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The levels of circulating long non-coding *RNA GAS5* in prostate carcinoma patients: a single-center study

Nivoi cirkulišuće *lncRNA GAS5* kod obolelih od karcinoma prostate: iskustvo jednog centra

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Abstract

Background/Aim. Prostate carcinoma (PCa) is second occurring carcinoma that affects the male population. Although PCa incidence rates are high, most cases have a favorable prognosis, with comfortable long-term life quality. The aim of the study was to compare long non-coding RNA (*lncRNA*) growth arrest-specific transcript 5 (*GAS5*) plasma levels between healthy individuals and patients with PCa, and also between PCa patients with different prognostic scores. **Methods.** The present study included a total of 40 patients with PCa and a control group of 20 healthy individuals. PCa patients were divided into two subgroups (20 patients each) based on the prognostic criteria of the American Joint Committee on Cancer. The patient data were collected and analyzed; *lncRNA GAS5* levels were quantified using the real-time polymerase chain reaction method. Statistical analysis was conducted using the IBM SPSS Statistics 26.0 computer program (IBM, USA, 2019). **Results.** The relative quantification of *lncRNA GAS5* expression levels showed down-regulation in PCa patients compared to healthy individuals; however, the difference was marginally statistically significant ($p = 0.056$). With further analysis of the given results, we concluded that the expression level of *lncRNA GAS5* was not significantly different in the first patient subgroup and the healthy individuals ($p = 0.268$). Patients from the second subgroup had significantly lower plasma levels of *lncRNA GAS5* than healthy individuals ($p = 0.033$). The difference in the level of *lncRNA GAS5* expression between patients with favorable prognoses (Group 1) and the ones with worse prognostic scores (Group 2) did not indicate statistical significance ($p = 0.275$). In both Group 1 ($p = 0.805$) and Group 2 ($p = 0.454$), the plasma levels of *lncRNA GAS5* were not significantly different in comparison to the age (≤ 65 vs. > 65 years). **Conclusion.** One of the main objectives of PCa research is identifying novel and more efficient biomarkers. Conducted research provides strong evidence about the significance of *lncRNAs GAS5* in PCa, as well as the correlation between decreased expression of *lncRNA GAS5* and poor prognosis in various tumors.

Key words:
biomarkers; prognosis; prostate neoplasms; rna, long noncoding.

Apstrakt

Uvod/Cilj. Karcinom prostate (KP) je po učestalosti drugi karcinom u muškoj populaciji. Premda je incidenca obolevanja od KP visoka, kod najvećeg broja bolesnika prognoza je povoljna, sa zadovoljavajućim kvalitetom života dugoročno. Cilj rada bio je da se utvrde razlike u nivoima *long non-coding RNA* (*lncRNA*) *growth arrest-specific transcript 5* (*GAS5*) u plazmi zdravih ispitanika i bolesnika sa KP, kao i između obolelih od KP u različitim prognostičkim stadijumima. **Metode.** U studiju je bilo uključeno 40 bolesnika sa KP i 20 zdravih osoba (kontrolna grupa). Bolesnici sa KP su, na osnovu prognostičkih kriterijuma *American Joint Committee on Cancer*, bili podeljeni u dve podgrupe (u svakoj po 20 bolesnika). Podaci o bolesnicima su prikupljeni i analizirani, a *lncRNA GAS5* je kvantifikovan korišćenjem metode lančane reakcije polimeraze u realnom vremenu. Statistička analiza podataka izvršena je pomoću programa IBM SPSS Statistics 26.0 (IBM, USA, 2019). **Rezultati.** Izmereni nivo ekspresije *lncRNA GAS5* bio je niži kod obolelih od KP u odnosu na zdrave osobe, mada granično statistički značajan ($p = 0,056$). Daljom analizom dobijenih podataka, utvrđeno je da razlika u nivou ekspresije *lncRNA GAS5* u prvoj podgrupi bolesnika i zdravih osoba nije bila

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statistički značajna (p = 0,268). Bolesnici iz druge podgrupe imali su značajno niže vrednosti *lncRNA GAS5* u odnosu na zdravu populaciju (p = 0,033). Razlika u nivou ekspresije *lncRNA GAS5* između bolesnika sa povoljnijom prognozom (Grupa 1) i bolesnika sa lošom prognozom (Grupa 2) nije bila statistički značajna (p = 0,275). U oba slučaja, u Grupi 1 (p = 0,805) i u Grupi 2 (p = 0,454), vrednosti *lncRNA GAS5* u plazmi nisu pokazale razliku u odnosu na starost bolesnika (≤ 65 vs. > 65 godina).

Zaključak. Jedan od glavnih ciljeva u istraživanju KP je pronalaženje novih i efikasnijih biomarkera. Sprovedeno istraživanje pruža jake dokaze o značaju *lncRNAs GAS5* u KP, kao i o povezanosti niskih vrednosti ekspresije *lncRNA GAS5* sa lošom prognozom kod različitih tumora.

Ključne reči:
biomarkeri; prognoza; prostata, neoplazme; rnk, duga nekodirajuća.

Introduction

Following lung cancer, prostate carcinoma (PCa) is the second leading carcinoma in males, accounting for 14.1% of all newly diagnosed cancers and 6.8% of all cancer-related deaths [1]. Its incidence in the countries with higher human development index (HDI) is 37.5 *per* 100,000 in comparison to 11.3 *per* 100,000 in countries with lower HDI [1]. The average age at the time of PCa diagnosis is 66, with the incidence and mortality increasing with age [2]. PCa is not an aggressive disease, but it usually metastasizes to bones and lymph nodes [3]. Despite high incidence, PCa has a favorable prognosis, with high quality of life [4]. The five-year survival rate for localized-stage cancer is 99.3%, with less than 6% progression to metastatic disease. The five-year survival rate for distant stage PCa has improved in the last decades and includes 32.3% of all patients [5].

There are more than 20 tumor markers currently used in tumor diagnostics, but only prostate-specific antigen (PSA) is used in prostate cancer [6]. In order to detect an early stage of asymptomatic PCa, PSA (normal serum level > 4.0 ng/mL) as a primary tumor marker is usually used in combination with digitorectal examination. However, studies have shown that levels of PSA in the serum are more specific for benign prostatic hyperplasia, so only 25% of people with increased levels of PSA will develop PCa [7]. There is a constant need for discovering novel biomarkers with higher specificity and sensitivity, which could be used in early diagnosis and follow-up of patients with PCa.

Nowadays, increased attention is directed toward examining the impact of long non-coding RNAs (*lncRNAs*) on cancer pathology [8–10]. Long non-coding RNAs, often called "genomic dark matter", are non-protein-coding transcripts with a length of more than 200 nucleotides. Their role in the human genome is mostly unknown, but novel studies have shown the involvement of *lncRNAs* in regulating cell proliferation and apoptosis. They can also interact with promoter or enhancer sequences to modulate gene expression and, consequently, act as tumor suppressors or oncogenes [10–13]. The finding, which indicates that *lncRNA* from tumor cells can be detected in the plasma, has prompted the idea of using *lncRNA* as a biomarker in cancer patients [9]. Indeed, accumulated knowledge on *lncRNA* has indicated their possible usage as diagnostic/prognostic markers and also as therapeutic targets in many tumors [14].

One of the well-known *lncRNAs* is growth arrest-specific transcript 5 (*GAS5*), a protein non-coding RNA of about 630 nucleotides, initially described as a tumor suppressor. This *lncRNA* is encoded by the *GAS5* gene (1q25), a member of the 5′-terminal oligopyrimidine (5′-TOP) gene family, comprised of 12 exons and 11 introns [15]. The *GAS5* introns are transcribed into 10 box C/D small nucleolar RNA (*snoRNA*) molecules involved in the epigenetic regulation of gene expression [9, 10]. Previous studies have not proven the connection between the expression levels of *lncRNA GAS5* and patients' age [16, 17]. Expression of *lncRNA GAS5* is decreased in growing tissue but increased in periods of dormancy, so its lower expression can predict a worse prognosis [18]. There are several tumors linked with lower expression of *lncRNA GAS5*, such as colorectal cancer, non-small-cell lung cancer (NSCLC), breast cancer, gliomas, and others [10]. Recent data have shown that the *lncRNA GAS5* levels were significantly downregulated in tissues of different tumors and patient plasma [19]. Furthermore, the *lncRNA GAS5* levels were reduced significantly in the PCa tissues and cell lines [20]. However, at this moment, published data on the expression of plasma *lncRNA GAS5* levels in PCa patients are deficient.

The aim of the study was to assess the variability of *lncRNA GAS5* plasma levels between healthy individuals and patients with PCa, between PCa patients with different prognostic scores, and the impact of patients' age on the *lncRNA GAS5* expression.

Methods

Type of study and patients

The present opservational, prospective, and case-control study included a total of 40 patients with PCa treated operatively or conservatively at the Clinic for Urology, Military Medical Academy (MMA), Belgrade, Serbia in 2021 and a control group of 20 healthy individuals. This research protocol was approved by the Ethics Committee of MMA (Approval No. from 26 April 2018), according to the principles of the Declaration of Helsinki. All of the participants signed an informed consent to participate in the study.

The PCa patients were divided into two subgroups (20 patients each) based on the prognostic criteria of the American Joint Committee on Cancer (AJCC) that includes Tumor, Nodes, Metastasis (TNM) classification, serum concentration of PSA, and tumor grade (Gleason score – GS) [21]. Group 1 included patients with favorable prognostic scores 1 and 2 according to AJCC criteria, while Group 2 included patients with AJCC unfavorable prognostic scores 3 and 4. Control

Table 1

Age, family history of prostate carcinoma, body mass index, and prostate-specific antigen level in the control group

Characteristic	Control group
Age (years)	37.2 ± 7.4
Family history of prostate carcinoma	
positive	1 (5)
negative	19 (95)
Body mass index	27.56 ± 3.67
Prostate-specific antigen (ng/mL)	0.90 ± 0.79

Results are shown as average ± standard deviation except family history, which is shown as numbers (percentages).

Table 2

Clinical characteristics of prostate carcinoma patients

Characteristic	Group 1	Group 2	p^1
Age (years)	64.5 ± 6.8	67 ± 5.9	0.189
Family history of prostate carcinoma			
positive	6 (30)	5 (25)	0.718
negative	14 (70)	15 (75)	
Body mass index	25.92 ± 3.49	27.14 ± 3.51	0.277
Prostate-specific antigen (ng/mL)			
≤10	13 (65)	7 (35)	0.058
>10	7 (35)	13 (65)	
Gleason grade			
1, 2, 3	20 (100)	17 (85)	0.231
4, 5	0 (0)	3 (15)	
Pathologic t stage			
2	20 (100)	3 (15)	**< 0.001**
3, 4	0 (0)	17 (85)	
Pathologic n stage			
N0	20 (100)	16 (80)	0.106
N1	0 (0)	4 (20)	
Involvement of bones, seminal vesicles, or locally advanced disease			
present	0 (0)	2 (10)	
absent	20 (100)	18 (90)	0.487

[1]*p*-values were calculated by chi-squared test or two-tailed Fisher exact test (when the characteristics were present in less than five patients); only values for body mass index and average age were compared using a two-tailed *t*-test.
Bolded value is statistically significant. Results are shown as numbers (percentages) except age and body mass index which are shown as average ± standard deviation.

group, Group 1, and Group 2 were matched with regard to PCa risk factors [family history, body mass index (BMI), smoking status, alcohol intake, and physical activity], excluding age. Individuals younger than 18 years of age and patients with the presence of other/secondary malignancies were not included in this study. Data about age, the presence of PCa in the family, BMI, and PSA level in the control group are presented in Table 1; clinical characteristics of PCa patients included in the present study are shown in Table 2. In parallel, the independent cohort for validation of *lncRNAs GAS5* quantification included 11 healthy subjects and 13 PCa patients (prognostic score 1–4). In the independent cohort, the average age of the healthy controls was 36.7 ± 8.6 years, while of the PCa patients, it was 66.2 ± 5.9 years.

Quantification of lncRNA GAS5

Peripheral blood was collected in tubes with ethylenediaminetetraacetic acid and delivered to the laboratory of the Center for Clinical Pharmacology, MMA. In order to separate plasma, blood was centrifuged at 1,200 g for 10 min at 4 ºC and subsequently at 12,000 g for 10 min at 4 °C. Plasma samples were stored at -40 ºC until RNA isolation (Isolate II RNA Mini Kit, Bioline, UK). Concentrations of total RNA were calculated after spectrophotometry at 260 nm (NanoPhotometer NP60, Implen, USA). In order to evaluate the integrity of isolated RNA, reverse transcription polymerase chain reaction (RT-PCR) and amplification of four different-in-length DNA sequences were employed for 30 randomly chosen samples (10 controls and 20 PCa).

The isolated total RNA (2 μg) was transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, UK) according to the manufacturer's instructions (25 ºC for 10 min; 37 °C for 120 min; 85 °C for 5 min; hold at 4 °C).

For further quantitative Real time-PCR (q-PCR) runs, 3 μL of cDNA was used. The qRT-PCR was performed using *GAS5* (sense: 5'-CTTGCCTGGACCAGCTTAAT-3', anti-

sense: 5'-AAGCCGACTCTCCATACCT-3') or housekeeping gene β-actin (sense: 5'-ACCCACACTGTGCCCATC TA-3', antisense: 5'-CGCAACCGCTCATTGCC-3') specific primers (Invitrogen, Thermo Fisher Scientific, UK) [22, 23], and Power SYBR® Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, UK), according to the manufacturer's instructions. The amplification was run through an initial denaturation (95 ºC for 5 min) followed by 50 cycles at 95 ºC for 15 sec and 60 ºC for 1 min on Step One Plus Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, USA). Relative quantification was performed by the comparative $2^{-\Delta\Delta}$Ct method, using healthy controls as calibrators.

Statistical analysis

The statistical analysis using the program IBM SPSS Statistics 26.0 (IBM, USA, 2019) was performed. All continuous variables were described in the form of mean ± standard deviation (SD). Comparisons of parametric variables between two groups were performed by independent samples *t*-test. Comparisons of parametric variables between the three groups were performed with the ANOVA test. The normality of data distribution was tested by the Kolmogorov-Smirnov test. Attributive variables were described as a proportion of the total number of patients. The Chi-squared (χ^2) test or Fischer's exact test was used for comparing categorical variables. All the analyses were evaluated at the level of statistical significance of $p < 0.05$.

Results

In order to validate the results of *lncRNA GAS5* expression in controls and PCa patients, we performed a validation study in an independent cohort (11 controls and 13 PCa patients with prognostic scores 1–4). As shown in Figure 1, levels of *lncRNA GAS5* were concordant between the validation cohort and the study population. In the validation cohort, levels of *lncRNA GAS5* were lower in PCa patients than in healthy subjects (0.78 ± 0.6 vs. 1.1 ± 0.41; $p = 0.055$).

The relative quantification of *lncRNA GAS5* levels in the plasma of PCa patients and healthy controls demonstrated a difference of marginal statistical significance ($p = 0.056$) between the groups, whereby *lncRNA GAS5* levels were lower in PCa patients (0.81 ± 0.52) than in controls (1.11 ± 0.59). Considering prognostic groups, levels of *lncRNA GAS5* were not significantly higher in the healthy controls compared with the patients from Group 1 (1.11 ± 0.59 vs. 0.91 ± 0.54; $p = 0.268$). However, patients from Group 2 had significantly lower plasma levels of *lncRNA GAS5* than the controls (0.72 ± 0.52 vs. 1.11 ± 0.59; $p = 0.033$). Group 1 had higher levels of *lncRNA GAS5* than Group 2, but the difference between the groups is not statistically significant (0.91 ± 0.54 vs. 0.72 ± 0.52; $p = 0.275$) (Figure 2).

The average age of the healthy controls was 37.2 ± 7.4 years, and of the PCa patients, it was 66.9 ± 6.5 years (64.5 ± 6.8 years for Group 1 and 67 ± 5.9 years for Group 2).

LncRNA GAS5 levels between the two prognostic groups of PCa patients (Group 1 vs. Group 2) were not significantly different ($p = 0.275$); *p*-values were obtained by independent samples *t*-test (Figure 3).

Plasma levels of *lncRNA GAS5* were not significantly different between the patients aged 65 or below and patients older than 65 years in both Group 1 (0.87 ± 0.94 vs. 0.94 ± 0.67; $p = 0.805$) and Group 2 (0.61 ± 0.5 vs. 0.79 ± 0.51; $p = 0.454$) (Figure 4). The *lncRNA GAS5* levels were not significantly different between Group 1 and Group 2 when analyses included only patients ≤ 65 years of age (0.82 ± 0.45 vs. 1.15 ± 0.75; $p = 0.247$) or patients older than 65 years (0.91 ± 0.54 vs. 0.91 ± 0.61; $p = 0.578$).

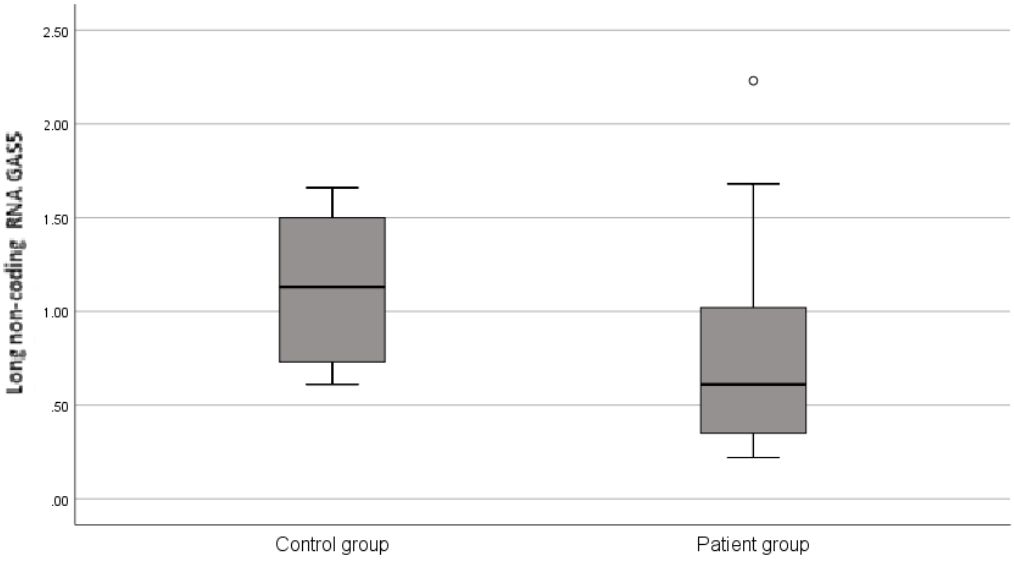
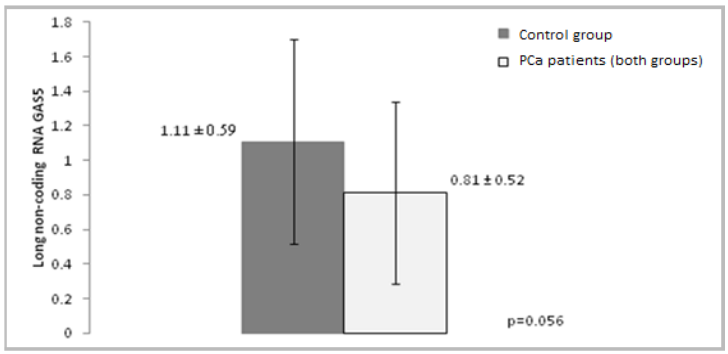
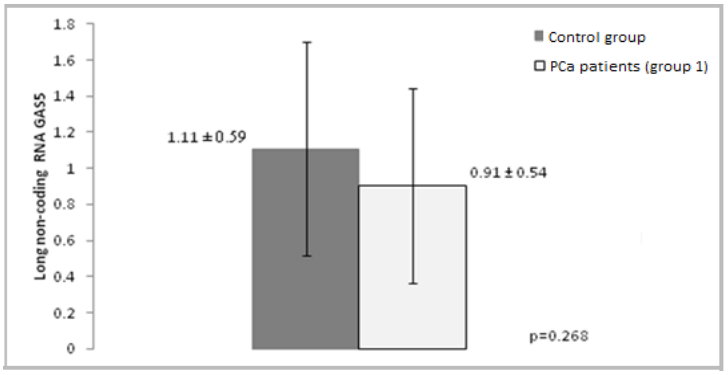


Fig. 1 – *LncRNA GAS5* levels in the independent cohort of healthy subjects and prostate carcinoma (PCa) patients. Levels of *lncRNA GAS5* were lower in PCa patients than in healthy subjects (0.78 ± 0.6 vs. 1.1 ± 0.41; $p = 0.055$). *p*-value was obtained by Mann-Whitney *U* test.



A)



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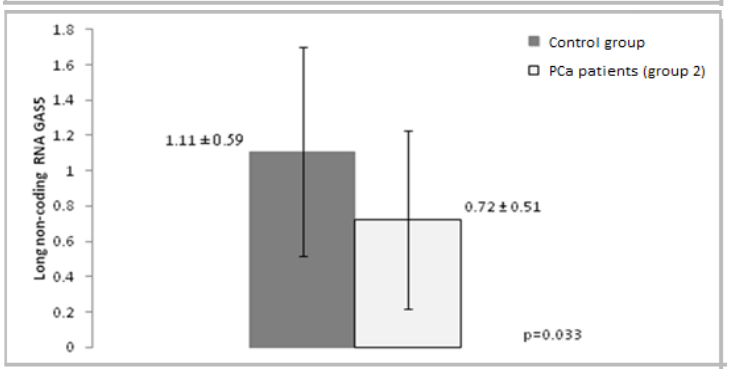

C)

Fig. 2 – *Lnc RNA GAS5* levels in prostate cancer (PCa) patients and controls: A) The difference in *lncRNA GAS5* levels between the controls and PCa patients was of marginal statistical significance ($p = 0.056$); B) The difference in *lncRNA GAS5* levels between controls and PCa patients from Group 1 (patients with favorable prognosis) was not statistically significant ($p = 0.268$); C) *LncRNA GAS5* levels between controls and PCa patients from Group 2 (patients with poor prognosis) were significantly different ($p = 0.033$).
***p*-values were obtained by independent samples *t*-test.**

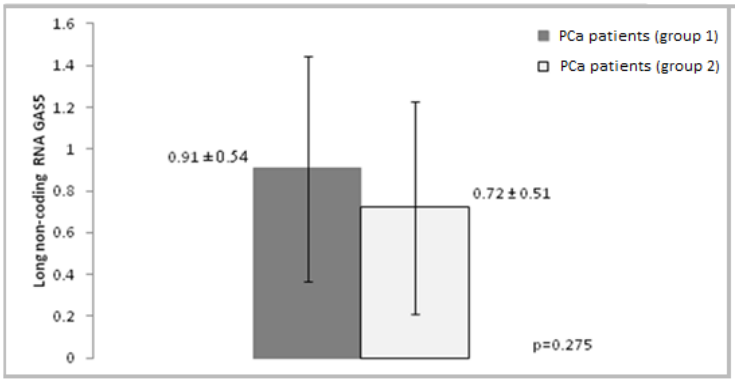

Fig. 3 – *LncRNA GAS5* levels in prostate carcinoma (PCa) patients with different prognostic scores (Group 1 vs. Group 2); $p = 0.275$.
***p*-value was obtained by independent samples *t*-test.**

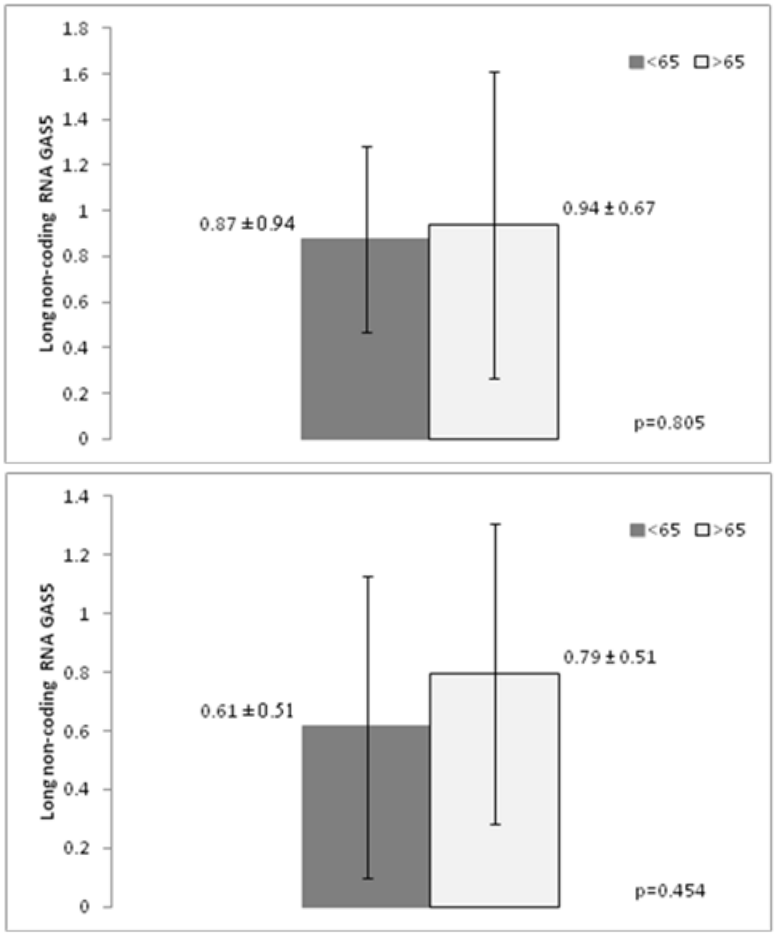

Fig. 4 – *LncRNA GAS5* levels in prostate carcinoma (PCa) patients of different age groups. Levels of *lncRNA GAS5* were not significantly different in patients ≤ 65 years compared to patients > 65 years of age nor in Group 1 (p = 0.805) (A), nor in Group 2 (p = 0.454) (B). p-values were obtained by independent samples t-test.

Discussion

As mentioned earlier, findings of relatively stable *lncRNAs* plasma concentration have implied the possible usage of circulating *lncRNAs* as a noninvasive diagnostic biomarker in different pathologic conditions. The research was conducted for various tumor types, including NSCLC [9], breast cancer [24], diffuse large B-cell lymphoma (DLBCL) [25], malignant mesothelioma [16], and many nonmalignant diseases, such as type 2 diabetes mellitus [26]. In this research, the expression of *lncRNA GAS5* was quantified in plasma samples of PCa patients.

We found that *lncRNA GAS5* expression levels were downregulated in the PCa patients compared to healthy individuals (p = 0.056); however, the difference was of marginal statistical significance. The possible reason for marginal statistical significance was the relatively small sample size. Similar results were obtained by Vesovic et al. [9] and Tan et al. [19] in NSCLC patients, as well as Han et al. [24] in breast cancer patients. However, Senousy et al. [25] reported that *lncRNA GAS5* expression was significantly downregulated ($p < 0.0001$) in the overall DLBCL patients compared to the control group. In patients with malignant mesothelioma, Weber et al. [16] reported a low sensitivity of 14% when *lncRNA GAS5* was used as a single marker, a combination of calretinin and mesothelin showed a sensitivity of 64%, but a panel composed of *lncRNA GAS5*, calretinin, and mesothelin reached a sensitivity of 73% (at a predefined specificity of 97%). Similarly, Tan et al. [19] found a strong association of *lncRNA GAS5* levels with carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) (p = 0.017 and p = 0.001, respectively) and suggested that a combination of circulating *lncRNA GAS5* levels with CEA and CA19-9 was more advantageous for the diagnosis of the early stage of NSCLC. On the other hand, in the research of Senousy et al. [25], a panel of HOX transcript antisense intergenic RNA (*HOTAIR*) and *lncRNA GAS5* demonstrated good results for response assessment in DLBCL patients treated with rituximab-cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP).

It is evident that the level of *lncRNA GAS5* expression varies across tumor types; the results of the present study indicate that *lncRNA GAS5* as a single marker was not significantly downregulated in PCa patients with favorable prognosis. However, we can only assume that in combination with PSA and other potential biomarkers, *lncRNA GAS5* could be used in PCa diagnostics.

Furthermore, our results showed that the level of *lncRNA GAS5* expression was not significantly different be-

tween patients with favorable prognosis and the healthy individuals ($p = 0.268$) nor between patients with different prognostic scores ($p = 0.275$). However, levels of *lncRNA GAS5* were significantly different between patients with AJCC unfavorable prognostic scores 3 and 4 and healthy individuals ($p = 0.033$). Vesovic et al. [9] did not observe a difference in *lncRNA GAS5* expression between early-stage tumor patients (TNM stage I/II) and healthy controls; however, there was an apparent difference in the TNM stage III patients. Moreover, they found that circulating *lncRNA GAS5* was a potent predictor of tumor size since patients with tumors > 3 cm had a significantly diminished expression compared to patients with tumors ≤ 3 cm. These results were expected since *lncRNA GAS5* is a well-known tumor suppressor.

The origin of *lncRNAs* in the bloodstream remains unexplained. Xue et al. [20] showed that *lncRNA GAS5* levels were reduced significantly in the PCa tissues compared to healthy tissue ($p < 0.05$); overexpression of *lncRNA GAS5* significantly decelerated tumor growth through the inactivation of a serine/threonine-specific protein kinase/the mammalian target of rapamycin (AKT/mTOR) signaling pathway ($p < 0.05$). In NSCLC patients, Tan et al. [19] showed that *lncRNA GAS5* decreased in NSCLC tissues compared to noncancerous tissues ($p < 0.001$). Furthermore, there was a weak connection between 55 paired plasma samples and cancer tissues obtained from the same individuals with NSCLC ($p > 0.05$). Despite the connection being weak, it could be concluded that changes in the levels of circulating nucleic acids were associated with tumor burden and malignant progression and that circulating *lncRNA GAS5* originated from the self-secretion, apoptotic, or necrotic tumor cells [27].

In our study, we also analyzed the patient's age and determined a statistically significant difference between the PCa patients and the healthy individuals ($p < 0.001$), which is expected considering that PCa is a common disease in older males. Furthermore, our patient age correlates with literature data, with cited average age at the moment of diagnosis being almost 66 years [24]. We found that the level of *lncRNA GAS5* expression does not differ significantly between tested patients below and over 65 years of age and also between patients younger and older than 65 years of age in Groups 1 and 2 (independent samples test, $p = 0.805$, $p = 0.454$, $p = 0.247$, and $p = 0.578$, respectively). Differing *lncRNA GAS5* expression between age groups has not been determined in other research so far, which correlates with our results [8, 21].

Conclusion

One of the main priorities in PCa research is the identification of novel biomarkers. Conducted research provides strong evidence about the significance of *lncRNAs* in PCa, as well as the correlation between decreased expression of *lncRNA GAS5* and poor prognosis in various tumors. The results of this study are based on a relatively small patient size. Future prospective studies on a larger scale could show the true value of the plasma *lncRNA GAS5* expression level in PCa patients either as a single marker or in combination with other biomarkers.

Acknowledgment

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Conflict of interest

The authors declare no conflict of interest.

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