhibition of colorectal cancer cell proliferation, invasion, and migration, and induction of epithelial-mesenchymal transition *via TFF1* silencing

Inhibicija proliferacije, invazije i migracije ćelija kolorektalnog karcinoma i indukcija epitelno-mezenhimalne tranzicije utišavanjem *TFF1* gena

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Abstract

Background/Aim. The identification of aberrant genes associated with colorectal cancer (CRC) and understanding their pathogenic mechanisms are important for early diagnosis and the determination of appropriate therapy. The aim of this study was to examine the impact of trefoil factor 1 (TFF1) on the proliferation, invasion, and migration of CRC cells. **Methods.** The expression of TFF1 in 40 CRC tissue samples was assessed using immunohistochemistry. Additionally, the expression of TFF1 in CRC cell lines HCT116 and Caco-2 was determined using real-time quantitative polymerase chain reaction (qPCR) and Western blot analysis. Then the Caco-2 cell line was transfected with a lentivirus-packaged small interfering ribonucleic acid to silence TFF1 expression. The cell counting kit-8 assay and cell colony formation assay were used to assess cell proliferation and colony formation capacity, while the Transwell assay and wound healing assay were used to assess cell invasion and migratory ability. Subsequently, the expression levels of E-cadherin and vimentin were assessed using qPCR and Western blotting to study the relationship between TFF1 expression and epithelial-mesenchymal transition. **Results.** The positive rate of TFF1 expression in CRC tissues was 35% (14/40). The HCT116 cell line demonstrated modest levels of TFF1 expression, while the Caco-2 cell line exhibited significant levels. Caco-2 cells expressed less TFF1 messenger ribonucleic acid and protein after *TFF1* silencing. The proliferative and colony-forming powers of Caco-2 cells, as well as their invasion and migratory capabilities, were subsequently seen to decrease. Furthermore, silencing *TFF1* expression led to an increase in E-cadherin expression and a decrease in vimentin expression. **Conclusion.** Caco-2 CRC cells can be inhibited from proliferating, migrating, and invading by silencing *TFF1* expression. TFF1 may play a significant role in the initiation, development, and metastasis of CRC by promoting epithelial-mesenchymal transition.

Key words:
cell movement; cell proliferation; colorectal neoplasms; gene expression regulation; immunohistochemistry; trefoil, factor-1.

Apstrakt

Uvod/Cilj. Identifikacija aberantnih gena povezanih sa kolorektalnim karcinomom (*colorectal cancer* – CRC) i razumevanje njihovih patogenih mehanizama je od značaja za ranu dijagnozu bolesti i određivanje odgovarajuće terapije. Cilj rada bio je da se ispita uticaj trefoil faktora 1 (TFF1) na proliferaciju, invaziju, i migraciju ćelija CRC. **Metode.** Ekspresija TFF1 u 40 uzoraka tkiva CRC procenjena je pomoću imunohistohemije. Pored toga, ekspresija TFF1 u CRC ćelijskim linijama HCT116 i Caco-2 određena je pomoću analize kvantitativne lančane reakcije polimerazom u realnom vremenu (*quantitative polymerase chain reaction* – qPCR) i *Western blot* analize. Zatim je Caco-2

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ćelijska linija transfektovana malom interferirajućom ribonukleinskom kiselinom upakovanom u lentivirus u cilju utišavanja ekspresije *TFF1*. Za procenu proliferacije ćelija i sposobnosti formiranja kolonija korišćeni su esej *cell counting kit-8* i test formiranja kolonija ćelija, dok su *Transwell* esej i test zaceljivanja rana korišćeni za procenu invazivne i migratorne sposobnosti ćelija. Nakon toga, nivoi ekspresije E-kaderina i vimentina procenjeni su pomoću qPCR i *Western blot* analize kako bi se ispitala veza između ekspresije TFF1 i epitelno-mezenhimalne tranzicije. **Rezultati.** Pozitivna stopa ekspresije TFF1 u tkivima CRC bila je 35% (14/40). U HCT116 ćelijskoj liniji detektovani su umereni nivoi ekspresije TFF1, dok su u Caco-2 ćelijskoj liniji zapaženi značajno viši nivoi ekspresije. U Caco-2 ćelijama zabeležen je smanjen nivo TFF1 informacione ribonukleinske kiseline i proteina nakon utišavanja *TFF1*. Zatim je uočeno da se smanjuje sposobnost proliferacije i formiranja kolonija kod Caco-2 ćelija, kao i njihove invazivne i migratorne sposobnosti. Štaviše, utišavanje ekspresije *TFF1* dovelo je do povećanja ekspresije E-kaderina i smanjenja ekspresije vimentina. **Zaključak.** Utišavanje ekspresije *TFF1* može inhibirati proliferaciju, migraciju i invaziju CRC ćelija Caco-2. Podsticanjem epitelno-mezenhimalne tranzicije TFF1 može igrati značajnu ulogu u nastanku, razvoju i metastaziranju CRC.

Ključne reči:
ćelija, pokreti; ćelija, proliferacija; kolorektalne neoplazme; geni, regulacija ekspresije; imunihistohemija; trefoil, faktor-1.

Introduction

Colorectal cancer (CRC) is one of the most prevalent gastrointestinal malignancies, ranking third in terms of global incidence among malignant tumors and second in terms of mortality [1–3]. Recent advancements in next-generation sequencing and related technologies have facilitated the identification of genes with aberrant expression patterns in colorectal tumor tissues. Trefoil factor 1 (TFF1), a small polypeptide characterized by the presence of a trefoil factor domain, is primarily synthesized and secreted by mucous secretory epithelial cells lining the digestive tract, where it plays a pivotal role in mucosal damage repair [4]. Expression of TFF1 is ubiquitous at sites of mucosal injury throughout the gastrointestinal tract. Following gastrointestinal mucosal injury, the *TFF1* gene experiences a rapid up-regulation, thereby contributing to the process of gastrointestinal mucosal repair and reconstruction [5]. Several studies have shown that TFF1 expression is up-regulated in CRC tissues, and TFF1 levels have a wide variability (47.1–89%) [6–10]. The up-regulated expression demonstrated that TFF1 may play an important role in the tumorigenesis of human CRC.

Overexpression of TFF1 has been observed in various tumors, including breast cancer, pancreatic cancer, pulmonary cancer, and bladder cancer. Its overexpression has been associated with tumor cell proliferation, apoptosis, metastasis, and angiogenesis [11–14]. However, the involvement of TFF1 in the pathogenesis and progression of CRC remains a subject of debate.

The aim of this study was to examine the expression levels of TFF1 in CRC and elucidate its biological functions and potential underlying mechanisms in this specific cancer type.

Methods

Research material

A total of 40 patient-derived CRC tissue specimens were collected, comprising 22 cases of colon cancer and 18 cases of rectal cancer in 2022. Notably, none of the patients had received neoadjuvant therapy, and there was no prior medical history of other malignancies. This study was approved by the Ethics Committee of the Second Hospital of Tianjin Medical University (No. KY2023K207, from October 26, 2023). The study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

RPMI-1640 medium and fetal bovine serum were acquired from Gibco (USA). Penicillin-streptomycin solution was obtained from Invitrogen (USA). Polyvinylidene fluoride membrane was purchased from Millipore (USA), and Matrigel was acquired from Corning (USA). Secondary antibodies were obtained from Cell Signaling Technology (USA). Sterile petri dishes, 6-well plates, and 96-well plates were purchased from Corning (USA). SYBR Green Fast quantitative real-time polymerase chain reaction (qPCR) Mix was purchased from Roche (Basel, Switzerland). The reverse transcription kit was purchased from Thermo (USA). The quantitative reverse transcription-PCR primers were designed by Shanghai Sangon Biological Engineering Co., Ltd. (China). The TFF1 antibody was supplied by Shanghai Santa Cruz Biotechnology Company (China). The marker for Western blot protein staining and Lipofectamine® 2000 were acquired from Invitrogen (USA). The packaging of small interfering ribonucleic acid (siRNA) and lentivirus was provided by Shanghai Jima Gene Co., Ltd. (China). The cell counting kit-8 (CCK-8) was acquired from Xi'an Mishu Biotechnology Co., Ltd. The human CRC cell line HCT116 and normal colon epithelial cell line CCD-18Co were provided by Zunyi Medical University, while the CRC cell line Caco-2 was obtained from Shanghai Zhongqiao Xinzhou Biological Co., Ltd.

Immunohistochemical analysis

The expression of TFF1 was detected using the streptavidin-peroxidase method. CRC tissues and adjacent non-tumor tissues were embedded in paraffin and sectioned. The tissue sections were baked at 60 °C for 1 hr, subsequently rewarmed to room temperature, and dewaxed in xylene for 30 min. After alcohol rehydration treatment, the tissue sections were washed three times with phosphate-buffered saline (PBS), then subjected to high-temperature

antigen retrieval using ethylenediaminetetraacetic acid, and again washed three times with PBS. The samples were then placed in a humidified chamber, where endogenous peroxidase activity was blocked with 4% H_2O_2 for 10 min at room temperature, followed by three PBS washes. Non-specific binding was blocked with 5% bovine serum albumin at 37 °C for 1 hr. The TFF1 antibody was appropriately diluted according to the manufacturer's instructions. Following the application of the primary antibody, the slides were incubated overnight at 4 °C, rinsed three times with PBS, treated with the secondary antibody, and then incubated at room temperature for 1 hr. After three additional washes with PBS, a 3,3'-diaminobenzidine color solution was applied, and the samples were incubated at room temperature for 5 min. Finally, the slides were counterstained with hematoxylin, dehydrated, cleared, and sealed. The scoring was performed as follows: under the microscope, the degree of cell staining and the proportion of positive cells were scored, respectively. Degree of cell staining: 0 – no coloration, 1 – light yellow, 2 – yellow, and 3 – brown. Proportion of positive cell scoring: ≤ 10% – 1 point, 10% to 50% – 2 points, 50% to 80% – 3 points, and ≥ 80% – 4 points. The degree of cell staining and the rate of positive cells were multiplied, and the result ≤ 1 was considered negative (-), 2–4 indicated weak positive (+), 5–8 positive (++), 9–12 strong positive (+++). Histomorphological analysis was performed using an Olympus BX53 microscope (Olympus Corporation, Tokyo, Japan) at a magnification of ×200. CellSens software (version 2.3, Olympus) was used for image acquisition. Five sections *per* tissue were analyzed.

Cell culture

The CRC cell lines HCT116 and Caco-2, as well as the normal colonic epithelial cell line CCD-18Co, were maintained in petri dishes with RPMI-1640 medium, 10% fetal bovine serum, and 1% penicillin–streptomycin solution at 37 °C in a humidified incubator containing 5% CO_2.

Quantitative real-time polymerase chain reaction

Total RNA was extracted using the TRIzol reagent (Invitrogen, USA), and reverse-transcribed into complementary DNA (cDNA) using a qPCR kit with the following primers: glyceraldehyde-3-phosphate dehydrogenase primer: 5′-ACA ACTTTGGTATCGTGGAAGG-3′(forward), 5′-GCCATCAC GCCACAGTTTC-3′(reverse), and TFF1 primer: 5′-CCCTC CCAGTGTGCAAATAGG-3′(forward), 5′-GAACGGTGTCG TCGAAACAG-3′(reverse).

For reverse transcription, 0.5 μg of total RNA was mixed with 5 μL of oligo deoxythymidine primer in a 200 μL ribonuclease (RNase)-free tube and brought to a final volume of 11.5 μL with RNase-free water. The mixture was heated at 70 °C for 5 min and then chilled on ice for 5 min. After brief centrifugation, 5 μL of 5 × avian myeloblastosis virus (AMV) buffer, 2.5 μL of deoxyribonucleoside triphosphates, 0.5 μL of RNase inhibitor, and 0.5 μL of AMV reverse transcriptase were added, and the volume was brought to 25 μL with RNase-free water. The reaction was carried out at 42 °C for 60 min to synthesize cDNA. For qPCR, the reaction system contained 20 μL of Hieff qPCR SYBR Green Master Mix, 10 μL of RNase-free water, 3 μL of forward primer, 3 μL of reverse primer, and 4 μL of cDNA template. After brief centrifugation, reactions were amplified under standard cycling conditions. The following PCR cycling conditions were used: 1 cycle of 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 30 s.

Western blotting analysis

Total protein was extracted using a protein extraction reagent, and the concentration of total protein was determined using the Bradford method. The proteins were then transferred onto a polyvinylidene fluoride membrane. After incubating with 5% skim milk, the membranes were incubated with primary and secondary antibodies. Finally, the protein bands were exposed and developed using an enhanced chemiluminescence system. The gray value of target bands was analyzed using ImageJ software.

Small ribonucleic acid transfection

The cellular samples were divided into three groups: the negative control (NC) group (non-targeting siRNA), the small interference transfection Group I (siRNA1 group), and the small interference transfection Group II (siRNA2 group). Caco-2 cells (1.0×10^5 cells/mL) were cultured in 6-well plates, and transfection was started when the cells were in good condition and had reached 80–90% cell density. Experiments were performed according to the Roche X-tremeGENE siRNA transfection kit. Under sterile conditions, 2 μg of the siRNA transfection sequence was mixed with 100 μL of Opti-MEM I. Separately, 10 μL of the transfection reagent was diluted in 100 μL of Opti-MEM I and incubated for 5 min at room temperature. The solutions were then mixed and incubated for 15 min at room temperature. Finally, the mixture was added to the 6-well plates. The volume was brought to 2 mL with culture medium and incubated at 37 °C with 5% CO_2.

The siRNA target sequence for TFF1 is as follows: siRNA1: 5′-AGUGGGUAUGUUUGGUAGGTT-3′, siRNA2: 5′-AUGGUAUAAGGAUAGAAGCTT-3′. After 72 hrs of transfection, RNA and protein were extracted and examined using Western blotting and qPCR to compare the silencing efficiency of the two short interfering RNA fragments.

Lentiviral transfection

The cellular specimens were divided into three groups: the blank group (human CRC cells without siRNA vehicle and medium), the NC short hairpin (sh)RNA group (CRC cells transfected with empty vehicle), and the TFF1 shRNA group (CRC cells transfected with specific TFF1 siRNA). Cells in good growth condition were selected and seeded in 6-well plates (5×10^5 cells/well) and incubated overnight. When the cell growth density reached 40–50%, the cells

were ready for transfection. For viral infection, 400 μL of complete medium containing polybrene (final concentration, 5 μg/mL) was prepared, and 20–100 μL of lentiviral stock solution was added. The culture medium in 6-well plates was replaced with the viral suspension, and blank and NC groups were established in parallel. After 24 hrs of incubation, the viral medium was replaced with fresh complete medium, and cells were maintained under standard culture conditions. The lentivirus carried a green fluorescent protein reporter gene, and transduction efficiency was monitored by fluorescence microscopy. When the proportion of fluorescent-positive cells reached 80%, the cells were either passaged or cryopreserved. To establish stable cell lines, untransduced cells were eliminated by puromycin selection using the resistance gene encoded by the viral vector. Subsequent experiments were performed once cells had recovered and reached optimal growth status.

Cell counting kit-8 assay

Cell proliferation capacity was evaluated by CCK-8 assay. Cells from each group were suspended at a density of 1×10^4 cells/mL, and 100 μL/well of the suspension was seeded into 96-well plates in triplicate. Measurements were taken at 0, 24, 48, 72, and 96 hrs. Ten microliters of CCK-8 solution was added to each well and incubated for 2 hrs at 37 °C. Subsequently, the optical density value was measured with a microplate reader (Varioskan LUX, Thermo Fisher Scientific).

Cell colony formation assay

Exponentially growing cells were harvested, counted, and seeded into 6-well plates at a density of 500 cells/well. After gentle shaking to evenly distribute the cells, the plates were incubated at 37 °C with 5% CO_2 for 10–14 days, during which the medium was replaced every 3 days. After cell clones became visible, the culturing process was concluded. The cells were then fixed with 4% paraformaldehyde and stained with 0.1% crystal violet solution. The number of cell colonies was subsequently quantified. Colonies containing ≥ 50 cells were counted under an optical microscope (Olympus BX53, Tokyo, Japan), and images were acquired with the DP74 imaging system using cellSens dimension software (version 2.3, Olympus).

Transwell assay

Matrigel was diluted with precooled RPMI-1640 culture medium at a 1 : 3 ratio. The mixture was then spread over the upper surface of the Transwell chamber. After incubating at 37 °C for 1 hr, 750 μL of RPMI-640 medium was added to the lower chamber. The transfected cells were suspended at a concentration of 1×10^5 cells/mL. Subsequently, 200 μL of the cell suspension was seeded into the upper layer of the Transwell chamber and cultured at 37 °C for 72 hrs. Cells that successfully traversed the membrane were stained with a 0.1% crystal violet solution, and the number of cells was counted using an inverted microscope.

Wound healing assay

Cell migratory ability was analyzed using a wound healing assay. The transfected cells were seeded in a 6-well plate. When the cell density reached 90%, traces were drawn using a 200 μL tip head. Photographs of the same wound site were taken at 0, 24, 48, and 72 hrs, and the migratory distance was measured using ImageJ software.

Expression of E-cadherin and vimentin

The expressions of E-cadherin and vimentin messenger (m)RNA and protein in Caco-2 cells were assessed using qPCR and Western blot. The techniques for qPCR and Western blot were identical to those previously described.

Statistical analysis

The statistical software SPSS version 25.0 was employed. The measurement results are reported as the mean ± standard deviation, and the *t*-test and analysis of variance were used to compare the groups. The value of $p < 0.05$ was considered statistically significant.

Results

Trefoil factor 1 expression in colorectal cancer tissues

Forty CRC tissue sections were collected and examined by immunohistochemistry staining. Results revealed that TFF1 expression was predominantly localized in the cytoplasm of cells in colorectal cancer tissues (Figure 1A), while no obvious TFF1 expression was detected in normal colonic epithelial tissues (Figure 1B) or in the negative control without primary antibody (Figure 1C). The positive rate of TFF1 expression in CRC tissues was determined to be 35% (14/40).

Trefoil factor 1 expression in colorectal cancer cell lines

Western blot and qPCR were used to compare the expression of the *TFF1* gene protein and mRNA in the CRC cell lines HCT116 and Caco-2, as well as the normal epithelial cell line CCD-18Co. The expression of TFF1 protein and mRNA in Caco-2 was higher than that in HCT116 cells and CCD-18Co ($p < 0.05$) (Figure 2A, B, and C). Therefore, Caco-2 cells were adopted for further investigation.

Inhibiting trefoil factor 1 expression

After being transfected with two siRNA sequences, the TFF1 expression in Caco-2 cells was detected by qPCR. The results revealed a significant decrease in the mRNA expression levels in the siRNA1 and siRNA2 groups compared to the control group ($p < 0.05$). Moreover, siRNA2 inhibited TFF1 expression more effectively than siRNA1. The siRNA2 sequence was therefore packaged into a lentivirus and

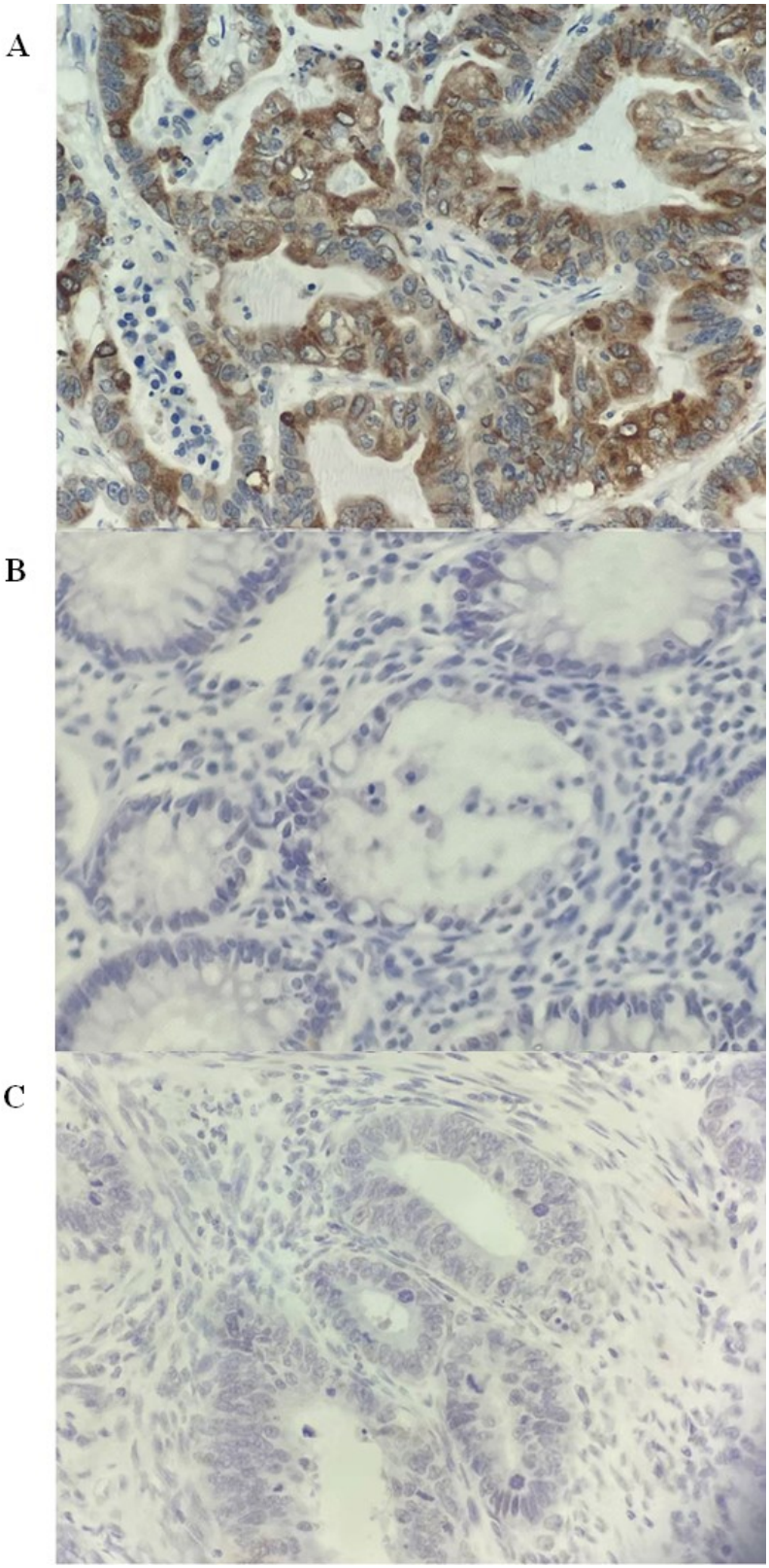

Fig. 1 – Expression of trefoil factor 1 (TFF1) protein in colorectal cancer tissues: A) positive immunohistochemistry (IHC) expression of TFF1 in colon cancer tissues (×200); B) negative IHC expression of TFF1 in normal colon epithelial tissues (×200), and C) negative IHC expression of TFF1 in normal colon epithelial tissues without primary antibody (×200).

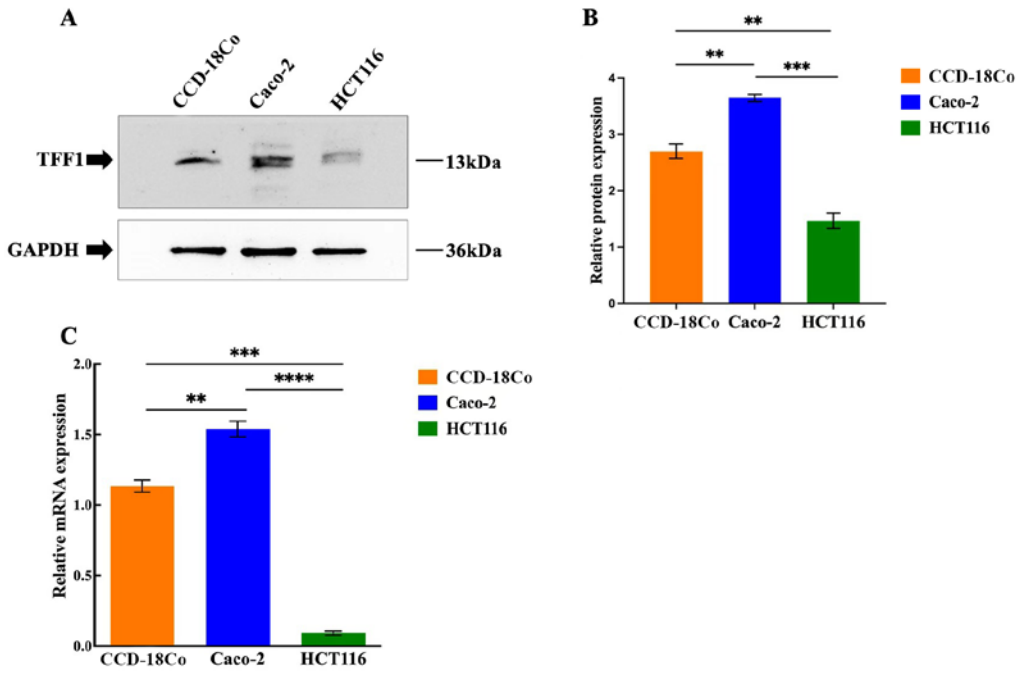

Fig. 2 – Trefoil factor 1 (TFF1) expression in colorectal cancer cell lines: A) Western blotting assay of TFF1 protein expression; B) grayscale analysis of Western blot results, and C) TFF1 mRNA expression detected by qPCR. GAPDH – glyceraldehyde-3-phosphate dehydrogenase; kDa – kilodalton; mRNA – messenger ribonucleic acid; qPCR – quantitative polymerase chain reaction.
Results are presented as mean ± standard deviation derived from triplicate analyses. *$p < 0.05$.

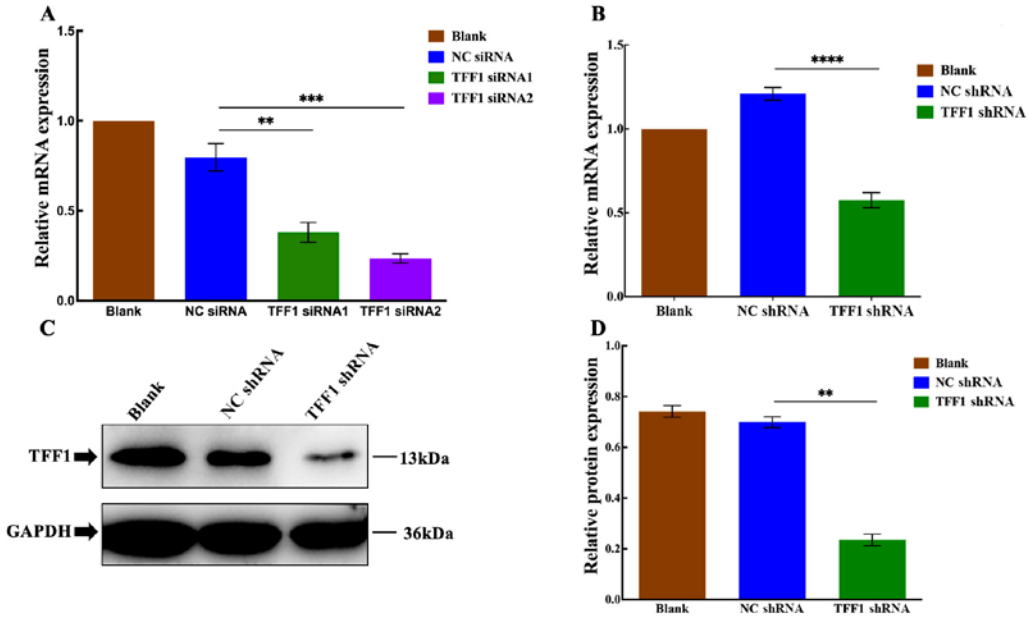

Fig. 3 – TFF1 mRNA and protein expression in cells after transfected with interfering siRNA sequences: A) qPCR detection of TFF1 mRNA after siRNA transfection; B) qPCR detection of TFF1 mRNA after lentiviral shRNA infection; C) Western blotting analysis of TFF1 protein expression, and D) relative TFF1 protein expression analyzed by grayscale scanning of Western blot bands.
NC – negative control; siRNA – small interfering ribonucleic acid; shRNA – short hairpin ribonucleic acid. For other abbreviations, see Figure 2.
Results are expressed as mean ± standard deviation of three independent experiments. *$p < 0.05$.

used to infect Caco-2 cells in subsequent experiments. The efficiency of lentivirus infection was validated using Western blot and qPCR. The TFF1 protein and mRNA levels demonstrated a substantial drop in the TFF1 shRNA group compared to the control group ($p < 0.05$) (Figure 3A, B, C, and D).

Proliferation ability analysis

Compared to the control group, the knockdown of TFF1 dramatically decreased CRC cell activity and diminished proliferative capacity ($p < 0.05$) (Figure 4A). Correspondingly, the results of the colony formation assay demonstrated a suppression of the colony formation ability in TFF1-knockdown cells ($p < 0.05$) (Figure 4B and C).

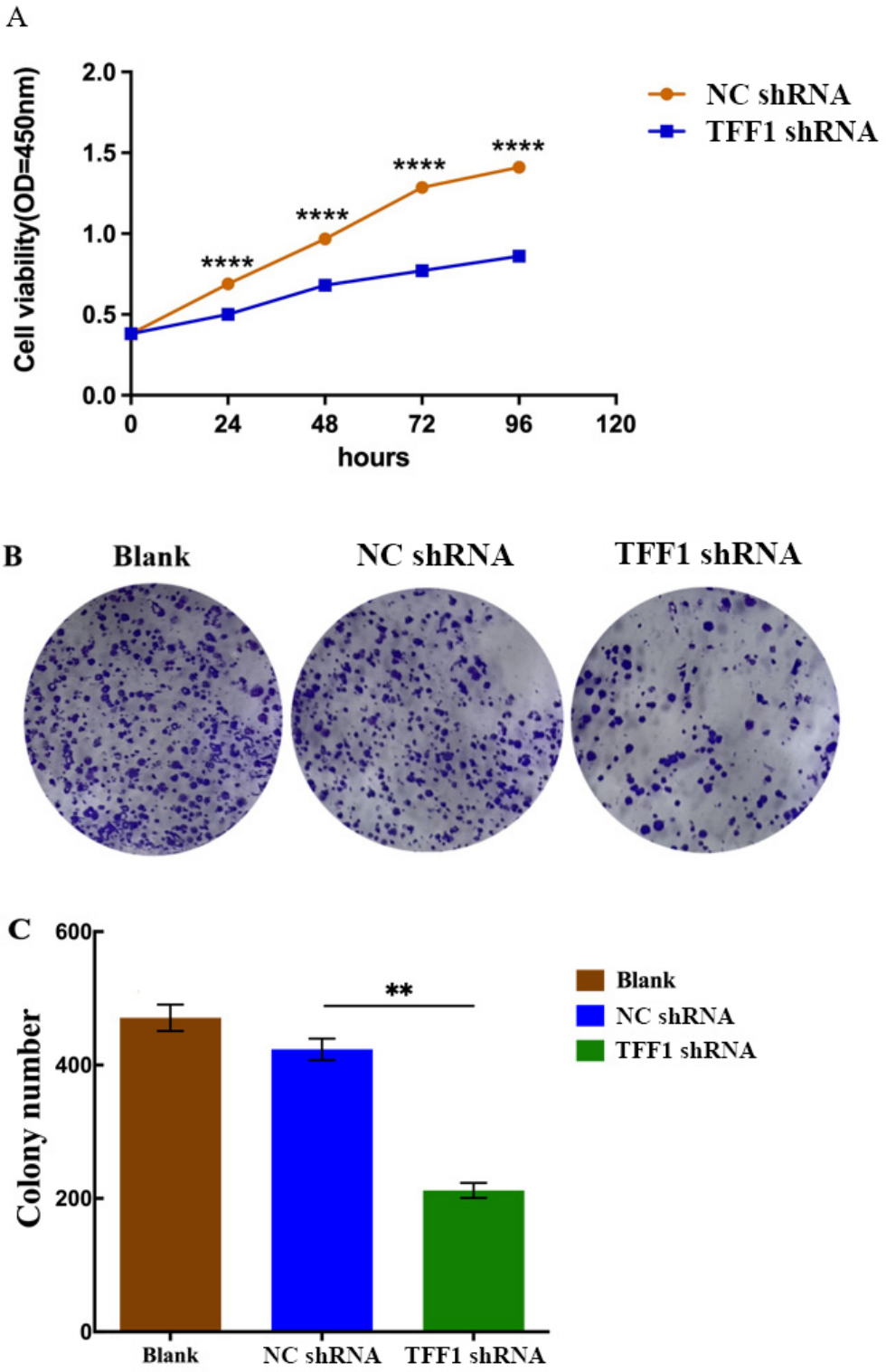

Fig. 4 – Proliferation ability analysis: A) cell counting kit-8 assay depicting cell viability [optical density (OD) = 450 nm]; B) representative images of colony formation assay (crystal violet staining, magnification ×200), and C) quantitative analysis of colony numbers from the colony formation assay.
For abbreviations, see Figures 1 and 3.
Results are shown as mean ± standard deviation of three independent experiments. *$p < 0.05$.

Invasion ability analysis

Cell invasion ability was evaluated using the Transwell assay. After culturing for 72 hrs, the number of transmembrane cells in the blank group, the NC shRNA group, and the TFF1 shRNA group was 167.329 ± 6.560, 143.190 ± 4.863, and 35.610 ± 5.728, respectively. The number of transmembrane cells in the TFF1 shRNA group was significantly lower than that in the blank and NC shRNA groups (all $p < 0.01$) (Figure 5A and B).

Migratory ability analysis cell

The migratory capacity of CRC cells was explored through a wound healing assay. The results demonstrated a substantial decrease in the migratory rate of the TFF1 shRNA group compared to the control group ($p < 0.05$) (Figure 6A and B). This result suggests that TFF1 knockdown reduces the migratory ability of CRC cells.

Relationship between trefoil factor 1 expression and epithelial-mesenchymal transition

The epithelial-mesenchymal transition (EMT) markers E-cadherin and vimentin were detected by qPCR and Western blotting analysis. The results demonstrated that the mRNA and protein expression levels of E-cadherin increased while the mRNA and protein expression levels of vimentin decreased following interference with *TFF1* gene expression (Figure 7A and B). These data imply that TFF1 is integrally involved in and enhances the EMT pathway in CRC.

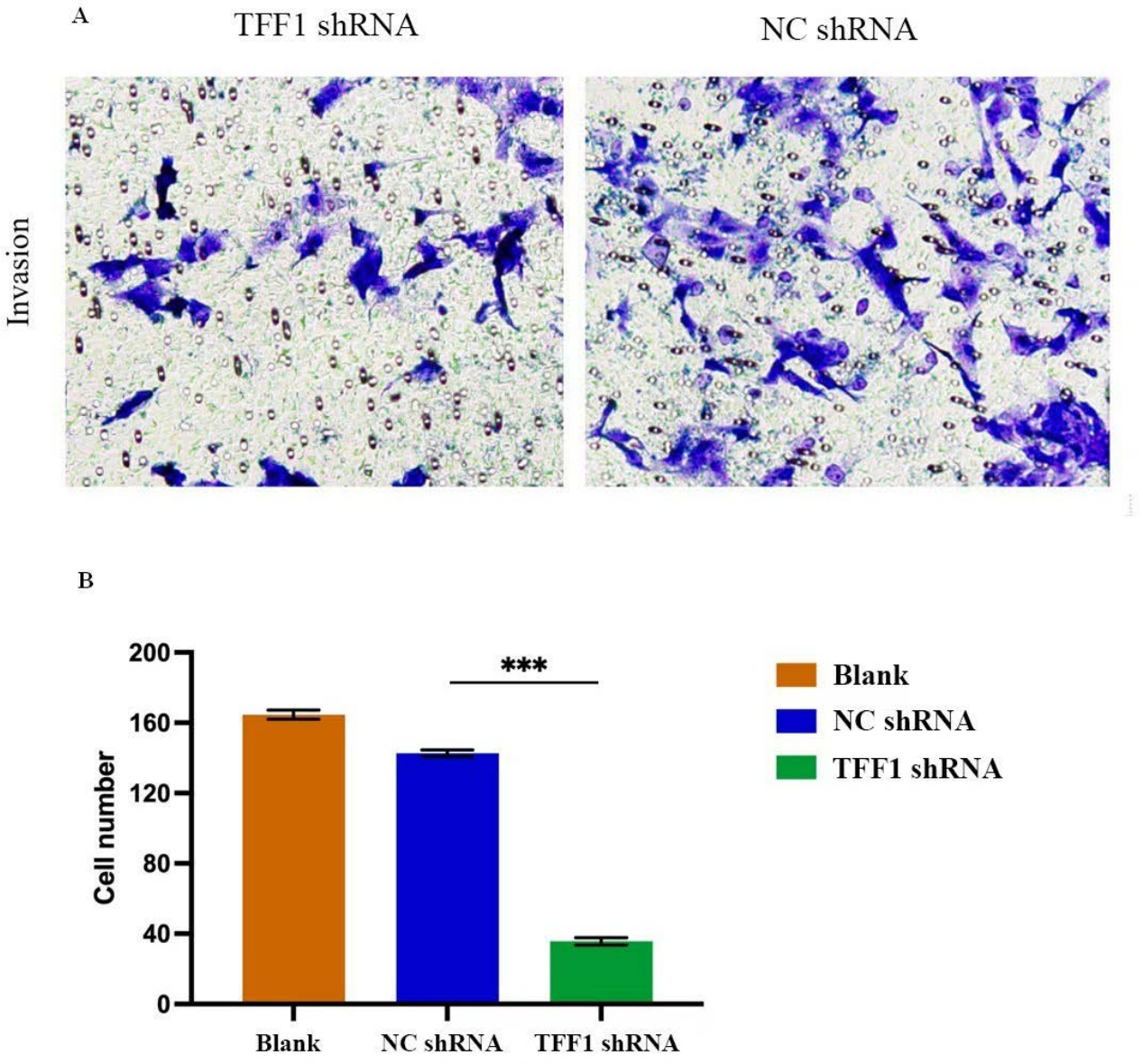

Fig. 5 – Invasion ability analysis: A) representative images of invasive cells (crystal violet staining, magnification ×200) and B) quantitative analysis of transmembrane cell numbers.
For abbreviations, see Figures 1 and 3.
Results are shown as mean ± standard deviation of three independent experiments. *$p < 0.05$.

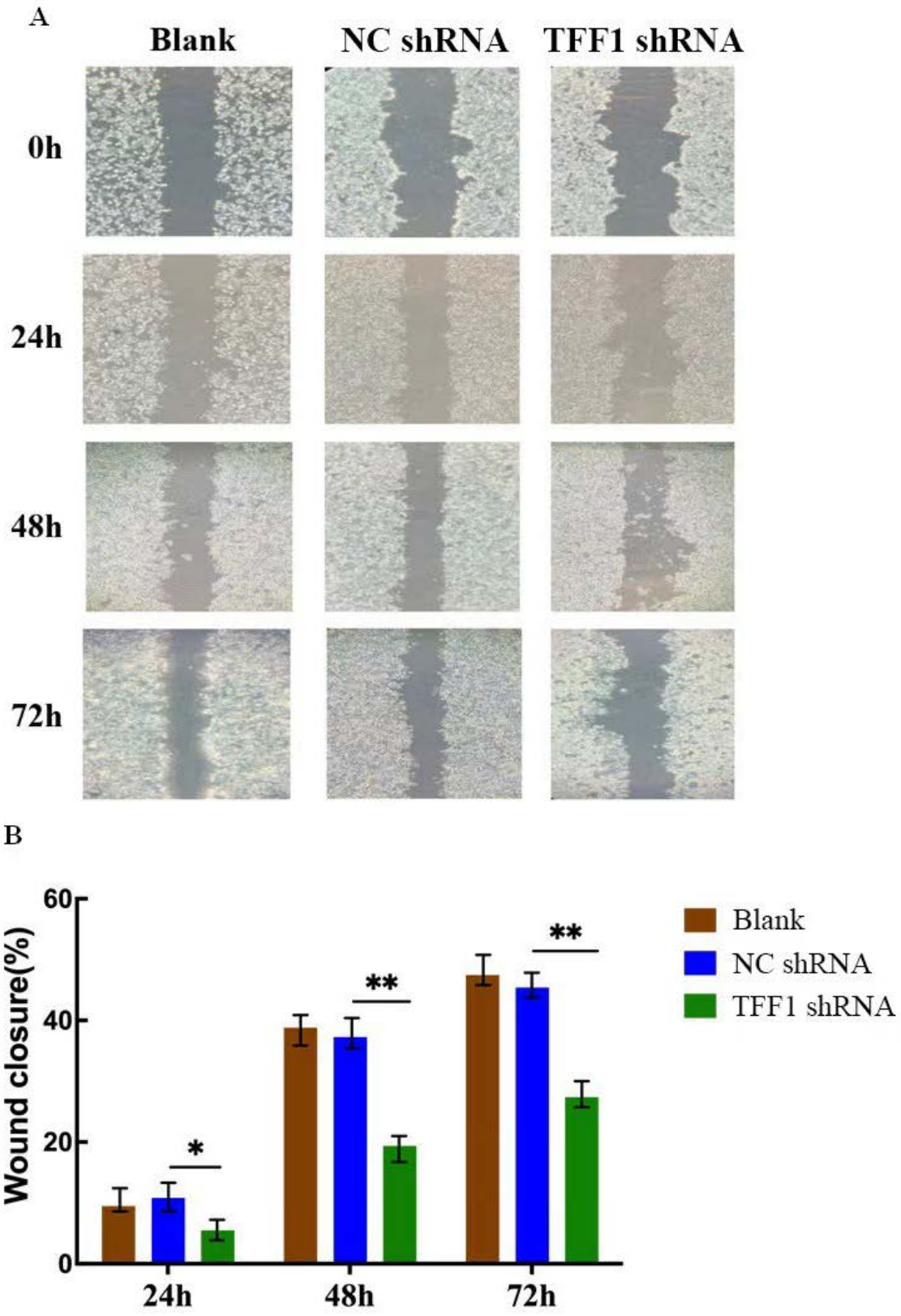

Fig. 6 – Cell migration capability analysis: A) representative images of wound healing and B) quantitative analysis of wound closure percentage.
For abbreviations, see Figures 1 and 3.
Results are shown as mean ± standard deviation of three independent experiments. *$p < 0.05$.

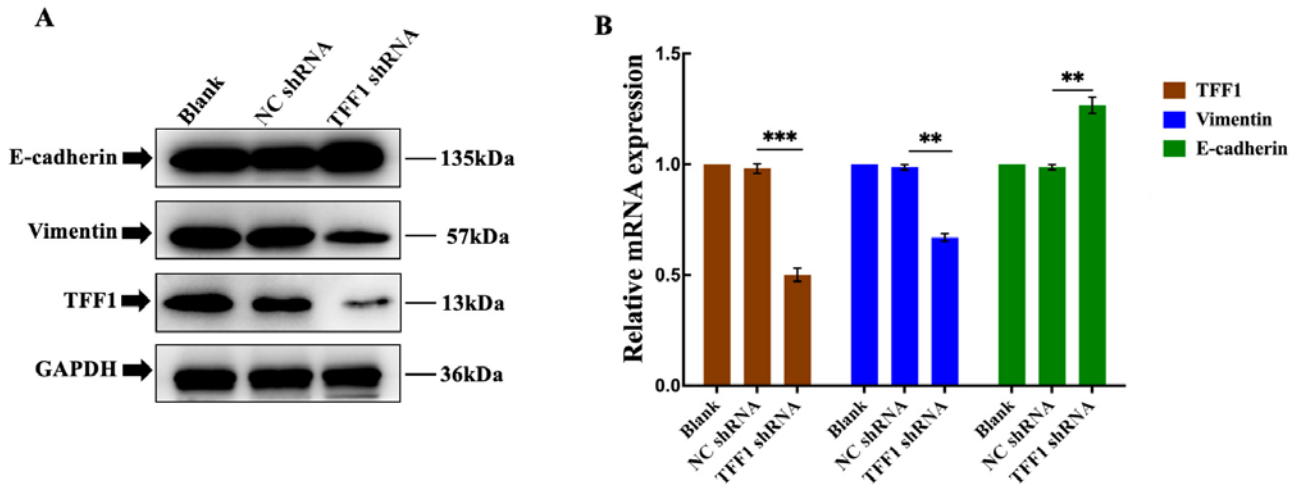

Fig. 7 – Relationship between TFF1 expression and epithelial-mesenchymal transition: A) Western blotting analysis of E-cadherin and vimentin protein expression and B) qPCR analysis of E-cadherin and vimentin mRNA expression. For abbreviations, see Figures 2 and 3. Results are expressed as mean ± standard deviation of three independent experiments. *$p < 0.05$.

Discussion

The identification of aberrant genes associated with CRC and a comprehensive understanding of its pathogenic mechanisms is of paramount importance for early disease diagnosis and the identification of potential therapeutic targets. The initiation and progression of CRC is a complex process influenced by various factors, including the activation of oncogenes, the inactivation of tumor suppressor genes, and the abnormal activation of signaling pathways. At present, well-defined genes in this context encompass *RAS*, *p53*, *APC*, dMMR, and so on [15–20].

The TFF family consists of a group of small polypeptide compounds, primarily secreted by gastrointestinal mucosal epithelial cells. The mammalian TFF family comprises three members, namely TFF1 (commonly known as breast cancer-associated peptide or pS2), TFF2 (spasmolysin or SP), and TFF3 (intestinal trefoil factor or ITF) [4, 5, 21, 22]. All these proteins share a common structural feature, the "clover" domain (P domain) [23].

As a critical member of the TFF family, *TFF1* is located on chromosome 21q, and its gene structure encompasses three exons, two introns, and two transcription promoters [24]. Presently, there is limited research concerning the influence of *TFF1* on the proliferation, invasion, and migration of CRC, as well as the underlying mechanisms.

In this study, immunohistochemical methods were used to assess 40 CRC tissue samples at the protein level, revealing an expression rate of TFF1 in CRC tissues amounting to 35% (14 out of 40), with 26 cases being negative, 12 being weak positive, 1 being positive, and 1 being strong positive. The 35% expression rate for TFF1 in this study is lower than 89% and 51% reported in the other two studies by the same method [6, 25]. This suggests that not all CRC cell lines exhibit up-regulation of *TFF1* gene expression, and the expression rate varies among populations in different regions.

Furthermore, Yusufu et al. [26] documented that the *TFF3* gene exhibits the potential to promote the malignant biological characteristics of CRC, including proliferation, migration, and invasion, by facilitating EMT. However, the relationship and underlying mechanism concerning TFF1 and the malignant biological behavior of CRC were not investigated in their study. Bossenmeyer-Pourié et al. [27] reported that TFF1 can lower cell proliferation by delaying G1-S cell phase transition in HCT116. TFF1 acts as a tumor suppressor in lung and gastric cancers, whereas it acts as a tumor promoter in CRC, ovarian cancer, and pancreatic cancer [28]. Therefore, it can exhibit a dual antiproliferative and antiapoptotic role [9, 27, 28]. In this study, two siRNAs were designed based on the coding sequence of TFF1, and the results indicated that siRNA2 had a stronger silencing effect on *TFF1* than siRNA1. Therefore, siRNA2 was incorporated into the lentivirus for functional evaluation in CRC cells. The functional assays unveiled that *TFF1* silencing effectively restrained the proliferation, colony-forming ability, invasion, and migration of CRC cells.

The process of EMT encompasses the conversion of tumor cells from an epithelial to a mesenchymal phenotype, which is regarded as a pivotal step in tumor cell invasion and dissemination [29–31]. This transition is characterized by the downregulation of epithelial markers and the upregulation of mesenchymal-related proteins. Notable markers of EMT include the downregulation of E-cadherin and the upregulation of N-cadherin or vimentin. The outcomes of this study revealed that TFF1 silencing exerts an inhibitory impact on EMT. In light of previous research by Yusufu et al. [26], which suggested that the *TFF3* gene enhances CRC malignancy by promoting EMT, it is plausible to hypothesize

that the *TFF1* gene may similarly contribute to CRC malignancy by mediating EMT.

Although some studies have investigated the TFF1 protein expression in human CRC [6–10], our study is the first to use RNA interference to silence *TFF1*, revealing that silencing *TFF1* can inhibit the proliferation, invasion, and migration of human CRC cells by suppressing EMT. The obtained results indicate that TFF1 may play a significant role in the tumorigenesis and development of human CRC, which further implicates that TFF1 can be regarded as a potential marker for poor prognosis in human CRC.

Conclusion

In summary, the findings indicate that silencing trefoil factor 1 can effectively suppress the proliferation, invasion, and migration of colorectal cancer Caco-2 cells, thereby suggesting that trefoil factor 1 may play a significant role in the initiation, progression, and metastasis of colorectal cancer by facilitating epithelial-mesenchymal transition.

Conflict of interest

The authors declare no conflict of interest.

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Nerve-sparing vs. non-nerve-sparing open radical prostatectomy: correlations between International Index of Erectile Function and corpus cavernosum electromyography

Otvorena radikalna prostatektomija sa i bez poštede neurovaskularnog snopa: korelacije između Internacionalnog indeksa erektilne funkcije i elektromiografije kavernoznih tela

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Abstract

Background/Aim. Erectile dysfunction (ED) increases with age, and the importance of sexual health has become more widespread in therapeutic practice. The aim of this study was to evaluate the correlation between subjective and objective measures of ED in patients undergoing nerve-sparing vs. non-nerve-sparing open radical prostatectomy for localized prostate cancer. **Methods.** This prospective controlled study included 50 patients with diagnosed prostate cancer and normal preoperative erectile function (EF). Patients were divided into nerve-sparing (n = 25) and non-nerve-sparing (n = 25) groups. EF was assessed preoperatively and at six months using the International Index of Erectile Function (IIEF) questionnaire, while corpus cavernosum electromyography recorded spontaneous smooth muscle activity. Statistical analysis included paired *t*-tests and Spearman's correlations. **Results.** Postoperative IIEF scores declined significantly in both groups (nerve-sparing: 22.04 ± 2.10 to 17.87 ± 3.83, $p < 0.001$; non-nerve-sparing: 21.67 ± 2.64 to 6.42 ± 1.51, $p < 0.001$). Bilateral nerve-sparing preserved superior EF compared to unilateral preservation (19.87 ± 2.80 vs. 14.13 ± 2.42, $p < 0.001$). No significant correlation was found between corpus cavernosum electromyography parameters (amplitude, mean wave, phase reversals) and IIEF scores ($p > 0.05$). **Conclusion.** Nerve-sparing open radical prostatectomy, particularly bilateral techniques, significantly reduces ED severity. However, corpus cavernosum electromyography did not correlate with patient-reported outcomes, suggesting its limited standalone utility in postoperative ED assessment.

Key words:
electromyography; erectile dysfunction; penile erection; prostatectomy; prostatic neoplasms.

Apstrakt

Uvod/Cilj. Erektilna disfunkcija (ED) se povećava sa godinama, a značaj seksualnog zdravlja postao je sve rasprostranjeniji u terapeutskoj praksi. Cilj rada bio je da se proceni korelacija između subjektivnih i objektivnih parametara ED kod bolesnika koji su podvrgnuti otvorenoj radikalnoj prostatektomiji, sa i bez poštede neurovaskularnog snopa, zbog lokalizovanog karcinoma prostate. **Metode.** Prospektivna kontrolisana studija obuhvatila je 50 bolesnika sa dijagnostikovanim karcinomom prostate i normalnom preoperativnom erektilnom funkcijom (EF). Bolesnici su podeljeni u grupu sa poštedom neurovaskularnog snopa (n = 25) i grupu bez poštede neurovaskularnog snopa (n = 25). EF je procenjivana preoperativno i nakon šest meseci korišćenjem upitnika za procenu Internacionalnog indeksa eretkilne funkcije (IIEF), dok je elektromiografija kavernoznih tela registrovala spontanu aktivnost glatkih mišića. Statistička analiza uključivala je upareni *t*-test i Spearmanove korelacije. **Rezultati.** Postoperativni rezultati IIEF su se značajno smanjili u obe grupe (poštedna tehnika: 22,04 ± 2,10 na 17,87 ± 3,83, $p < 0,001$; nepoštedna tehnika: 21,67 ± 2,64 na 6,42 ± 1,51, $p < 0,001$). Bilateralnom poštedom neurovaskularnog snopa očuvana je superiornija EF u poređenju sa unilateralnom tehnikom (19,87 ± 2,80 vs.14,13 ± 2,42, $p < 0,001$). Nije nađena značajna korelacija između parametara elektromiografije kavernoznog tela (amplituda, srednji talas, fazni preokreti) i rezultata IIEF ($p > 0,05$). **Zaključak.** Otvorena radikalna prostatektomija sa poštedom neurovaskularnog snopa, naročito bilateralna tehnika, značajno smanjuje težinu ED. Međutim,

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elektromiografija kavernoznih tela nije korelirala sa ishodima koje su prijavili bolesnici, što ukazuje na njenu ograničenu samostalnu primenljivost u postoperativnoj proceni ED.

Ključne reči:
elektromiografija; erektilna disfunkcija; penis, erekcija; prostatektomija; prostata, neoplazme.

Introduction

Erectile dysfunction (ED) can be defined as an occasional or persistent inability to achieve and sustain a sufficient penile erection for satisfying sexual intercourse. The significance of sexual health, particularly ED, has become more prevalent in therapeutic practice over the past three decades. The prevalence of ED increases with age, affecting 40% of men aged 40 to 70 years and impacting millions of men globally [1, 2]. The cause of ED is multifactorial, involving a complex interaction of vascular, neurological, hormonal, and psychological variables. Moreover, vascular irregularities in the penile blood supply and erectile tissue are significantly linked to cardiovascular disease and its risk factors. A previous meta-analysis revealed ED as an independent predictor of cardiovascular events [3–5]. Vascular variables primarily influence ED at the local level, but neurogenic factors may affect ED at all levels of the nervous system, encompassing local supply *via* the autonomic nervous system, the genital apparatus, and extending to the spinal, supraspinal, and higher cerebral centers [1]. The European Association of Urology Guidelines on Sexual and Reproductive Health recommend redefining the etiology of ED into a binary categorization of primary organic and primary psychogenic, given that most cases are of mixed etiology. Over the past two decades, extensive information has emerged concerning the relationship between several modifiable and unmodifiable risk factors and the pathophysiology of ED. Prevalent etiologies of ED encompass psychological disorders (depression, anxiety, and stress), neurological disorders (stroke, Alzheimer's disease, spinal cord injury), hormonal imbalances, prostate problems [such as radiation and/or surgery for prostate cancer (PCa)], and cardiovascular diseases. Certain medications used for chronic conditions (such as antidepressants, antihistamines, and antihypertensives) or lifestyle factors (including alcohol or substance misuse and obesity) may precipitate ED [6, 7]. Among men, PCa ranks as the second most prevalent solid tumor and the fifth leading cause of cancer-related death. Age, race, familial history, and genetic mutations are recognized non-modifiable risk factors for PCa, but metabolic syndrome, obesity, and smoking are considered potential modifiable risk factors. The largest age-standardized incidence rates are observed in Western and Northern Europe, Australia/New Zealand, South and North America, and Southern Africa [8–11]. Standard treatment modalities for clinically localized PCa include active monitoring/surveillance, radical prostatectomy (RP), radiotherapy, and brachytherapy. As a standard surgical approach for localized prostate cancer, RP involves excision of the entire prostate gland along with adequate adjacent tissue to ensure negative margins [8, 12, 13]. The International Index of Erectile Function (EF)-5 (IIEF-5) questionnaire is a commonly used assessment for evaluating ED of several origins, particularly in patients post-RP. The IIEF has five domains: EF, orgasmic function, sexual desire, intercourse satisfaction, and overall satisfaction [14, 15]. Among the various surgical options for PCa, RP can be conducted using numerous approaches, with open RP (ORP) and laparoscopic RP (LRP) being predominant until the introduction of robot-assisted RP (RARP). Following RP, patients face a considerable risk of postoperative complications, most notably ED. Postoperative ED is a significant problem for patients that can impact quality of life and mental well-being, perhaps affecting the choice to pursue surgical intervention [16, 17]. Corpus cavernosum (CC) electromyography (EMG) – CC-EMG measures the spontaneous electrical activity of cavernous smooth muscles during flaccidity, reflecting integrated sympathetic tone. Unlike invasive needle electrodes, surface electrodes provide reliable recordings without disrupting tissue integrity. CC-EMG potentials diminish during erection, validating their association with cavernous smooth muscle contractility [18].

The aim of this study was to evaluate the correlations between subjective and objective parameters of ED in patients who underwent nerve-sparing vs. non-nerve-sparing ORP, for localized PCa.

Methods

The study was conducted at the Clinic of Urology, University Clinical Center of Vojvodina, Novi Sad, Serbia, between June 2021 and July 2022. The study was approved by the Ethics Committee of the University Clinical Center of Vojvodina (No. 00-02/595, from November 20, 2009) and conducted in accordance with the Declaration of Helsinki. Prior to their acceptance into the study, all patients were required to provide informed consent.

The study's inclusion criteria were as follows: male aged > 40 years; localized PCa [pT1b-T3a, Gleason score ≤ 7, prostate-specific antigen (PSA) < 20 ng/mL]; normal preoperative EF (IIEF-5 score ≥ 17). The study's exclusion criteria were as follows: declining to sign the informed consent form; metastatic PCa; presence of neurological disorders or diabetes mellitus; previous chemotherapy or pelvic radiotherapy; PSA recurrence during follow-up; technical failures in EMG recording.

Patients were divided into two groups: 25 patients undergoing nerve-sparing group (NSG) ORP (either unilateral or bilateral neurovascular bundle preservation) and 25 patients treated with the non-nerve-sparing group (NNSG) technique based on established oncological criteria. All patients underwent diagnostic procedures, including transrectal ultrasound prostate scans and PSA level laboratory findings.

PCa was diagnosed after transrectal ultrasound-guided prostate biopsy. Pelvic computed tomography scan and skeletal scintigraphy were performed to exclude metastatic progression of disease. Each patient underwent ORP, performed by a single surgeon.

Using the IIEF-5 questionnaire administered preoperatively and at six-month postoperative follow-up, EF was clinically evaluated. The questionnaire consists of five questions, each rated on a five-point scale. A maximum of 25 points can be attained, and a score of 21 indicates ED. The severity of ED is classified into four grades: severe (1–7 points), moderate (8–11 points), mild to moderate (12–16 points), and mild (17–21 points) [14].

CC-EMG recordings were performed using the Solar® urodynamic system with Neuro Module (Medical Measurement Systems, Netherlands), following the methodology described by Roaiah et al. [18]. Surface electrodes were placed on the mid-shaft of the penis (over both corpora cavernosa) after standard skin preparation with abrasive gel (NuPrep®, Weaver and Co., USA) and alcohol disinfection to reduce impedance. A reference electrode was positioned on the ipsilateral knee. Recordings were conducted in a quiet, temperature-controlled environment (22–24 °C) with patients in a supine position (30° trunk elevation) to minimize sympathetic arousal artifacts. Each 20-min flaccid-state recording captured spontaneous electrical activity, which was subsequently analyzed for three key parameters: wave amplitude (average, maximal, and minimal values expressed in μV), wave complexity (number of peaks *per* complex and phase reversals or "turns"), and baseline slow-wave activity patterns.

Statistical analysis

Statistical analyses were performed using the SPSS 26.00 software. Continuous variables were expressed as mean ± standard deviation (SD) for normally distributed data or median with interquartile range for non-parametric distributions, and normality was assessed using Shapiro-Wilk testing. Group comparisons utilized Student's *t*-test for parametric data and Mann-Whitney *U* test for non-parametric datasets. Paired comparisons employed the Wilcoxon signed-rank test, while associations between parameters were evaluated using Spearman's correlation coefficient (ρ). A two-tailed p-value < 0.05 was considered statistically significant throughout all analyses.

Results

The baseline demographic characteristics of the analyzed patient groups showed no statistically significant variation in age distribution between NSG and NNSG (Table 1). The average age in NSG was 62.59 ± 4.51 years (range: 55–70), whereas in NNSG, it was 62.90 ± 3.10 years (range: 58–67). These findings confirm demographic homogeneity and allow valid comparison of functional outcomes between the groups.

The distribution of preoperative and postoperative Gleason scores between the study groups showed that preoperative analysis showed no significant difference in Gleason score 6 vs. Gleason score 7 distribution between NSG (64% vs. 36%) and NNSG (44% vs. 56%) ($p > 0.05$). Similarly, postoperative Gleason scores revealed no statistically significant differences ($p > 0.05$), confirming oncological comparability across the cohorts (Table 2).

The descriptive parameters of EF evaluation before and after RP in NSG and NNSG showed that the mean value in NSG patients was 22.04 ± 2.10 preoperatively and 17.87 ± 3.83 postoperatively. Both numbers demonstrate maintained EF in all participants. Student's *t*-test revealed a very significant difference in EF evaluation pre- and post-RP in NSG ($t = 5.053$, $p = 0.000$), indicating considerably lower values postoperatively. The mean score among NNSG patients was

Table 1

Baseline demographic characteristics of the study groups

Age	Group		*p*-value
	nerve-sparing	non-nerve-sparing	
Years (mean ± SD)	62.59 ± 4.51	62.90 ± 3.10	> 0.05
Range (min–max)	55–70	58–67	–

SD – standard deviation; min – minimum; max – maximum.

Table 2

Preoperative and postoperative Gleason score distribution in nerve-sparing and non-nerve-sparing groups

Gleason score category	Group (n = 25)		*p*-value
	nerve-sparing	non-nerve-sparing	
Preoperative			
Gleason score 6	16 (64)	11 (44)	> 0.05
Gleason score 7	9 (36)	14 (56)	
Postoperative			
Gleason score 6	14 (56)	13 (52)	> 0.05
Gleason score 7	11 (44)	12 (48)	
Total	25 (100)	25 (100)	–

All values are given as numbers (percentages)
n – number.

21.67 ± 2.64 prior to the procedure and 6.42 ± 1.51 after the intervention, therefore substantiating the incidence of ED in NNSG patients pre-RP. Student's *t*-test for dependent samples revealed a very significant difference in EF evaluation before and after RP in NNSG (t = 14.488, p = 0.000), indicating considerably lower values post-intervention (Table 3).

The non-parametric correlation coefficients (Spearman's correlation coefficient ρ, significance level p) between the evaluation of EF values (IIEF-5 score) and the observed CC-EMG characteristics (amplitude, mean wave, turn) pre- and post-RP in the cohort of NSG patients showed that no significant correlation was identified among the observed parameters (Table 4).

The preoperative EF (IIEF-5) scores in NSG did not exhibit a significant difference between the unilateral and bilateral subgroups (p = 0.459). A statistical analysis of the average score post-surgery revealed a significant difference among the patient subgroups (t = 4.897, p = 0.000), with notably elevated values in the cohort with bilateral sparing (Table 5). A comparison of postoperative scores to preoperative values indicated a considerably higher change in the unilateral NSG (Mann-Whitney Z = 3.608, p = 0.000).

No significant difference (p = 0.933) was seen in preoperative ED scores between patients undergoing nerve-sparing procedures (mean = 4.65, median = 5.00, SD = 0.49) and those not undergoing nerve-sparing procedures (mean = 4.67, median = 5.00, SD = 0.49). The postoperative ED score values indicate a statistically significant difference (Mann-Whitney Z = 4.829, p = 0.000) between NSG (mean = 3.96, median = 4.00, SD = 0.93) and NNSG patients (mean = 1.33, median = 1.00, SD = 0.49).

Figure 1 displays the mean score values [95% confidence interval (CI)] post-surgery for both NSG and NNSG patients.

Figure 2 presents the ED score values alongside the 95% CI pre- and post-RP in NSG patients.

Table 3

Assessment of erectile function (IIEF-5 score) pre- and post-radical prostatectomy in the nerve-sparing and non-nerve-sparing groups

Groups	Mean ± SD	Median (Min–Max)
Nerve-sparing		
preoperative	22.04 ± 2.10	22 (17–25)
postoperative	17.87 ± 3.83	17 (11–23)
Non-nerve-sparing		
preoperative	21.67 ± 2.64	22 (17–25)
postoperative	6.42 ± 1.51	6 (5–9)

IIEF – International Index of Erectile Function; SD – standard deviation; Min – minimum; Max – maximum.

Table 4

Correlation between IIEF-5 questionnaire and CC-EMG findings, pre- and post-radical prostatectomy, in the nerve-sparing group of patients

Characteristics of CC-EMG	IIEF-5 score			
	preoperative		postoperative	
	Spearman's ρ	*p*-value	Spearman's ρ	*p*-value
Average amplitude	0.024	0.913	0.027	0.904
Maximal amplitude	-0.164	0.455	0.057	0.795
Mean wave	0.090	0.683	0.081	0.713
Turn (phase reversals)	-0.055	0.804	0.223	0.306

IIEF – International Index of Erectile Function; CC-EMG – corpus cavernosum electromyography.

Table 5

Assessment of erectile function (IIEF-5 score) pre- and post-radical prostatectomy, in unilateral and bilateral subgroups of the nerve-sparing group patients

Subgroups	IIEF-5 score			
	preoperative		postoperative	
	mean ± SD	median (min–max)	mean ± SD	median (min–max)
Unilateral	22.50 ± 1.41	22 (21–25)	14.13 ± 2.42	14 (11–17)
Bilateral	21.80 ± 2.40	22 (17–25)	19.87 ± 2.80	22 (16–23)
Total	22.04 ± 2.10	22 (17–25)	17.87 ± 3.83	22 (11–23)

IIEF – International Index of Erectile Function; SD – standard deviation; min – minimum; max – maximum.

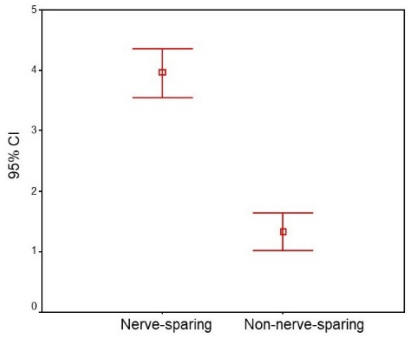

Fig. 1 – Assessment of erectile function (ED score) post radical prostatectomy in nerve-sparing vs. non-nerve-sparing groups. ED – erectile dysfunction; CI – confidence interval.

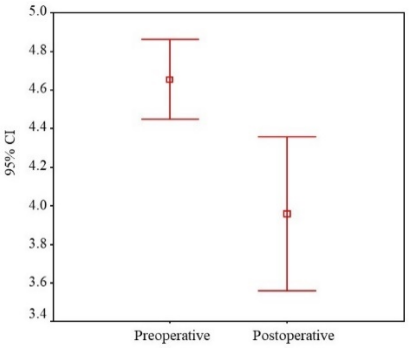

Fig. 2 – Assessment of erectile function (ED score) pre- and post-radical prostatectomy in the nerve-sparing group. For abbreviations, see Figure 1.

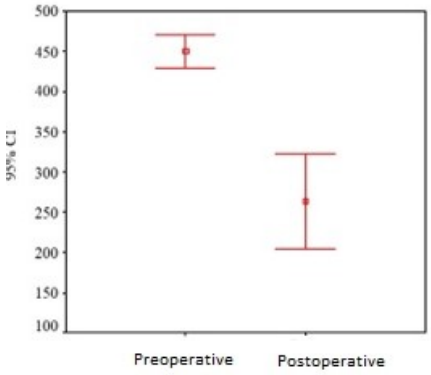

Fig. 3 – Electromyographic parameters of corpus cavernosum function (average amplitude) pre- and post-radical prostatectomy in the non-nerve-sparing group. For abbreviations, see Figure 1.

Figure 3 illustrates the mean amplitude values together with the 95% CI for NNSG patients, both pre- and post-surgery.

Discussion

The objective of RP is to entirely excise the tumor while minimizing surgical morbidity, such as urine incontinence and reduced sexual function. RP is the gold standard therapy for localized PCa and produces excellent oncological results. Systematic reviews indicate that RARP is superior to LRP in decreasing ED, yet comparable to ORP. However, some studies have found no difference in ED outcomes between the two surgical methods. Urologists frequently assert that the surgeon's expertise is more critical than the surgical technique employed in RP regarding functional results, particularly EF [12, 13, 17, 19]. In our institution, both ORP and LRP are routinely performed; however, ORP was selected for this study based on the surgeon's preference.

This prospective controlled study evaluated the effect of RP, with or without neurovascular bundle preservation, on postoperative EF in patients with localized PCa. Employing

both subjective (IIEF-5 questionnaire) and objective (CC-EMG) evaluation methodologies, we wanted to clarify the processes behind post-prostatectomy ED and the prospective protective function of nerve-sparing surgical approaches. The findings provide valuable insights into the functional outcomes associated with different surgical approaches while highlighting the potential role of electrophysiological testing in postoperative evaluation.

Our data show that ED is a common and substantial outcome of RP. The NSG cohort had a statistically significant reduction in EF at six months postoperatively; however, it retained scores predominantly within the "mild" dysfunction range. In contrast, individuals in the NNSG cohort had a significant decline in EF, with the majority transitioning into the "severe" ED category. The dorsal penile nerve plays a dual role in EF through both sensory innervation and autonomic connections with cavernous nerves. Its anatomical proximity to the prostatic apex makes it susceptible to surgical injury during RP, potentially contributing to postoperative ED through combined sensory and neurovascular mechanisms. This vulnerability may explain functional outcomes even in nerve-sparing procedures [20].

No significant preoperative variation in IIEF-5 scores was seen within groups, indicating comparable baseline EF. This highlights that the postoperative decrease is mostly due to the surgical method, rather than inherent functional disparities. Moreover, subgroup analysis indicated that bilateral NSG was markedly more effective than unilateral preservation in maintaining EF, underscoring the need for whole neurovascular preservation when possible. In our study, EMG analysis of corpus cavernosum function did not reveal any statistically significant correlation with subjective EF (IIEF-5), either preoperatively or postoperatively. The role of CC-EMG in evaluating EF post-RP remains incompletely defined. Although previous work by Roaiah et al. [18] suggested that EMG could provide insight into the functional status of smooth muscle tissue within the corpora cavernosa, our data suggest limited clinical utility in this context, particularly when evaluated in isolation. Several factors may explain this lack of correlation. First, the flaccid-state CC-EMG recordings may be less sensitive to subtle functional changes. Second, the neurogenic component of EF, though important, may be overwhelmed by vascular or psychogenic elements post-surgery. These findings align with the comprehensive review of Kyriazis et al. [21], which demonstrated that bilateral nerve-sparing techniques, particularly intrafascial approaches, optimize EF preservation. The observed functional decline even in nerve-sparing cases supports the findings of Yildiz et al. [20] regarding dorsal penile nerve vulnerability during apical dissection. This research highlights the necessity for athermal techniques and accurate nerve mapping to reduce neuropraxia while ensuring oncological safety [18–23].

This study confirms that bilateral nerve-sparing techniques better preserve EF post-prostatectomy, with outcomes aligning with established evidence. Younger patients (< 60 years) with good preoperative function showed superior recovery, while CC-EMG parameters lacked correlation with subjective outcomes, suggesting limited clinical utility. In the study of Prabhu et al. [24], long-term data indicate functional decline, particularly in older patients, underscoring the need for individualized preoperative counseling. Postoperative EF recovery is a protracted process, often requiring years rather than months. While nerve-sparing approaches, particularly bilateral preservation, correlate with better outcomes, even optimal techniques cannot guarantee immediate functional restoration [25, 26].

This study's limitations include a reduced sample size, shortened follow-up duration, and the omission of additional influential factors, including body mass index, smoking status, and prior psychiatric comorbidities. Although a six-month follow-up provides valuable insights into the early postoperative recovery of EF, long-term evaluation (12–24 months) would offer a more comprehensive picture and should be considered in future studies. Despite these constraints, our findings further endorse the application of nerve-sparing methodologies in PCa surgery, which is clinically justified. Furthermore, they propose that although EMG may possess theoretical significance in evaluating cavernous nerve or smooth muscle function, its clinical applicability remains unclear without a more robust link to subjective or functional results.

Conclusion

This study suggests that nerve-sparing methods, especially bilateral preservation, substantially enhance the recovery of erectile function following radical prostatectomy. Although corpus cavernosum electromyography provided objective electrophysiological data, its association with patient-reported IIEF-5 scores proved limited, suggesting that these methods are complementary rather than interchangeable in post-surgical evaluation. These findings underscore the importance of surgical precision in preserving neurovascular bundles, while highlighting the need for further research to improve functional assessment methods. The observed discrepancy between expected and obtained corpus cavernosum electromyography findings may partly reflect the limited number of patients in each subgroup.

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Development of a personalized sound therapy system and its therapeutic effect on subjective tinnitus patients

Razvoj personalizovanog sistema zvučne terapije i njegov terapijski efekat na pacijente sa subjektivnim tinitusom

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Abstract

Background/Aim. Tinnitus is a prevalent auditory condition that significantly affects the quality of life. Despite the variety of treatments available, their effectiveness is inconsistent. The aim of this study was to evaluate the impact of a newly developed sound therapy system on tinnitus patients, specifically in terms of changes in audiology, tinnitus-related tests, and tinnitus psychoacoustic characteristics. **Methods**. A total of 100 patients with tinnitus were included in the study. They were divided into two groups: the control group (CG) (n = 48) and the treatment group (TG) (n = 52). Various demographic characteristics of the patients were recorded. All patients had a thorough audiological assessment, which included pure tone audiometry and acoustic immittance testing, as well as tinnitus-specific evaluations, such as tinnitus frequency matching, tinnitus loudness matching, minimum masked level (MML), and residual inhibition (RI) examinations. In addition, psychoacoustic characteristics of tinnitus were assessed, including Tinnitus Handicap Inventory (THI), Hospital Anxiety and Depression Scale (HADS), Pittsburgh Sleep Quality Index (PSQI), and Visual Analog Scale (VAS) scores. **Results.** There were no significant differences in various demographic characteristics between CG and TG before acoustic therapy. Additionally, there were no significant differences between the groups in audiometric assessments, tinnitus-related tests, psychological acoustic characteristics before treatment, or in the distribution of tinnitus frequencies. The hearing threshold level in TG was notably lower than in CG (p = 0.0238). Tinnitus loudness was significantly reduced in TG (p = 0.0256). MML in TG tended to improve (p = 0.0532), although this difference did not reach statistical significance and showed a positive trend. Among the subscales of THI, TG demonstrated significant improvements. PSQI scores also indicated they were significantly lower in TG than in CG (p = 0.0238). The total score on HADS (16.58 ± 2.89 vs. 16.67 ± 2.76; p = 0.8730) and the total score on VAS (4.19 ± 1.48 vs. 4.22 ± 1.37; p = 0.9160) did not show a significant difference. **Conclusion.** Acoustic therapy significantly improves patients' hearing, reduces tinnitus loudness, and improves sleep quality. Our acoustic therapy system is an effective strategy for alleviating chronic subjective tinnitus.

Key words:
audiometry; hearing tests; quality of life; surveys and questionnaires; tinnitus.

Apstrakt

Uvod/Cilj. Tinitus je rasprostranjeno auditivno stanje koje značajno utiče na kvalitet života. Uprkos nizu dostupnih tretmana, njihova delotvornost je varijabilna. Cilj rada bio je da se proceni uticaj novorazvijenog sistema zvučne terapije na pacijente sa tinitusom, posebno u smislu promena u audiologiji, testova povezanih sa tinitusom i psihoakustičkih karakteristika tinitusa. **Metode.** U studiju je bilo uključeno 100 pacijenata sa tinitusom. Podeljeni su u dve grupe, kontrolnu grupu (KG) (n = 48) i terapijsku grupu (TG) (n = 52). Zabeležene su različite demografske karakteristike pacijenata. Svi pacijenti imali su temeljnu audiološku procenu, koja je obuhvatila audiometriju čistih tonova i testiranje akustičke imitanse, kao i procene specifične za tinitus, kao što su određivanje frekvencije tinitusa, određivanje jačine tinitusa, ispitavanja minimalnog maskirajućeg nivoa (*minimum masked level* – MML) i rezidualne inhibicije (RI). Pored toga, procenjene su psihoakustičke karakteristike tinitusa, uključujući rezultate Upitnika umanjenja sposobnosti zbog tinitusa (*Tinnitus Handicap Inventory* – THI), Bolničke skale za anksioznost i depresiju (*Hospital Anxiety and Depression Scale* – HADS), Pitsburškog indeksa kvaliteta sna (*Pittsburgh Sleep Quality Index* – PSQI) i Vizuelne analogne skale (*Visual Analog Scale* – VAS). **Rezultati.** Nije bilo značajnih razlika u različitim demografskim karakteristikama između KG i TG pre akustičke terapije. Dodatno, nije bilo značajnih razlika između grupa u audiometrijskim procenama, testovima povezanim sa tinitusom, psihoakustičkim karakteristikama tinitusa pre tretmana, niti u distribuciji frekvencija tinitusa. Prag sluha u TG bio je znatno niži nego u KG

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(p = 0,0238). Jačina tinitusa značajno se smanjila u TG (p = 0,0256). MML u TG imao je tendenciju poboljšanja (p = 0,0532), iako ova razlika nije dostigla statističku značajnost i pokazala je pozitivan trend. Među podskalama THI, u grupi TG je pokazano značajno poboljšanje. Takođe, rezultati PSQI pokazali su da su skorovi u TG bili značajno niži nego u KG (p = 0,0238). Ukupni rezultati na skalama HADS (16,58 ± 2,89 vs. 16,67 ± 2,76; p = 0,8730) i VAS (4,19 ± 1,48 vs. 4,22 ± 1,37; p = 0,9160), nisu pokazali značajnu razliku. **Zaključak.** Akustička terapija značajno poboljšava sluh pacijenata, smanjuje jačinu tinitusa i poboljšava kvalitet sna. Naš sistem akustičke terapije je efikasna strategija za ublažavanje hroničnog subjektivnog tinitusa.

Ključne reči:
audiometrija; sluh, ispitivanje; kvalitet života; ankete i upitnici; tinitus.

Introduction

Tinnitus is a prevalent auditory condition characterized by the perception of sound without external acoustic stimulation, with most cases classified as subjective, meaning the sounds are perceived only by the individual [1, 2]. Epidemiological studies across 16 countries reveal that the majority of research (38.5%) has been conducted in Europe, with nearly half (48.7%) of the studies published since 2010 [3]. Reported prevalence rates vary widely, ranging from 5.1% to 42.7%, while studies using consistent definitions report a narrower prevalence of 11.9% to 30.3% [4]. Data indicate that tinnitus prevalence increases with age, and males tend to have a higher incidence compared to females [5]. Recent research suggests that 9.6% of adults in the USA experienced tinnitus within the past 12 months, while in China, tinnitus patients constitute 7.5% of those seeking treatment at otolaryngology clinics [6]. Persistent tinnitus can lead to significant psychological distress, including depression and anxiety, severely impacting quality of life and causing difficulties with sleep, concentration, and emotional well-being. Comorbidities such as hypertension, diabetes, and arteriosclerosis further exacerbate the overall health burden [7–9]. Due to the lack of standardized treatment protocols, ineffective or poorly regulated treatments can lead to increased healthcare costs [10, 11]. The 2014 clinical practice recommendations from the American Academy of Otolaryngology-Head and Neck Surgery do not endorse pharmaceutical therapies, including antidepressants, anticonvulsants, and anti-anxiety drugs. Instead, cognitive behavioral therapy and the use of hearing aids are considered effective management strategies [12], particularly for patients with associated hearing impairment.

In recent years, personalized and effective acoustic therapy (AT) protocols for tinnitus patients have become a primary focus of clinical research worldwide [13–15]. Various acoustic therapies, including natural sounds [15], broadband noise [16], tailored notched music training [17], and frequency discrimination training [18], have been developed and studied. Although numerous studies have demonstrated the efficacy of acoustic therapies and conducted systematic analyses, they have concluded that more large-scale, multi-center, randomized controlled studies are necessary. Additionally, it remains unclear which specific AT protocols are most effective for different tinnitus profiles or how different therapies compare when treating the same type of patient [19–21]. Tinnitus is primarily attributed to reduced inhibitory neural modulation, overactivity, and synchronous firing of neurons at edge frequencies due to decreased auditory input [22–24]. New acoustic therapies aim to reverse these abnormal neurophysiological activities. For instance, acoustic coherence reset neuromodulation involves phase resetting and random presentation of multiple frequencies surrounding the tinnitus pitch, aiming to desynchronize aberrant neuronal activity and achieve long-term relief or elimination of tinnitus perception after treatment cessation [25]. A recent study has shown that auditory coordinated reset neuromodulation significantly reduces tinnitus symptoms, lowers Visual Analog Scale (VAS) loudness and tinnitus questionnaire scores, and maintains its efficacy over extended periods [26]. Brain wave pattern changes indicate that auditory coordinated reset neuromodulation normalizes abnormal brain activity associated with tinnitus. Acoustic therapies improve tinnitus through two main mechanisms: by correcting pathological synchronous neural activity to reduce or eliminate tinnitus perception, and by helping patients understand and habituate to their tinnitus, thereby diverting attention away from it [27, 28].

Therefore, developing effective AT systems holds significant clinical importance for individuals with tinnitus. In this study, to reduce the costs of AT and provide convenient and effective treatment for patients, an AT system was developed. Patients were evaluated using tinnitus history questionnaires, the Tinnitus Handicap Inventory (THI), the Self-Rating Anxiety Scale, the Self-Rating Depression Scale, and VAS. Despite various studies demonstrating the effectiveness of AT, a comprehensive review concluded that more large-scale, multi-center, controlled studies are necessary.

The aim of this study was to evaluate the impact of a newly developed sound therapy system on tinnitus patients, specifically in terms of changes in audiology, tinnitus-related tests, and tinnitus psychoacoustic characteristics, with an understanding of the specific mechanisms by which the therapy improves tinnitus symptoms and its effect on patients' quality of life.

Methods

Patients diagnosed with subjective tinnitus who attended the Otolaryngology outpatient clinic, West China Hospital, Sichuan University, Chengdu, Sichuan, China, from June 2022 to September 2023 were included. The initial diagnosis was confirmed by the primary physician. The patients met the inclusion and exclusion criteria for this trial. Patients are informed about the content, objectives, risks, and benefits of the study. The study was approved by the Ethics Committee

of the University Research Ethics Review Committee, West China Hospital, Sichuan University (No. 2022/34/STS/45, from February 21, 2022), in accordance with the Declaration of Helsinki.

Following the acquisition of informed permission, the patients were randomly assigned to two groups by computer-generated randomization. Both groups were assessed before therapy and at 2 weeks, 1 month, 2 months, and 3 months after treatment.

Based on previous tinnitus treatment studies, the sample size was estimated using the Global Tinnitus Handicap Questionnaire, similar to THI, as the primary outcome measure. The significance level was set at 0.05, with an assumed dropout rate of 20%. This clinical trial enrolled a total of 112 patients. Twelve patients withdrew due to transportation difficulties and were unable to complete follow-up assessments. Therefore, 100 patients completed the full follow-up.

Inclusion criteria

The study included participants with subjective tinnitus with normal hearing or mild sensorineural hearing loss, which has been persistent for at least 6 months, and those aged between 18 and 80 years.

Exclusion criteria

Participants were excluded if they had objective tinnitus (such as heart murmurs, carotid artery bruits, or vascular sounds causing tinnitus), acoustic neuroma, paragangliomas, or other head and neck tumors or otological diseases, including chronic otitis media, cholesteatoma, or auditory neuropathy. Moreover, patients were excluded if they had chronic diseases (cardiovascular diseases, endocrine disorders) or malignancies that cause physical discomfort affecting the patient's mood, or if they had been diagnosed with psychiatric disorders.

Patient randomization

An independent research assistant, not involved in clinical activities, was appointed to manage the study. Based on the estimated sample size, participants were sequentially numbered starting from 1. This numbering also served as the enrollment order for the participants. Computer-generated random sampling was used to assign participants to the groups. Participants were assigned to the control group (CG) based on the numbers obtained from the random sampling, while the remaining participants were assigned to the treatment group (TG). The researchers implementing the AT and the outpatient physicians enrolling the participants were blinded to the group assignments. The enrolling physicians were only aware that the participants were receiving AT, but not the specific treatment protocol. Participants were informed about the background, purpose, assessment metrics, and follow-up times of the clinical trial, but were not told the specific AT protocol. To ensure consistency in measurements and assessments, a single researcher was designated to implement the AT throughout the entire clinical trial. Blinding was maintained until the end of the trial.

Sound therapy system treatment

During the AT process, participants were required to wear in-ear or over-ear headphones in a quiet environment (at home or a specific workplace). They were allowed to engage in relaxing activities such as walking, reading, or browsing the internet. However, they were instructed to avoid exposure to other external sounds and to focus on listening to the recorded stimuli.

The personalized sound therapy used in this study was an original protocol developed by our research team. It consisted of broadband noise enriched with frequency components matching each individual's tinnitus pitch, with a frequency spectrum ranging from 100 Hz to 12 kHz. The sound intensity was adjusted to be 5–10 decibels (dB) below the tinnitus loudness level (as determined during tinnitus loudness matching) and maintained below 65 dB sound pressure level to ensure auditory safety and comfort. Each session lasted 30 min, with three sessions a day (morning, afternoon, and evening) conducted continuously for a period of three months.

Participants in CG received standard counselling. They were exposed to neutral, non-customized broadband relaxation sounds (white noise) at comfortable listening levels (< 65 dB sound pressure level) for the same duration and frequency as TG. These sounds were not frequency-tailored to the tinnitus pitch and served as a placebo-like auditory intervention.

Patient adherence was monitored through daily listening logs maintained by participants and verified during each follow-up visit. Participants were instructed not to use any additional sound therapy, hearing aids, or tinnitus-masking devices during the study period. Compliance rates exceeding 85% were considered adequate for inclusion in the final analysis.

The choice of auditory therapy ear

If the participant has unilateral tinnitus, the affected ear is designated as the treatment ear. If the participant has bilateral tinnitus, the more severe side is selected as the treatment ear. If both sides have equivalent severity, the ear with poorer hearing is defined as the treatment ear. If the participant has cranial tinnitus, the ear with poorer hearing is designated as the treatment ear.

Follow-up record

Participants were followed up at the hospital at 2 weeks, 1 month, 2 months, and 3 months after treatment, with a total of four visits, to assess changes in tinnitus frequency, loudness, pitch, and timbre. If participants' tinnitus symptoms showed no improvement after three months of treatment, a new AT plan was reviewed and formulated for

them at the end of the trial. After three months of regular treatment, the patients came to the centers for follow-up appointments. This included comprehensive audiological assessments such as pure-tone audiometry and tympanometry. Additionally, they underwent tests related to tinnitus, including tinnitus frequency matching, tinnitus loudness matching, minimum masking level (MML), and residual inhibition (RI) testing. Patients also completed the following assessment scales: the THI, Hospital Anxiety and Depression Scale (HADS), Pittsburgh Sleep Quality Index (PSQI), and VASs [VAS-numeric (VASn) and VAS-intensity/frequency (VASif)].

Sound healing system effect

The THI scores and VAS ratings for loudness and distress within each group were assessed before treatment and at 2 weeks, 1 month, 2 months, and 3 months after treatment. These THI scores and VAS ratings for loudness and distress were also compared between the two groups at these time points. Additionally, the effectiveness rates of the two AT protocols were compared. Using THI scores as the standard, a THI score reduced to 16 or below, or a THI score reduction of 17 or more, was considered adequate.

Statistical analysis

All statistical analyses were performed using the SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA). Continuous variables were first tested for normality using the Shapiro-Wilk test. Normally distributed data were presented as mean ± standard deviation and compared between groups using the independent-sample *t*-test. Non-normally distributed data were expressed as median (interquartile range) and analyzed using the Mann-Whitney *U* test. Categorical variables were expressed as numbers (percentages) and compared using the Chi-square (χ^2) or Fisher's exact test, as appropriate. Comparisons between CG and TG were performed using the paired *t*-test or the Wilcoxon signed-rank test, depending on the data distribution. A two-tailed value of $p < 0.05$ was considered statistically significant. Graphs were generated using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA).

Results

Demographic characteristics of patients

To verify whether there were differences in the demographic characteristics of CG and TG before AT, a detailed statistical analysis was performed (Table 1). The trial had 100 clinical subjects, with 48 patients assigned to CG and 52 to TG. The results showed that, in terms of age, the mean age of patients in CG was 41.8 ± 16.6 years, and the mean age of patients in TG was 42.1 ± 15.7 years, with no statistically significant variation observed between the two groups ($p = 0.850$). Further analysis revealed no significant difference in age stratification between the two groups (under 60 years of age and 60 years old and above). In CG, 23 individuals were younger than 60 years, while 25 were 60 years old or above. In TG, 22 people were younger than 60 years, and 30 were 60 years old or above.

In CG, 26 (54.2%) patients were male and 22 (45.8%) female. In TG, males accounted for 24 (46.2%) patients and females accounted for 28 (53.8%); there was no significant difference in sex ratio between the two groups ($p = 0.504$). In

Table 1

Demographic characteristics of patients

Characteristics	Groups		*p*-value
	control (n = 48)	treatment (n = 52)	
Age, years	41.8 ± 16.6	42.1 ± 15.7	0.850
< 60	23	22	
≥ 60	25	30	
Sex			0.504
male	26 (54.2)	24 (46.2)	
female	22 (45.8)	28 (53.8)	
Duration of illness			0.631
6 months–4 years	19 (39.6)	17 (32.7)	
> 4 years	29 (60.4)	35 (67.3)	
Comorbidities			0.589
no	32 (54.2)	34 (46.2)	
yes	16 (45.8)	18 (53.8)	
Tinnitus side			0.771
left	22 (45.8)	26 (50.0)	
right	26 (54.2)	26 (50.0)	
Tinnitus-related sleep problems			0.591
no	25 (52.1)	29 (55.8)	
yes	23 (47.9)	23 (44.2)	

n – number.

***Note:* Continuous variables were expressed as mean ± standard deviation and compared using an independent-sample *t*-test. Categorical variables were presented as numbers (percentages) and compared using the Chi-square (χ^2) test.**

The value of $p < 0.05$ was considered statistically significant.

CG, 19 (39.6%) patients had a disease course of 6 months to 4 years, while 29 (60.4%) had a disease course longer than 4 years. In TG, the disease course ratio was 17 (32.7%) vs. 35 (67.3%), respectively, and the difference was not statistically significant ($p = 0.631$).

It was shown that 32 (54.2%) patients in CG had no comorbidities, while 16 (45.8%) had. In TG, comorbidity rates were 34 (46.2%) vs. 18 (53.8%), respectively, with no significant difference ($p = 0.589$). In CG, 22 (45.8%) patients with tinnitus experienced tinnitus on the left side and 26 (54.2%) on the right. In TG, 26 (50.0%) patients reported left-sided tinnitus and 26 (50.0%) right-sided tinnitus, with no statistically significant difference between the groups ($p = 0.771$).

Finally, in terms of tinnitus-related sleep problems, 25 (52.1%) patients in CG had no sleep problems, while 23 (47.9%) had sleep problems. In TG, the rates were 29 (55.8%) vs. 23 (44.2%), respectively, and the difference was not statistically significant ($p = 0.591$).

There were no notable differences in various demographic characteristics between CG and TG before AT, indicating that the two groups were comparable prior to treatment.

Audiological assessments, tinnitus-related tests, and psychological acoustic characteristics of tinnitus

Detailed statistical analyses were conducted to compare differences in audiometric assessments, tinnitus-related tests, and psychological acoustic characteristics between CG and TG before treatment (Table 2). The results show that, regarding hearing levels (HLs), 27.1% of patients in CG had normal hearing, while 72.9% had some degree of hearing loss. In TG, these proportions were 23.1% and 76.9%, respectively, with no statistically significant difference ($p = 0.810$). These findings suggest that both groups have similar HLs. Concerning tinnitus frequency, 33.3% of patients in CG had tinnitus frequencies below 1 kHz, 25.0% had frequencies between 1 kHz and 3 kHz, and 41.7% had frequencies above 4 kHz. In TG, these proportions were 26.9%, 34.6%, and 38.5%, respectively, with no statistically significant difference ($p = 0.599$). The results indicate no significant difference in the frequency distribution of tinnitus between the groups. Regarding tinnitus loudness, the mean loudness in CG was 16.89 ± 5.14 dB sensation level (SL), while in TG, it was 15.99 ± 6.49 dB SL, with no statistically significant difference ($p = 0.441$). These data suggest that both groups do not differ significantly in tinnitus loudness.

For the tinnitus MML, the average value in CG was 12.23 ± 3.49 dB SL. In contrast, the mean value in TG was 13.19 ± 4.49 dB SL, with no statistically significant difference ($p = 0.234$). This outcome indicates that the two groups do not differ significantly in their MML for tinnitus.

Regarding RI, 29.2% of patients in CG showed no changes or rebound, while 70.8% experienced complete or partial occlusion. In TG, these proportions were 21.2% vs. 78.8%, respectively, with no statistically significant differ-

Table 2

Audiological assessments, tinnitus-related tests, and psychological acoustic characteristics of tinnitus

Characteristics	Groups		*p*-value
	control (n = 48)	treatment (n = 52)	
Listening level (dB HL)			0.81
normal	13 (27.1)	12 (23.1)	
hearing loss	35 (72.9)	40 (76.9)	
Tinnitus frequency, kHz			0.599
< 1	16 (33.3)	14 (26.9)	
1–3	12 (25.0)	18 (34.6)	
> 4	20 (41.7)	20 (38.5)	
Tinnitus loudness (dB SL)	16.89 ± 5.14	15.99 ± 6.49	0.441
Tinnitus minimum masking level (dB SL)	12.23 ± 3.49	13.19 ± 4.49	0.234
Residual inhibition			0.944
no changes or bounces	14 (29.2)	11 (21.2)	
full or partial occlusion	34 (70.8)	41 (78.8)	
THI score			0.943
functionality	12 (25.0)	14 (26.9)	
catastrophic	20 (41.7)	21 (40.4)	
emotion	16 (33.3)	15 (28.8)	
PSQI score			0.691
sleep well	18 (37.5)	16 (30.8)	
poor sleep	30 (62.5)	36 (69.2)	
Total HADS score	16.67 ± 2.76	16.58 ± 2.89	0.873
VAS score	4.22 ± 1.37	4.19 ± 1.48	0.916

dB HL – decibels hearing level; dB SL – decibels sensation level; THI – Tinnitus Handicap Inventory; PSQI – Pittsburgh Sleep Quality Index; HADS – Hospital Anxiety and Depression Scale; VAS – Visual Analog Scale.

All values are given as numbers (percentages) or mean ± standard deviation.

***Note:* Continuous variables were tested for normality using the Shapiro-Wilk test. Normally distributed data were compared using the independent-sample *t*-test. Non-normal data were analyzed using the Mann-Whitney *U* test. Categorical variables were compared using the Chi-square (χ^2) test. The value of $p < 0.05$ was considered statistically significant.**

ence (p = 0.944). The RI results suggest that the two groups do not differ significantly. Concerning the THI, 25.0% of patients in CG exhibited functional impairment, 41.7% exhibited catastrophic impairment, and 33.3% exhibited emotional impairment. In TG, these proportions were 26.9%, 40.4%, and 28.8%, respectively, with no statistically significant difference (p = 0.943). These findings indicate that the two groups do not differ significantly in THI scores.

Regarding PSQI, 37.5% of patients in CG reported good sleep, while 62.5% reported poor sleep. In TG, these proportions were 30.8% vs. 69.2%, respectively, with no statistically significant difference (p = 0.691). The PSQI data suggest that the two groups do not differ significantly in sleep quality.

For the total HADS score, the average score in CG was 16.67 ± 2.76. In contrast, in TG, it was 16.58 ± 2.89, with no statistically significant difference (p = 0.873). These results indicate that the two groups do not differ significantly in anxiety or depression levels. For the VAS score, the average score in CG was 4.22 ± 1.37, while in TG, it was 4.19 ± 1.48, with no statistically significant difference (p = 0.916). The VAS findings show that the two groups do not differ significantly in tinnitus loudness ratings.

Overall, CG and TG showed no significant differences in audiometric assessments, tinnitus-related tests, and psychological acoustic characteristics before treatment, indicating that the two groups were comparable in these measures.

The frequency of ringing in the ear

A detailed statistical analysis was conducted to compare the differences in tinnitus frequency distribution between CG and TG (Table 3). The results show that both groups have similar distributions across various tinnitus frequency ranges, including low frequency (500 Hz), medium frequency (500–3,000 Hz), and high frequency (> 3,000 Hz). The percentages of patients with tinnitus at different frequencies were comparable between the two groups, ranging from 3.8% to 12.5%. Overall, the two groups did not show any significant differences in tinnitus frequency distribution, indicating that they are comparable in this aspect.

The patients' hearing tests, tinnitus-related examinations, and Tinnitus Psychoacoustic Questionnaire scores after sound therapy

To assess the effects of sound therapy on patients' hearing tests, tinnitus-related assessments, and the Tinnitus Psychoacoustic Questionnaire scores, data from the CG and TG were compared. The results indicated significant improvements across multiple measures following sound therapy (Table 4). Specifically, the listening level was significantly lower in TG (18.63 ± 6.52 dB HL) in comparison to CG (22.87 ± 11.80 dB HL; p = 0.0238). Similarly, tinnitus loudness was significantly reduced in TG (12.29 ± 4.19 dB SL) compared to CG (16.89 ± 5.14 dB SL; p = 0.0256). The MML for tinnitus showed a trend toward improvement in TG (10.19 ± 2.11 dB SL) compared to CG (12.23 ± 3.49 dB SL; p = 0.0532). Additionally, TG demonstrated significant improvements in the THI subscales – functional (8.98 ± 2.66 vs. 14.12 ± 8.97; p = 0.0123), catastrophic (5.28 ± 3.44 vs. 7.12 ± 4.97; p = 0.0324), and emotional (8.28 ± 4.56 vs. 12.22 ± 6.97; p = 0.0231). The PSQI score was significantly lower in TG (3.28 ± 1.56) compared to CG (5.97 ± 2.17; p = 0.0238). However, there were no significant differences observed in the total HADS score (16.58 ± 2.89 vs. 16.67 ± 2.76; p = 0.8730) or VAS score (4.19 ± 1.48 vs. 4.22 ± 1.37; p = 0.9160). The VAS tinnitus loudness exhibited a trend towards improvement (3.22 ± 0.37 vs. 4.97 ± 2.14; p = 0.0521), whereas no significant difference was found in VAS tinnitus annoyance (4.16 ± 2.49 vs. 4.15 ± 2.03; p = 0.6510).

Table 3

Frequency of tinnitus between the control and treatment groups

Frequency range of ringing	Frequency (kHz)	Group		*p*-value
		control (n = 48)	treatment (n = 52)	
Low (500 Hz)	0.125	2 (4.2)	2 (3.8)	0.74
	0.25	2 (4.2)	2 (3.8)	
	0.5	3 (6.25)	4 (7.7)	
Medium (500–3,000 Hz)	0.63	2 (4.2)	4 (7.7)	0.62
	0.75	2 (4.2)	3 (5.8)	
	1	2 (4.2)	2 (3.8)	
	1.5	2 (4.2)	2 (3.8)	
	2	2 (4.2)	3 (5.8)	
	2.5	3 (6.25)	3 (5.8)	
	3	3 (6.25)	2 (3.8)	
	4	3 (6.25)	4 (7.7)	
	5	3 (6.25)	3 (5.8)	
High (> 3,000 Hz)	6	4 (8.3)	4 (7.7)	0.68
	6.5	4 (8.3)	5 (9.6)	
	7	5 (10.4)	4 (7.7)	
	8	6 (12.5)	5 (9.6)	

n – number. All values are given as numbers (percentages).
***Note:* Group differences were analyzed using the Chi-square (χ^2) test. The value of $p < 0.05$ was considered statistically significant.**

Table 4

Patient hearing tests, tinnitus-related examinations, and Tinnitus Psychoacoustic Questionnaire scores after sound therapy

Parameters	Group		*p*-value
	control (n = 48)	treatment (n = 52)	
Listening level (dB HL)	22.87 ± 11.80	18.63 ± 6.52	0.0238
Tinnitus loudness (dB SL)	16.89 ± 5.14	12.29 ± 4.19	0.0256
Tinnitus minimum masking level (dB SL)	12.23 ± 3.49	10.19 ± 2.11	0.0532
THI score			
functionality	14.12 ± 8.97	8.98 ± 2.66	0.0123
catastrophic	7.12 ± 4.97	5.28 ± 3.44	0.0324
emotion	12.22 ± 6.97	8.28 ± 4.56	0.0231
PSQI score	5.97 ± 2.17	3.28 ± 1.56	0.0238
Total HADS score	16.67 ± 2.76	16.58 ± 2.89	0.873
VAS score	4.22 ± 1.37	4.19 ± 1.48	0.916
VAS tinnitus loudness	4.97 ± 2.14	3.22 ± 0.37	0.0521
VAS tinnitus annoyance	4.15 ± 2.03	4.16 ± 2.49	0.651

For abbreviations, see Table 2. All values are given as mean ± standard deviation.
***Note:* Data normality was assessed using the Shapiro-Wilk test. Between-group comparisons were conducted using the independent-sample *t*-test for normally distributed data or the Mann-Whitney *U* test for non-normal data.**
The value of $p < 0.05$ was considered statistically significant.

Discussion

Tinnitus is a condition with a high incidence rate that can significantly impact patients' daily lives, work, studies, and social interactions [29]. Currently, there is a lack of effective treatment options for chronic subjective tinnitus. In this study, an AT system was developed to examine the therapeutic effects on chronic subjective tinnitus. The THI scale score was used as the primary evaluation measure, while the HADS scale, PSQI scale, VAS score, and objective tinnitus loudness served as secondary measures. Results showed that after treatment, the THI score decreased significantly, and the objective loudness of tinnitus was lower, with these differences being statistically significant. This indicates that our developed AT system effectively improved tinnitus.

Tinnitus treatment includes masking therapy, tailor-made notch music therapy, and frequency resolution training [30–32]. Acoustic treatment is suitable for all types of tinnitus, especially those with hearing loss [33]. It offers advantages such as high safety and simplicity of operation, although its efficacy remains debated, mainly due to the lack of objective evaluation methods [34]. Currently, the assessment of tinnitus treatment success primarily relies on several commonly used subjective scales [35]. Among these, VAS of tinnitus loudness and THI are the most widely used and well-established assessment tools [36, 37]. These scales measure not only the perceptual intensity of tinnitus but also its impact on the patient's everyday life. Additionally, the Self-Rating Anxiety Scale is extensively utilized in psychological clinics to evaluate patients' anxiety levels [38, 39]. By using these scales collectively, the effectiveness of tinnitus treatment can be assessed comprehensively and accurately across multiple dimensions, thereby guiding clinical practice more effectively.

A previous study has shown that the severity of ringing is not correlated with ringing frequency, and that low and medium frequencies are the same [40]. In this study, there is no statistical difference between the number of people experiencing high-frequency ringing and those without it. Therefore, the ear-ringing patients were divided into groups based on the main tone frequency: low frequency (< 500 Hz), medium frequency (500–3,000 Hz), and high frequency (> 3,000 Hz). These groups received different types of AT before and after treatment to ensure there was no difference in the intensity of ringing in the ears before treatment, i.e., whether the effect of AT differs among patients with tinnitus in different frequency segments.

The effects of AT on patient hearing tests, tinnitus-related tests, and Tinnitus Psychoacoustic Questionnaire scores were systematically evaluated by comparing data from CG and TG. The results showed that sound therapy led to significant improvements in several measures. Firstly, the hearing threshold level in TG was notably lower than in CG, indicating that AT can effectively lower patients' hearing thresholds and enhance hearing sensitivity. Secondly, tinnitus loudness was significantly reduced in TG, further confirming the therapy's effectiveness in alleviating tinnitus symptoms. Additionally, MML in TG tended to improve, although this difference did not reach statistical significance and showed a positive trend. Among the subscales of THI, TG demonstrated significant improvements in the functional score, catastrophizing, and emotional score. This indicates that sound therapy is effective not only on a physiological level but also significantly reduces tinnitus-related distress on a psychological level.

PSQI scores also showed that TG was significantly lower than CG, indicating that sound therapy can improve patients' sleep quality. However, the total score on HADS, and the total score on VAS did not show a significant difference, suggesting that sound therapy had a limited effect on overall anxiety and depression levels. Finally, VAS tinnitus loudness showed an improvement trend, but VAS tinnitus annoyance showed no significant difference. This may sug-

gest that AT is more effective in reducing tinnitus loudness. However, its role in alleviating tinnitus distress requires further study.

Although this study has achieved some success in evaluating the effectiveness of AT in tinnitus patients, there are still some limitations. First, the relatively small sample size may result in lower statistical significance for some outcomes, and a larger sample size may improve the reliability and external validity of the results. Second, the age distribution of the two groups was very similar, and the sex ratio and disease duration were comparable, which may have limited the ability to generalize the study findings. In addition, the proportion of comorbidities was identical between the two groups, and the distribution of tinnitus side and tinnitus-related sleep problems was not significantly different between the two groups. These factors may affect the general applicability of the findings. Therefore, future research with larger samples, more diverse populations, within-group comparisons, and comprehensive outcome measures is warranted to validate and extend these findings.

This study demonstrated that AT significantly improves patients' hearing, reduces tinnitus loudness, and enhances sleep quality. However, its effects on anxiety, depression, and tinnitus-related distress remain to be further clarified.

Future studies should investigate the long-term efficacy of AT and assess its applicability across diverse patient populations. Additionally, performing within-group comparisons (pre- and post-treatment) in both treatment and control cohorts could provide a more precise evaluation of treatment effects. It is also possible that the therapy administered to CG exerted partial effects on tinnitus perception, which may explain the lack of statistically significant differences between groups in some measures. Incorporating these approaches in future research would provide a more comprehensive understanding of the efficacy of AT.

Conclusion

Overall, the results of this study support the use of sound therapy as an effective intervention for enhancing hearing and reducing the adverse effects of tinnitus, thereby improving the overall quality of life for patients.

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Conflict of interest

The authors declare no conflict of interest.

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Correlation between clinically and histopathologically measured tumor width in basal cell carcinoma

Korelacija između kliničke i histopatološke izmerene širine tumora kod bazocelularnog karcinoma kože

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Abstract

Introduction/Aim. Basal cell carcinoma (BCC) is the most common malignant skin tumor in fair-skinned populations. Although it has low metastatic potential, it can be highly locally invasive and, in addition to impairing function, may even pose a threat to life itself. The majority of BCCs can be successfully reated by classical surgical excision with appropriate safety margins. The aim of this study was to evaluate the correlation between clinical and histopathological tumor width in excised BCCs. **Methods.** A prospective clinical and histopathological study included 45 subjects consecutively examined at the Department of Dermatologic Surgery and Skin Tumors, University Clinical Center of Vojvodina, Novi Sad, Serbia, with a total of 60 primary BCC specimens obtained by classical surgical excision. Medical history was recorded, and dermatological examination was performed. Prior to the excisional biopsy, an incision was made along the edge of the clinically visible tumor to the level of the papillary dermis, and the standard recommended safety margin was added. Histopathological assessment involved microscopic analysis at various magnifications. The histopathological tumor width was measured using a millimeter ocular. The total clinically estimated tumor width was determined as the distance between the two stated skin incisions, and the distance from the incision to the histopathological tumor margin was also recorded. **Results.** A statistically significant correlation was found between clinically determined and microscopically measured total tumor width ($p < 0.05$). The mean clinically estimated width was 9.55 ± 5.13 mm, while the histopathological width was 7.98 ± 4.75 mm. The statistically determined difference between the clinically marked and microscopically measured tumor width was less than 2.0 mm in 96.7% of cases, i.e., in 88.4% of cases, this difference in assessment was 1.0 mm or less. **Conclusion.** There is a positive correlation between the clinically assessed and histopathologically verified width of the BCC.

Key words:
carcinoma, basal cell; dermoscopy; histological techniques; skin neoplasms.

Apstrakt

Uvod/Cilj. Bazocelularni karcinom (*basal cell carcinoma* – BCC) je najčešći maligni tumor kože kod ljudi svetle puti. Iako ima nizak metastatski potencijal, lokalno može biti visoko invazivan, te pored funkcije dela tela na kome se nalazi može ugroziti i sam život. Većina BCC-a se može uspešno lečiti klasičnom hirurškom ekscizijom uz odgovarajuće sigurnosne margine. Cilj rada bio je da se proceni korelacija između kliničke i patohistološke širine kod ekscidiranih BCC-a. **Metode.** Prospektivna klinička i histopatološka studija obuhvatila je 45 bolesnika pregledanih na Odeljenju za dermatohirurgiju i tumore kože, Univerzitetskog kliničkog centra Vojvodine, Novi Sad, Srbija, gde je analizirano ukupno 60 uzoraka primarnih BCC-a dobijenih klasičnom hirurškom ekscizijom. Uzeti su anamnestički podaci i obavljen je dermatološki pregled. Pre ekscizione biopsije, načinjen je rez duž ivice klinički vidljivog tumora do nivoa papilarnog dermisa i dodata standardno preporučena sigurnosna margina. Histopatološka obrada uključila je mikroskopsku analizu na različitim uveličanjima. Histopatološka širina tumora merena je milimetarskim okularom. Ukupna klinički procenjena širina tumora određena je kao razdaljina između dva navedena reza na koži, a zabeležena je i razdaljina između reza i histopatološke margine tumora. **Rezultati.** Utvrđena je statistički značajna korelacija između klinički određene i mikroskopski izmerene ukupne širine tumora

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($p < 0,05$). Prosečna klinički procenjena širina bila je 9,55 ± 5,13 mm, dok je histopatološka širina bila 7,98 ± 4,75 mm. Statistički utvrđena razlika između klinički obeležene i mikroskopski izmerene širine tumora bila je u 96,7% slučajeva manja od 2,0 mm, tj. u 88,4% slučaja ova razlika u proceni bila je 1,0 mm ili manja. **Zaključak.** Postoji pozitivna korelacija između klinički procenjene i histopatološki verifikovane širine BCC-a.

Ključne reči:
karcinom, bazocelularni; dermoskopija; histološke tehnike; koža, neoplazme.

Introduction

Basal cell carcinoma (BCC) is a slow-growing malignant skin tumor of epidermal origin, representing the most common malignant tumor in fair-skinned populations [1, 2]. It accounts for approximately 80% of non-melanoma skin cancers, whereas squamous cell carcinoma makes up the remaining 20% [3]. Although BCC shows a very low tendency for metastases (0.0028–0.55%), if left untreated, it can be locally destructive, compromising the function and esthetics of the region and even endangering life itself [4]. The incidence of BCC is constantly increasing, and it is associated with significant morbidity and high cost of treatment [5].

The etiology and pathogenesis of BCC are complex. Apart from genetic characteristics, exposure to ultraviolet (UV) radiation stands out as a significant environmental risk factor [6]. From a molecular perspective, BCC is linked to mutations in keratinocyte progenitor cells [7]. Most of these mutations occur due to deoxyribonucleic acid damage induced by UVB radiation. In almost all cases of BCC, there is an activation of the hedgehog signaling pathway [3]. Certain genodermatoses and autoimmune diseases, as well as immunosuppression, are associated with a higher incidence of BCC.

There are several clinical types of BCC. The most frequent are nodular and superficial, while others include sclerosing, ulcerated (ulcus rodens), and destructive (ulcus terebrans) types. According to the World Health Organization classification, other subtypes, which are mainly based on histological findings and are difficult to differentiate clinically, include micronodular, fibroepithelial, basosquamous and metatypical, keratotic, cystic, infundibulocystic, and adenoid subtypes [4]. In individuals with darker skin phototypes, BCC can have a different clinical presentation and can frequently present as pigmented BCC, where dermoscopy, as an ancillary diagnostic method, is especially useful [8].

Given that BCC is characterized by local tissue destruction and a very low rate of metastasis, the tumor, node, metastasis (TNM) staging system provides limited value in its assessment. The T stage is nonspecific, while N and M status are negative in more than 99% of cases [4].

Factors used to assess the risk of recurrence include tumor localization, size, histopathological subtype, and whether the tumor is primary or recurrent [9]. Localizations with a higher incidence of tumor occurrence and recurrence serve as important indicators for the diagnosis and prognosis of BCC. The high-risk area for BCC recurrence, the "H zone", includes the following regions: nose, eyebrows, upper lids, lips, mandibular angles, earlobes, preauricular region, hands, feet, and genital area. The second, so-called "M zone", involves the regions of the remaining parts of the face, scalp, neck, and pretibial region. These are the zones with a moderate risk of recurrence, while the "L zone" includes the trunk and extremities (excluding hands, feet, and pretibial region) and shows a lower likelihood of BCC recurrence. Another important prognostic factor of the risk of recurrence is the tumor size. The likelihood of recurrence in the "H zone" is higher for tumors with a diameter of over 6.0 mm, while in the "M zone" it applies to tumors larger than 10.0 mm. Moreover, larger BCCs are more likely to exhibit subclinical spread beyond the clinically visible tumor margins and thus, the risk of tumor recurrence on the resection margin of the histopathological specimen is much higher in larger tumors. Regarding histopathological subtype, a high risk of recurrence is observed in the following subtypes of BCC: sclerosing, infiltrating, metatypical, and micronodular, while a low risk is typical for superficial, nodular, adenoid, tubercular, infundibulocystic, cystic, and fibroepithelial (Pinkus tumor) subtypes [4].

Mohs micrographic surgery is the most effective treatment for BCC, especially for recurrent tumors and for those located in high-risk, cosmetically sensitive areas [10]. The most common treatment is surgical excision. The majority (more than 95%) of BCCs can be definitely treated with a classical surgical excision with appropriate safety margins [4]. Punch or excision biopsy provides a definite diagnosis by determining the histological characteristics of a lesion [9]. Non-surgical topical treatment (i.e., imiquimod, 5-fluorouracil), photodynamic therapy, superficial radiation therapy, and surgical curettage with electrodessication are reserved for low-risk BCCs or for patients in whom other therapeutic modalities are contraindicated. Systemic therapy is indicated in cases of locally advanced or metastatic BCCs. Targeted therapy with inhibitors of the hedgehog signaling pathway is currently in clinical use [4].

According to current clinical guidelines, a safety margin of 3.0–5.0 mm is generally recommended for BCCs at low risk of recurrence, whereas margins greater than 5.0 mm are advised for high-risk tumors [4]. Others, however, suggest that the safety margin of 6.0 mm should be sufficient for both tumor variants [9], even for those 5.0 to 10.0 mm in size [11].

The study is based on the assumption that clinical assessment of BCCs can reliably predict histopathological outcomes regarding tumor size. We postulate that in dermatology settings, clinical estimation of tumor width and margin position will significantly correlate with

histopathological width and histopathological margin position, respectively.

The aim of this study was to evaluate the correlation between clinical and histopathological tumor width in excised BCCs.

Methods

Clinical study

The study was conducted at the University Clinical Center of Vojvodina, Novi Sad, Serbia. This prospective clinical and histopathological study included 60 specimens of both low- and high-risk primary BCCs, taken from 45 subjects consecutively examined at the Department of Dermatologic Surgery and Skin Tumors by an attending dermatologist. The subjects who had immunocompromising diseases at the same time and/or were receiving immunosuppressive therapy were excluded from the study, as were the subjects with genodermatoses accompanied by multiple BCCs, as well as those with tumors larger than 25 mm and recurrent tumors. The study was approved by the Ethics Committee of the Clinical Center of Vojvodina (No. 00-05/277, from April 30, 2012). Written informed consent was obtained from all participants prior to their inclusion in the study.

The following patient data were registered: sex, age, personal and family history of previous BCC, possible presence of multiple BCCs, and absence of other genetic syndromes. The clinical examination included a complete skin examination and dermoscopy. As for the tumors, we recorded the size (measured by a millimeter ruler) and localization of each BCC.

Clinically assessed tumor width was marked with a No. 15 surgical scalpel to make it visible on a histopathological specimen by placing an incision to the level of the papillary dermis. A classical surgical excision with 3 mm safety margins was performed, oriented along relaxation tension lines of the skin and adjoining anatomical structures. These markings were necessary for later measurements. The specimens were placed into a formalin solution and further processed using routine histopathological preparation, given that histopathological analysis remains the "gold standard" for confirming clinically established diagnoses of BCC. Histopathological evaluation involved microscopic analysis at various magnifications (×40, ×100, ×200, ×400). We measured histopathological tumor width using a millimeter ocular. The total clinically estimated tumor width was measured as the distance between the two stated incisions.

Specimens were divided into three groups according to both clinically and histopathologically measured tumor width: 0–10.0 mm, 10.1–20.0 mm, and 20.1 mm or more. Additionally, the distance of the incision from the histopathological tumor margin was measured. This third measure was applied to adequately verify whether the clinically estimated tumor width corresponded to the true, histopathologically confirmed width.

The data obtained were analyzed in comparison with each other and in relation to whether the incision was made within or outside the histopathologically verified tumor. Furthermore, the results were compared based on whether the difference between the clinically marked and histopathologically determined tumor width in the excised BCC specimen was greater or less than 2.5 mm. All these procedures and measurements were needed to determine the correlation between the clinically estimated and histopathologically determined tumor width.

Statistical analysis

Statistical analysis was performed using SPSS version 21 software. Descriptive statistics were primarily employed. Using univariate analysis, we determined which anamnestic, clinical, and histopathological factors showed a statistically significant difference between the corresponding groups. Odds ratios with corresponding 95% confidence intervals and *p*-values were calculated. A *p*-value of less than 0.05 was considered statistically significant.

Results

The study involved 45 subjects and histopathological analysis of 60 primary BCC tumors, both with low and high risk of recurrence.

The sample included 45 subjects, 26 (57.8%) females and 19 (42.2%) males.

The sex ratio was 1.37 in favor of females. The mean age of the study population was 63 years (range 28–85). The largest number of subjects (40.0%) was in the 40–59 age range. Subjects in the range of age 60–79 years comprised 37.8% of the sample. Those aged 80 years or above made up 17.8%, and those aged 20–39 years accounted for 4.4% of subjects.

Out of the total number of subjects, 40.0% had a history of BCC at another site, whereas 60.0% had no history of prior BCC. Additionally, 48.9% of subjects had more than one BCC. Regarding the histopathological subtype of tumor, nodular (51.7%) and superficial (40.0%) BCC comprised the largest percentage, while other subtypes accounted for 8.0% of the samples. The largest number of tumors in the specimens (65.0%) were located in the head and neck region. The next frequent localization was the trunk and shoulders (31.7%), and only 3.2% of our specimens were localized elsewhere.

Most tumors (78.4%) were up to 10.0 mm wide, 18.3% were 10.1–20.0 mm wide, and only 3.3% were wider than 20.1 mm (Table 1).

The mean clinically measured width of the sampled tumors was 9.55 ± 5.13 mm, while the mean histopathological tumor width was 7.98 ± 4.75 mm ($n = 60$) (Table 2).

The mean difference between the clinically measured and histopathologically verified tumor width was 1.57 mm, with a 95% confidence interval of 1.0–2.13 mm.

Further statistical analysis determines that the mean distance from the incision to the histopathologically verified

Table 1

Distribution of histopathological tumor width in the study population

Histopathological tumor width, mm	n (%)
0–10.0	47 (78.4)
10.1–20.0	11 (18.3)
≥ 20.1	2 (3.3)
Total	60 (100)

n – number of excised tumors.

Table 2

Mean clinical and histopathological tumor width with standard deviation (SD) in the study population

Tumor width, mm (n = 60)	Mean ± SD
Clinical	9.55 ± 5.13
Histopathological	7.98 ± 4.75

n – number of excised tumors.

tumor width was 0.62 mm (range 0.10–2.50 mm). Of 60 samples, two (3.3%) tumors had an incision distance ≥ 2.0 mm, whereas in 96.7% of tumors, the incision distance was less than 2.0 mm. More precisely, in 88.4% of the samples, the incision was 1.0 mm or less from the histopathologically verified tumor width. Regarding incision placement, 25.0% of incisions were made within the tumor, whereas 75.0% were made outside the tumor. In more than 95.0% of cases, the incision was at a distance of less than 1.5 mm.

Discussion

In our conditions, following clinical and dermoscopic examination, we performed excisional biopsy for tumors smaller than 25 mm, with a 3 mm safety margin, serving both as a diagnostic (establishing a definite histopathological type) and treatment procedure. Demographic characteristics from our study show that the mean age of subjects with BCC was 63 years, consistent with published data [1], with a female-to-male sex ratio of 1 : 1.37. Although these data support a higher incidence of this type of carcinoma in males, the sample size is too small to make the difference in trends statistically significant. Still, the findings align with global trends of increasing BCC incidence in females. This is most likely because women are increasingly exposed to risk factors by assuming traditionally "male" tasks. Furthermore, following current tanning trends involving natural and/or artificial sources of UV radiation represents a serious problem that should be addressed through adequate primary prevention measures. Similar preventive efforts are needed regarding exposure to different household chemical agents, as well as to various chemicals present in hair-care and styling products [12]. Our study also reveals that 40.0% of subjects report a history of BCC, while clinical examination shows one or more BCCs in 48.9% of subjects, which aligns with findings from another study (46.7%) [13].

Regarding the skin tumor localization in our study, the largest percentage of carcinoma (65.0%) is excised from the region of head and neck, which aligns with the results of other researchers, and with the known fact that this region bears the highest risk of BCC occurrence [4]. We are aware that a punch biopsy is the first and mandatory procedure in the most developed countries when establishing a definite diagnosis of BCC, but, because of the insufficient financial resources and the burden of healthcare, we opt to plan further treatment only for high-risk histopathological type of tumor [9]. For high-risk histopathological subtypes of BCCs and/or those located in the H-zone and/or recurrent tumors, we perform additional Mohs micrographic surgery after excisional biopsy [10].

The most common pathohistological subtype of tumor, according to our study, is the nodular type (51.7%), followed by the superficial type (40.0%), while only 8.0% account for other BCC subtypes. In comparison with the data from another study [13], where a nodular BCC is also the most common one (57.1%), a superficial BCC (19.5%) and other subtypes (14.1%) are present in larger proportions. This is likely due to the relatively small sample size in our study.

Our research shows a potential bias in the measurement of histopathological tumor width, as the study was conducted within a single healthcare institution. It should be noted that all measurements were made by dermato-oncology subspecialists, and since other institutions in our country do not provide comparable conditions, this approach can be considered representative in the given context. The result's authenticity would have been better if histopathological tissue processing had included a horizontal section. However, complete circumferential margin assessment is performed only in micrographic surgery, unlike the conventional vertical-section histopathological analysis applied in this study. Mohs micrographic surgery is both efficient and cost-effective, offering the highest cure rates, and is considered the gold standard for treating high-risk nonmelanoma skin cancers in most developed countries [5].

The methodology of our study, which involves placing an incision in the papillary dermis before excision, enables us to avoid the difference in tumor width measurement between clinical and histopathological assessments, which

results from specimen tissue contraction. On the other hand, this could be considered a shortcoming when the results are compared to another study [11].

Statistical analysis in this study reveals a significant correlation between the clinically estimated tumor width and the actual histopathological tumor width ($p < 0.05$). This finding emphasizes the importance of precise clinical margin assessment in standard surgical excision, which remains the most effective treatment for primary BCCs. Accurate estimation of safety margins is essential for optimizing cure rates while minimizing morbidity [10]. Our results support the continued use of excisional biopsy as a reliable and sufficient diagnostic and therapeutic approach for all primary BCCs with low-risk histopathological subtypes [11].

In 95.0% of the specimens, the difference between the clinically and histopathologically measured tumor widths was up to 1.5 mm, while 96.7% of specimens showed a difference of less than 2.0 mm. The single specimen in which the distance from the incision to the tumor exceeded 2.0 mm (specifically 2.40 mm) was a nodular BCC located in the so-called "cancerization field". This skin area typically contains numerous precancerous lesions; thus, clinically, the lesion cannot be exactly differentiated from carcinoma that is not clinically visible, although it may be present in a histopathological analysis. This circumstance indirectly affects the above-mentioned statistics.

The results of our study favor the views expressed in the study that suggest that it is actually needed to consider a potential reduction of the current safety margins [9]. In our study, a uniform safety margin of 3.0 mm is applied for both low and high-risk BCCs, and histopathological analysis confirms this margin as adequate in all cases. However, our recommendation of a 3.0 mm safety margin applies specifically to low-risk tumors. In order to determine whether this margin is sufficient for high-risk BCCs as well, long-term prospective studies with extended follow-up are necessary to evaluate the rate of recurrence at excision sites.

Conclusion

This study shows a positive correlation between the clinically assessed and histopathologically verified basal cell carcinoma width. Returning to the initial observation that the incidence of basal cell carcinoma continues to rise, it is evident that appropriate surgical treatment (with adequate safety margins) and prevention of recurrence will remain the focus of interest for a long time. In perspective, clinical guidelines should consider a decrease of safety margins during surgical treatment of basal cell carcinomas at a low risk of recurrence, to achieve maximal tissue salvage, and at the same time, minimize recurrence rates and resource consumption.

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Primeri referenci:

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Balint B. From the haemotherapy to the haemomodulation. Beograd: Zavod za udžbenike i nastavna sredstva; 2001. (Serbian)

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Tabele

Sve tabele pripremaju se sa proredom 1,5 na posebnom listu. Obeležavaju se arapskim brojevima, redosledom pojavljivanja, u levom uglu (**Tabela 1**), a svakoj se daje kratak naslov. Objašnjenja se daju u fus-noti, ne u zaglavlju. Svaka tabela mora da se pomene u tekstu. Ako se koriste tuđi podaci, obavezno ih navesti kao i svaki drugi podatak iz literature.

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