BJMG 28 (2), 2025 • 41-48
DOI: 10.2478/bjmg-2025-00015

ORIGINAL ARTICLE

THE ASSOCIATION OF *ACSL1* AND *UCP2* 3' UTR POLYMORPHISMS WITH THE CLINICOPATHOLOGICAL CHARACTERISTICS OF PATIENTS WITH COLORECTAL CANCER IN SERBIA

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ABSTRACT

Cancer cells rely upon fatty acids (FA) for proliferation, survival, and metastasis. Overexpression of long-chain acyl CoA synthetase 1 (ACSL1), responsible for long-chain FA synthesis, can increase cell invasion and proliferation. Uncoupling protein-2 (UCP2) promotes FA metabolism over glucose utilization in colorectal cancer cells. We aimed to investigate the association of *UCP2* 45bp ins/del and *ACSL1* rs8086 polymorphisms with the clinicopathological characteristics in patients with colorectal cancer. The study included 183 patients with colorectal cancer, 110 (60.1%) men and 73 (39.9%) women, with an average age of 62.1 years. Clinicopathological characteristics: tumor location, histological differentiation, presence of lymph node metastases, presence of distant metastases, and stage of the disease were collected for all patients. Rs8086 polymorphism genotypes were detected by Real-time PCR using TaqMan® SNP Genotyping assay, while genotyping of 45 bp ins/del polymorphism was performed using the PCR method. Distant metastases were more frequent in carriers of the *ACSL1* TT genotype than carriers of the CC or CT genotype (p=0.046). Also, carriers of the *UCP2* 45 I allele (II+ID genotype) had distant metastases more frequently than patients with the DD genotype (p=0.029). It has also been observed that there is a statistically significant association of *ACSL1* and *UCP2* genotype with cancer localization (p=0.006 and p=0.017, respectively). The results suggest that *ACSL1* rs8086 and *UCP2* 45 bp ins/del polymorphisms are associated with the occurrence of distant metastases and tumor location in patients with colorectal cancer.

Keywords: colorectal cancer, *ACSL1, UCP2*, gene polymorphism, distant metastases

INTRODUCTION

Colorectal cancer (CRC) is a disease that affects millions of people worldwide. It is the third most commonly diagnosed type of cancer, and the second most common cause of death in both genders [1]. It is well known that intensively proliferating cancer cells can have unique metabolic patterns. Altered lipid metabolism is increasingly recognized as an important feature of colorectal cancer (CRC). The lipid metabolic pathways affecting CRC cells are synthesis, desaturation, elongation, and mitochondrial oxidation of the fatty acids (FAs) [2]. Although CRC can depend on glycolysis, colon cancer cells adapt to low glucose conditions and survive through shifting from glycolysis to oxidative phosphorylation (OXPHOS) [3, 4]. Cancer cells rely upon fatty acids (FA) for proliferation, survival, and metastasis and use them for the production of energy and membrane maintenance [5]. Fatty acids can trigger signals necessary for tumorigenesis, enabling cancer cells to migrate and generate distant metastasis [6]. The serum levels of FAs can potentially be used as predictive biomarkers in colorectal cancer tumors [7].

Fatty acid biosynthesis and β-oxidation are found to be increased in many cancers [5]. Both pathways require a common initial step known as fatty acid activation by acyl-CoA synthetase (ACS) [8,9]. Fatty acid activation,

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catalyzed by acyl-CoA synthetases (ACSs), is key in metabolizing extracellularly derived and *de novo*-synthesized fatty acids [10]. It has been observed that ACSs are frequently deregulated in cancer cells, which leads to the altered distribution and number of intracellular FAs [11]. In colorectal cancer, three main acyl-CoA synthetase long chain (ACSL) isoforms, ACSL1, ACSL4, and ACSL6, are upregulated [10]. Overexpression of ACSL1 in colorectal cancer cells can increase cell invasion and proliferation, and it has been associated with poor clinical outcomes [12]. A functional polymorphism rs8086 has been detected in the 3'-UTR region of the *ACSL1* gene. It causes substitution of cytosine (C) with thymine (T), and it has been previously shown that the TT genotype correlates with higher *ACSL1* mRNA levels [12].

Most cellular reactive oxygen species (ROS) in mitochondria are produced through OXPHOS. A significant increase in oxidative stress markers has been observed in patients with CRC, suggesting a potential role for ROS in colon tumorigenesis and tumor progression [2,4]. ROS can influence the process of tumorigenesis by inducing DNA damage and nuclear genome instability and by increasing proliferation, survival, and migration of cells. Conversely, ROS can also function as anti-tumorigenic agents, since they can induce cellular senescence and cell death. The mechanisms by which cells respond to ROS depend on the location of ROS production and the concentration of different ROS species [13].

Uncoupling protein-2 (UCP2) is a mitochondrial inner membrane anion carrier which acts as a sensor of mitochondrial oxidative stress and a negative regulator of ROS production [14]. Loss of UCP2 function may result in increased generation of ROS [14]. Also, UCP2 participates in several metabolic processes, such as regulating food intake, insulin secretion, and immune responses, and acts as a metabolic switch that promotes fatty acid metabolism over glucose utilization. However, the precise mechanism by which UCP2 modulates these processes and mitochondrial functions remains unclear [15]. One of the polymorphisms of the *UCP2* gene is a 45bp ins/del polymorphism located in the 3'UTR region of exon eight at position +3474. This polymorphism's biological function is still unclear, but the location in the 3′UTR suggests that it may influence mRNA processing or stability of the transcript, affecting protein expression [16].

Considering the importance of discovering new biomarkers that could help predict disease progression, we aimed to investigate the association of *UCP2* 45bp ins/del and *ACSL1* rs8086 polymorphisms with the clinicopathological characteristics of the disease.

MATERIAL AND METHODS

Patients

This study included 183 patients with colorectal cancer who underwent surgical treatment at the Clinic for Digestive Surgery, Clinical Center of Serbia, Belgrade, between 2014 and 2016. All patients included in the study were over 18 years of age and had the diagnosis of colorectal adenocarcinoma confirmed by standard histopathological analyses. Exclusion criteria were previous presence of any malignant diseases and pregnancy.

A signed informed consent form was obtained from each patient, and the study protocol was approved by the Ethics Committee of the Faculty of Medicine, University of Belgrade, Serbia.

Information about age, gender, and clinicopathological characteristics of patients: tumor location, histologic differentiation, preoperative serum carcinoembryonic antigen (CEA) level, presence of lymph node metastases, presence of distant metastases, and stage of the disease. The postoperative stage of the disease was determined by tumor–node–metastasis (TNM) classification by the American Joint Committee on Cancer (AJCC, 8th edition) [17].

Genotyping

Molecular-genetic analyses were performed at the Institute of Human Genetics, Faculty of Medicine, University of Belgrade. Genomic DNA was extracted from 5mL peripheral blood by salting-out method immediately after sample collection, and the samples were stored at -20ºC [18]. The purity and the concentration of the isolated DNA were determined by measuring the absorbance at 260 nm and 280 nm on the spectrophotometer following DNA isolation and prior to genotyping.

Genotypes for *ACSL1* rs8086 polymorphism were detected by Real-time polymerase chain reaction using a standardized TaqMan® SNP Genotyping assay. Polymerase chain reaction (PCR) amplification included an initial step at 95ºC for 10 minutes, 40 cycles of 95ºC for 15 seconds, and 60ºC for 1 minute. PCR and post-PCR fluorescence analyses were performed on the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA), and the results were analyzed using the Applied Biosystems 7500 software v2.0.6 (Applied Biosystems, Foster City, CA).

Genotyping analysis of *UCP2* ins/del polymorphism included PCR and polyacrylamide gel electrophoresis. PCR amplification resulted in 412-bp (del) or 457-bp (ins) long fragments, as previously described [16]. DNA fragments were visualized under UV light, after staining the gel with Sybr®safe DNA dye.

Statistical analysis

Categorical variables are presented by numbers and percentages. Chi-square or Fisher's test was used to analyze the distribution of categorical variables depending on the genotype. Univariate and multivariate logistic regression analysis was also performed using gender and age of patients as covariates. The level of statistical significance was set at $p<0.05$. The power of the study was estimated by G Power 3.1.9.2 using the data obtained for the primary outcome (presence or absence of distant metastases). For a chosen effect size of 0.3, an error of the first type (α) of 0.05, and a power of 90% for Df=2, the required sample size was 172 participants. Statistical tests were performed using the SPSS 17 statistical package (SPSS Inc. Chicago, USA).

RESULTS

Our study included 183 patients with colorectal cancer, with an average age of 62.1. One-hundred-and-ten (60.1%) were men, and 73 (39.9%) were women. Their main demographic features and clinicopathological characteristics are presented in Table 1.

Genotype and allele frequencies of ACSL1 rs8086 and UCP2 45bp ins/del polymorphisms are presented in Table 2. The results of the association analysis between *ACSL1* and *UCP2* genotypes and clinicopathological characteristics are presented in Tables 3 and 4.

There was no statistically significant association between the analyzed polymorphisms and patient age at the time of diagnosis, stage of the disease, tumor grade and presence of lymph node metastases. Carriers of *ACSL1* rs8086 CC genotype had rectal localization of cancer significantly more often than patients with CT or TT genotype (p=0.006, OR=2.390 95% CI 1.278-4.496). Also, in patients that are carriers of the *UCP2* gene II genotype, colon cancer was statistically more frequent than in patients with DD or ID genotype (p=0.017, OR=5.535, 95% CI 1.411-21.711). Although the result is statistically significant, the number of patients with II genotype is small. Only three patients (27.3%) with II genotype developed rectal cancer while 8 (72.7%) had colon cancer. We have also found that the analyzed polymorphisms are associated with the presence of distant metastases. Multiple logistic regression analysis has shown that distant metastases were significantly more frequent in TT genotype carriers than carriers of CC or CT genotype (β=0.822, p=0.040). Age and gender were used as covariates based on the results of univariate logistic regression analysis (Table 5). In the case of UCP2 polymorphism, carriers of *UCP2* 45 I allele (II+ID genotype) had distant metastases more frequently than patients with DD genotype (p=0.040, OR=4.120, 95% CI 1.093-15.534). This result remained significant after logistic multiple regression analysis (β=1.510, p=0.036) (Table 5). Multiple regression analysis has also confirmed that the age and gender of patients can influence the pres-

Table 1. Demographic and clinicopathological characteristics of colorectal cancer patients.

Characteristics	Colorectal cancer patients, n (%)
Sex	
Male	110 (60.1)
Female	73 (39.9)
Age (range 19-86 years)	
≤50 years	23 (12.6)
>50 years	160 (87.4)
Tumor location	
Colon	65 (35.5)
-right	36 (19,7)
-left	29 (15,8)
Rectum	118 (64.5)
Differentiation	
WD	120 (69.8)
MD	43 (25.0)
PD	11 (5.2)
Preoperative serum CEA (ng/mL)	
≤6	51 (65.4)
>6	27 (34.6)
AJCC stage	
I	29 (15.8)
II	41 (22.4)
III	79 (43.2)
IV	34 (18.3)
Lymph node metastasis	
Negative	87 (47.5)
Positive ≤3 lymph nodes	49 (26.8)
>3 lymph nodes	47 (25.7)
Distant metastasis	
Negative	167 (91.3)
Positive	16 (8.7)

WD – well-differentiated; MD – moderately-differentiated, PD – poorly-differentiated; CEA- serum carcinoembryonic antigen; AJCC- American Joint Committee on Cancer

Table 2. Frequencies of *ACSL1* rs8086 and *UCP2* 45bp ins/del genotypes and alleles.

Genotypes	Frequency %	Alleles	Frequency %
rs8086			
CC	47.54	**C**	69.94
CT	44.81	**T**	30.06
TT	7.65		
45bp ins/del			
DD	52.87	**D**	73.28
ID	40.80	**I**	26.72
II	6.33		

Table 3. Genotype frequencies of *ACSL1* rs8086 polymorphism in relation to clinicopathological characteristics of patients with colorectal cancer.

variables		***ACSL1* rs8086**					
		Dominant model			**Recessive model**		
		CC n (%)	CT+TT n (%)	p-value	CC+CT n (%)	TT n (%)	p-value
Age (years)	≤50	9 (10.3)	14 (14.6)	0.388[a]	21 (12.4)	2 (14.3)	0.690[b]
	>50	78 (89.7)	82 (85.4)		148 (87.6)	12 (85.7)	
Tumor location	colon	22 (25.3)	43 (44.8)	0.006[a]	61 (36.1)	4 (28.6)	0.773[b]
	rectum	65 (74.7)	53 (55.2)		108 (63.9)	10 (71.4)	
AJCC stage	I+II	36 (41.4)	34 (35.4)	0.407[a]	65 (38.5)	5 (35.7)	0.839[a]
	III+IV	51 (58.6)	62 (64.6)		104 (61.5)	9 (64.3)	
Differentiation	WD+MD	75 (93.8)	86 (93.5)	0.942[a]	147 (93.0)	14 (100.0)	0.603[b]
	PD	5 (6.3)	6 (6.5)		11 (7.0)	0 (0.0)	
Lymph node metastasis	negative	45 (51.7)	42 (43.8)	0.281[a]	82 (48.5)	5 (35.7)	0.356[a]
	positive	42 (48.3)	54 (56.3)		87 (51.5)	9 (64.3)	
Distant metastasis	negative	82 (94.3)	85 (88.5)	0.172[a]	156 (92.3)	11 (78.6)	0.110[b]
	positive	5 (5.7)	11 (11.5)		13 (7.7)	3 (21.4)	

AJCC- American Joint Committee on Cancer; WD – well-differentiated; MD – moderately- differentiated, [a] Chi-square test, [b] Fisher's exact test

Table 4. Genotype frequencies of *UCP2* 45bp ins/del polymorphism in relation to clinicopathological characteristics of colorectal cancer patients.

variables		*UCP2* 45 bp ins/del					
		Dominant model			Recessive model		
		DD n (%)	ID+II n (%)	p-value	DD+ID n (%)	II n (%)	p-value
Age (years)	≤50	13 (14.1)	9 (11.0)	0.532[a]	21 (12.9)	1 (9.1)	1.000[b]
	>50	79 (85.9)	73 (89.0)		142 (87.1)	10 (90.9)	
Tumor location	colon	27 (29.3)	34 (41.5)	0.095[a]	53 (32.5)	8 (72.7)	0.010[b]
	rectum	65 (70.7)	48 (58.5)		110 (67.5)	3 (27.3)	
AJCC stage	I+II	39 (42.4)	28 (34.1)	0.265[a]	64 (39.3)	3 (27.3)	0.534[b]
	III+IV	53 (57.6)	54 (65.9)		99 (60.7)	8 (72.7)	
Differentiation	WD+MD	80 (90.9)	72 (96.0)	0.196[a]	141 (92.8)	11 (100.0)	1.000[b]
	PD	8 (9.1)	3 (4.0)		11 (7.2)	0 (0.0)	
Lymph node metastasis	negative	44 (47.8)	40 (48.8)	0.900[a]	78 (47.9)	6 (54.5)	0.667[a]
	positive	48 (52.2)	42 (51.2)		85 (52.1)	5 (45.5)	
Distant metastasis	negative	89 (96.7)	72 (87.8)	0.025[a]	150 (92.0)	11 (100.0)	1.000[b]
	positive	3 (3.3)	10 (12.2)		13 (8.0)	0 (0.0)	

AJCC- American Joint Committee on Cancer; WD - well differentiated; MD – moderately differentiated, PD – poorly differentiated, a Chi-square test, b Fisher's exact test

Table 5. Univariate and multivariate analysis of the association between genotypes and clinical factors and the occurrence of distant metastases in colorectal cancer patients.

Covariates	**Univariate analysis**			**Multivariate analysis**		
	OR	95% CI	p-value	OR	95% CI	p-value
Age (≤50 vs >50 years)	0.266	0.083-0.852	**0.026**	0.187	0.044-0.793	**0.023**
Gender	2.069	0.734-5.830	0.169	4.396	1.069-18.085	**0.040**
Differentiation (WD+MD vs PD)	0.973	0.116-8.135	0.980			
ACSL1 rs8086 (CC+CT vs TT)	1.809	0.900-3.636	0.096	2.749	1.020-7.414	**0.046**
UCP2 45 bp ins/del (DD vs ID+II)	4.120	1.093-15.534	**0.037**	5.094	1.179-22.016	**0.029**

WD - well differentiated; MD – moderately differentiated; PD – poorly differentiated

Table 6. Overview of the *ACSL1* rs8086 and *UCP2* 45 bp ins/del gene polymorphisms and gene expression functional relevance in colorectal cancer.

Gene (polymorphism)	Functional effect of the polymorphism	Functional relevance
ACSL1 (rs8086)	The TT genotype correlates with higher *ACSL1* mRNA levels [12]	*ACSL1* knockdown suppresses anchorage-independent growth and reduces the migration of cancer cells [5,10] Presence of the TT genotype is associated with the clinical outcome of colon cancer patients with stage II or III of the disease. TT genotype decreases disease-free survival, with a 3-fold higher risk of relapse [12]
UCP2 (45 bp ins/del)	Possible effect on mRNA processing and transcriptional activity [16]	I allele may be associated with a decreased risk of CRC development [28] Higher *UCP2* expression may promote cancer growth and survival [24] Overexpression of *UCP2* could be associated with tumor aggressiveness [29] In patients with gastric adenocarcinoma tumors with high *UCP2* expression were poorly differentiated, larger, had advanced T and N stages, increased number of infiltrated lymph nodes, lower apoptotic index and shorter disease-free survival [30]

ence of distant metastases. Patients older than 50 years of age had distant metastases less frequently than younger patients (p=0.023, OR=0.187; 95% CI 0.044-0.793), while women had distant metastases more frequently than men (p=0.04, OR=4.396, 95% CI 1.069-18.085).

DISCUSSION

Identifying gene polymorphisms that could represent new biomarkers of disease progression is of great importance for patients with colorectal cancer. It could significantly improve the prognostic ability and facilitate the development of effective targeted treatments, thus improving patients' survival rates.

ACSLs, necessary for activating long-chain fatty acids, are often deregulated in cancer cells. They can promote ungoverned cell growth, evade programmed cell death, and facilitate tumor invasion and migration [8,19]. So far, it has been found that *ACSL1* is upregulated in colon, breast, and liver tissue [8]. Also, it has been shown that *ACSL1* knockdown suppresses anchorage-independent growth and reduces the migration of cancer cells [5,10]. Vargas et al. showed that polymorphism rs8086 in the 3' UTR of the *ACSL1* gene affects gene expression, possibly through the change of miRNA binding affinity and mRNA stability. Patients' carriers of the TT genotype had higher gene expression than carriers of the CC or CT genotype [12]. The increased expression of the *ACSL1* gene in patients with the TT genotype may promote growth and migration of cancer cells.

The frequency of genotypes and alleles obtained in our study did not differ from the frequency in the general European population (https://www.ncbi.nlm.nih.gov/snp/rs8086); based on this, we can assume that rs8086 is not a susceptibility factor for colorectal cancer [20]. However, our results show that *ACSL1* rs8086 polymorphism is associated with a higher risk for the presence of distant metastases in patients with CRC. In the previous study of Vargas et al., TT genotype was significantly associated with the clinical outcome of patients with colon cancer that had stage II or III of the disease. Patients, carriers of the TT genotype, had significantly decreased disease-free survival, with a 3-fold higher risk of relapse [12]. To our knowledge, this is the only study dealing with the association of rs8086 polymorphism and clinical outcomes of patients with colon cancer that has been published so far.

The expression of *UCP2*, coding for a member of the mitochondrial anion carrier proteins family, changes during the course of the disease. While it is repressed during the process of tumorigenesis, leading to increase of oxidative stress in cancer cells, the expression of *UCP2* increases in the later stages of cancer development. Due to the increased *UCP2* expression, cancer cells are protected from apoptosis by negative regulation of mitochondrial ROS production [21]. Another possible mechanism of UCP2 action in tumor cells, besides regulating reactive oxygen species production, is the regulation of glucose and fatty acid oxidation metabolism, which is directly associated with tumor cell proliferation [4]. Pecqueur et al. have shown that UCP2 +/+ provides the persistence of FA catabolism, which, on one side, leads to reduced cell proliferation. On the other hand, it enhances resistance to glucose starvation [22]. So far, it is not clear how the *UCP2* expression is affected by the ins/del polymorphism. The location of this polymorphism in the 3'UTR region of the gene suggests that it may influence mRNA processing or transcript stability [16]. Also, positive linkage disequilibrium between the -866G/A (rs659366) polymorphism minor allele and the 45bp I allele has been observed. The minor allele (A allele) possibly has higher transcriptional

activity than the wt allele. Other functional variants also have modulating functions, and their interaction leads to a complex pattern of associations [23]. Li et al. have found the association of *UCP2* -866G/A with the survival rate after surgery. Patients with the GG genotype had the highest survival rate. The G allele has lower *UCP2* mRNA expression when compared with the A allele. This suggests that higher *UCP2* expression may promote cancer growth and survival [24]. It is possible that the presence of the I allele causes an increase in *UCP2* expression, which provides better modulation of colorectal cancer cells metabolism and enables their survival, proliferation, and migration.

Our results show that the frequencies of *UCP2* ins/del genotypes and alleles are in line with those published for other European populations [25-27], which suggests that this polymorphism does not represent a susceptibility factor for colorectal cancer. However, regarding the association with clinicopathological characteristics, we observed that carriers of the I allele have a higher risk of developing distant metastases. Marques et al. (2017) have shown that I allele could be associated with a decreased risk of CRC development. However, they did not find an association of the ins/del polymorphism with tumor location, TNM stage risk, relapse risk, and death risk [28]. To the best of our knowledge, no other studies are investigating this polymorphism's association with the clinicopathological characteristics of patients with CRC. Kuai et al. investigated the association between the expression of *UCP2* in colon cancer tissue and clinicopathological characteristics of the disease. They observed that *UCP2* expression was significantly higher in patients with cancer stages III and IV in comparison with the patients with cancer stages I and II. Also, they have shown that *UCP2* expression is associated with the development of colon cancer metastasis. Over-expression of *UCP2* could be associated with tumor aggressiveness, which makes UCP2 a potential target for colon cancer therapy [29]. Horimoto et al. have shown that *UCP2* expression is increased in colon cancer and correlates with the degree of neoplastic changes along the adenoma-carcinoma sequence [14]. In the study of Kyriazanos et al., 69 cases of gastric adenocarcinoma were analyzed, and it has been observed that tumors with high *UCP2* expression were poorly differentiated, larger, had advanced T and N stages, increased number of infiltrated lymph nodes, lower apoptotic index and shorter disease-free survival [30].

Overview of the *ACSL1* rs8086 and *UCP2* 45 bp ins/del gene polymorphisms and gene expression functional relevance in colorectal cancer has been presented in Table 6.

We also have found that tumor site specificity depends on the *UCP2* and *ACSL1* genotype. Carriers of the *UCP2* I allele had an increased risk for the development of colon cancer in comparison to rectal cancer while carriers of ACSL1 CC genotype had increased risk of developing rectal cancer. It is known that the biology and histopathology of the colon and rectum are distinct. Evidence shows that there is a difference in site-specific molecular pathways and enzyme expression patterns, but more data is necessary to better understand possible differences between the molecular biology of colon and rectal cancers [31, 32]. Our study has several limitations, however. This is a single center retrospective study with no prospective validation performed in the same or other populations at this moment. While the total number of patients is not small, when divided into groups based on genotype and factors analyzed, some of the groups appeared to be relatively small. Also, the lack of patient follow-up data after the surgery did not allow us to further explore the impact of the analyzed polymorphisms over time.

Gene polymorphisms analysis has a great potential for use in clinical settings since it is easy to perform, there is no need of invasive techniques and it is cost effective. Polygenic risk score (PRS) development may become a basis for personalized treatment. It may help clinicians to recognize high-risk patients earlier and provide personalized treatment, potentially with more aggressive strategy. However, it is still considered only investigational. Main challenges for application are small effect sizes, missing heritability and lack of knowledge regarding gene-gene and gene-environment interactions [33].

In conclusion, our results suggest that *ACSL1* rs8086 and *UCP2* 45bp ins/del polymorphisms may contribute to the occurrence of distant metastases and tumor location in patients with colorectal cancer. Subsequently these polymorphisms, as components of a polygenic risk score, might become biomarkers for tumor location and occurrence of distant metastases. Additional studies on a larger number of patients, are needed to confirm the results obtained here and to further investigate the potential importance of these genetic variants for patients with colorectal cancer.

Ethics Committee approval:

The study was approved by the Ethics Committee Faculty of Medicine, University of Belgrade (1322/V-10).

Conflict of interest:

The authors declare no conflict of interest.

Funding:

The project was funded by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia, grant no. 451-03-47/2023-01/ 200178 and 451-03-66/2024-03/200110.

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