Association of *SIRT1* single gene nucleotide polymorphisms and serum *SIRT1* levels with laryngeal squamous cell carcinoma patient survival rate

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Abstract.
BACKGROUND: *SIRT1* is a multifunctional protein, possibly essential in tumorigenesis pathways, which can act both as a tumor promoter and tumor suppressor depending on the oncogenes, specific to particular tumors. Pathogenesis of laryngeal cancer is multifactorial and the association of *SIRT1* expression with the clinical characteristics and prognosis of LSCC has not been fully identified.
OBJECTIVES: The study aimed to evaluate associations between single gene nucleotide polymorphisms (SNPs) of *SIRT1* (rs3818292, rs3758391, and rs7895833), serum *SIRT1* levels, and 5-year survival rate in patients with laryngeal squamous cell carcinoma (LSCC).
METHODS: The study involved 302 patients with LSCC and 409 healthy control subjects. The genotyping of SNPs was performed using RT-PCR, and serum *SIRT1* levels were determined by the ELISA method.
RESULTS: Our study found significant differences in genotype distributions of *SIRT1* rs3758391 polymorphisms between the study groups. *SIRT1* rs3758391 T/T genotype was associated with the increased LSCC development odds (OR = 1.960 95% CI = 1.028–3.737; $p = 0.041$). Carriers of *SIRT1* rs3758391 T/T genotype had statistically significantly increased odds of LSCC development into advanced stages under the codominant and recessive genetic models (OR = 2.387 95% CI = 1.091–5.222; $p = 0.029$ and OR = 2.287 95% CI = 1.070–4.888; $p = 0.033$, respectively). There were no statistically significant differences in serum *SIRT1* levels between the LSCC and control groups. However, LSCC patients with *SIRT1* rs3818292 AG genotype demonstrated a tendency to significantly lower *SIRT1* serum levels than controls ($p = 0.034$). No statistically significant associations between *SIRT1* (rs3818292, rs3758391, and rs7895833) SNPs and the 5-year survival rate of LSCC patients were found.
CONCLUSION: The present study indicated a statistically significant association between the *SIRT1* rs3758391 T/T genotype and increased LSCC development odds. LSCC patients with *SIRT1* rs3818292 AG genotype showed a tendency to manifest with lower *SIRT1* serum levels. No associations between *SIRT1* SNPs and the 5-year survival rate of LSCC patients were detected.

Keywords: *SIRT1*, laryngeal cancer, five-year survival rate, SIRT serum level

1. Introduction

Laryngeal squamous cell carcinoma (LSCC) is one of the most common malignancies of the upper aerodigestive tract, with high mortality and poor prognosis for patients. In 2020, 184615 new cases of LSCC were

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diagnosed, and 99840 patients died from this cancer worldwide [1]. World Health Organization has estimated that the incidence and mortality of LSCC will have increased from 52.4% to 64.5%, respectively, by 2040 [2]. The most significant increment is projected in countries with a low Human Development Index [2]. This cancer occurs much more frequently in men than in women. According to The Global Cancer Observatory, the age-standardized incidence rate of laryngeal cancer is 3.6/100,000 people among males and 0.49/1,00,000 people among females per year [1]. This can be explained by the fact that men are more likely to have harmful habits. Laryngeal cancer is a multifactorial disease; however, it is generally accepted that tobacco and high-intensity alcohol consumption are the main risk factors for LSCC [3,4,6,7]. Epidemiological studies have consistently linked pro-inflammatory dietary intake, human papillomavirus infection, environmental and occupational exposures with increased risk of LSCC [8–14]. A rising focus on identifying genetic and epigenetic factors to develop new diagnostic tools and treatment for laryngeal cancer can be seen [15]. Several studies have shown an essential role of gene variants, especially in combination with toxins, such as alcohol and cigarette smoke, in the etiopathology and the overall risk/survival rate of LSCC [15].

Despite the rather evident symptoms (hoarseness, dysphonia, dyspnea, and swallowing dysfunction) that may onset quite early in patients with laryngeal malignancies, in many cases, cancer already is at the advanced stage (III or IV) of the disease when diagnosed. Unfortunately, modern treatment strategies do not achieve improvement in therapeutic outcomes and survival [16,17]. Therefore, to implement effective strategies for the prevention and early diagnosis, LSCC needs to be determined from epidemiological and clinical data and modern diagnostic methods based on genetic discoveries.

A silent information regulator two homolog 1 (*SIRT1*) is a nicotinamide adenine dinucleotide (NAD+)-dependent enzyme which plays a critical role in a wide variety of cellular processes, such as genomic stability, glucose, and lipid metabolism, gene transcription, oxidative stress, and aging process via autophagy [18–26]. Dysregulation of *SIRT1* is involved in the pathogenesis of diabetes, neurodegenerations, depression, *anorexia nervosa*, and cardiovascular diseases and has been proposed as a therapeutic target [18,19,21,25,27]. Moreover, *SIRT1* is a multifunctional protein, possibly essential in tumorigenesis pathways, which can act both as a tumor promoter and tu-

mor suppressor depending on the oncogenes specific to particular tumors [28]. Meta-analyses carried out by Wang et al. in 2017 and Sun et al. in 2019 revealed that overexpression of *SIRT1* indicates a poor prognosis for patients with various cancers [29,30]. Ye et al. in their study, have demonstrated the inhibition of proliferation, invasion, and migration of lung cancer through downregulation of *SIRT1* expression and activation of cell autophagy [31]. Several studies have established an association between *SIRT1* expression and a reasonable prognosis of head and neck tumors [32,33]. The pathogenesis of laryngeal cancer is multifactorial, and the association of *SIRT1* expression with the clinical characteristics and prognosis of LSCC has not been fully identified.

Genetic variants may cause different *SIRT1* expressions, and it could play an essential role in laryngeal carcinogenesis. However, just one study has investigated the involvement of *SIRT1* single nucleotide polymorphism (SNP) in laryngeal cancer and has not found any associations between LSCC and *SIRT1* rs12778366 gene polymorphism [34].

The study aimed to evaluate associations between SNPs of *SIRT1* (rs3818292, rs3758391, and rs7895833), serum *SIRT1* levels, and the 5-year survival rate of patients with LSCC.

2. Materials and methods

2.1. Ethics statement

This case-control study was conducted at the Department of Otorhinolaryngology, Lithuanian University of Health Sciences (LUHS), Kaunas, Lithuania. The research protocol was approved by the Kaunas Regional Ethics Committee for Biomedical Research (authorization number BE-2-37). All procedures performed in the study followed the institution's ethical standards, the Helsinki declaration, and its later amendments or similar ethical standards. Informed consent was obtained from all individual participants included in the study.

2.2. Study protocol/design

Study population. A total study group consisted of 711 subjects: 302 patients with LSCC and 409 healthy individuals as a control group. The LSCC patient group included 292 (96.7%) males and 10 (3.3%) females. The control group included 393 (96.1%) males and 16 (3.9%) females. The LSCC patient and control groups

Table 1
Demographic characteristics of the study

Characteristic	Group		p value
	LSCC n = 302	Control group n = 409	
Male, n (%)	292 (96.7)	393 (96.1)	0.673*
Female n (%)	10 (3.3)	16 (3.9)	
Age years; mean (SD)	63.2 (8.1)	63.3 (11.6)	0.146**
Smoking ***, n			< 0.001
Yes	124 (41.1)	33 (8.1)	
No	4 (1.3)	156 (38.1)	
Alcohol consumption***, n			< 0.001
Yes	101 (33.4)	102 (24.9)	
No	27 (8.9)	87 (21.3)	
Stage, n (%)		–	–
I	110 (36.4)		
II	66 (21.9)		
III	53 (17.5)		
IV	73 (24.2)		
T, n (%)		–	–
1	113 (37.4)		
2	63 (20.9)		
3	59 (19.5)		
4	67 (22.2)		
N, n (%)		–	–
0	252 (83.4)		
1	17 (5.6)		
2	33 (10.9)		
M, n (%)		–	–
0	299 (99.0)		
1	2 (0.7)		
2	1 (0.3)		
G, n (%)		–	–
0	1 (0.3)		
1	89 (29.5)		
2	187 (61.9)		
3	25 (8.3)		

*Pearson Chi-Square, **Student's t test SD – standard deviation, T – tumor size, M – metastasis, N – metastasis to the neck lymph nodes, G – tumor differentiation grade, ***Data about smoking and alcohol consumption were collected from 128 LSCC patients and 189 control group subjects.

were adjusted by sex and age. Demographic characteristics of the study groups are presented in Table 1.

LSCC group. A detailed otorhinolaryngological examination, including flexible endoscopy and/or video laryngostroboscopy, and neck palpation was performed for all LSCC patients at the Out-patient Office in the Department of Otorhinolaryngology, LUHS. Peripheral venous blood samples were collected before preparation for general anesthesia and direct microlaryngoscopy with biopsy. The diagnosis of LSCC was histologically confirmed at the Department of Pathology, LUHS. The final diagnosis of LSCC was based on clinical data and the results of histological examination and laryngeal

and neck CT or MRI data. The staging of LSCC was accomplished as described by the American Joint Committee on Cancer [35].

Healthy controls. Subjects who were consulted at the Out-patient Office of the Department of Otorhinolaryngology, LUHS, and selected for surgical treatment (tympanoplasty, ossiculoplasty, tympanostomy, nasal bones reposition, septoplasty, rhinoseptoplasty, uvulopalatopharyngoplasty, or radiofrequency thermoablation of soft palate) for disorders not associated with the development of LSCC or other cancerogenic disease were enrolled in the present study as a healthy control group. The peripheral venous blood samples from these patients were collected from the same catheter that was used to induce general anesthesia. Individuals with no diagnosed oncological diseases who attended the family doctor's consultation for general health check-ups and had a peripheral blood sample collection were also included in this study as healthy controls. All patients with the diagnosed another type and localization of cancer, acute or chronic infectious disease, individuals using psychomotor suppressants and anti-epileptic drugs, and persons younger than 18 years old were excluded from this study.

Survival rate. Data on the LSCC group mortality rate, including the survival period after diagnosis of LSCC and the cause of death, were collected from the Lithuanian State Register of Death Cases and Their Causes.

2.3. DNA extraction, genotyping, and enzyme-linked immunosorbent assay

We extracted DNA samples from peripheral venous blood using the DNA salting-out method. The genotyping of all three SNPs was performed using TaqMan® Genotyping assays (Applied Biosystems Foster City, CA, USA): *SIRT1* (rs3818292, rs3758391, and rs7895833) according to manufacturer's instructions using the real-time polymerase chain reaction (PCR) method. *SIRT1* serum levels were assessed in 15 control subjects and 24 LSCC patients. The *SIRT1* level in serum of LSCC patients was determined using the commercial enzyme-linked immunoassay (ELISA) kit for human *SIRT1* (Human *SIRT1* ELISA Kit, Abcam, Cambridge, United Kingdom), according to the manufacturer's instructions, and the optical density was immediately measured at 450 nm wavelength using a microplate reader (Multiskan FC microplate photometer, Thermo Scientific, Waltham, MA)). The *SIRT1* level was calculated according to the standard curve; standard curve sensibility range: 0.63–40 ng/mL, sensitivity 132 pg/mL.

2.4. Quality control of genotyping

The repetitive analysis of 5% randomly chosen samples was performed for all SNPs to confirm the same rate of genotypes from initial and repetitive genotyping.

2.5. Statistical analysis

Statistical analysis was performed applying the SPSS/W 20.0 software (Statistical Package for the Social Sciences for Windows, Inc., Chicago, Illinois, USA).

The data of subjects' age were presented as the mean with standard deviation (SD) and median with interquartile range (IQR). The Student t-test was performed to compare the average age of the study groups and the Mann Whitney U test was used to compare the serum *SIRT1* levels between study groups. We used the sample size formula to calculate the minimum number in each group of serum *SIRT1* levels. Estimation of sample size power for comparing two means was used to calculate the study power setting. Hardy-Weinberg equilibrium analysis was performed to compare the observed and expected frequencies of *SIRT1* rs3818292, rs3758391, and rs7895833. The frequencies of genotypes and alleles, gender, age, distribution of LSCC stage, tumor size, neck lymph nodes, metastasis, and tumor differentiation grade are described using absolute numbers with percentages. The distributions of the genotypes and alleles of *SIRT1* rs3818292, rs3758391, rs7895833 in the LSCC and control groups were compared using the χ^2 test. Binomial logistic regression analysis was performed to estimate the impact of the genotypes on LSCC development. The odds ratios and 95% confidence intervals are presented. The best genetic model selection was based on the Akaike Information Criterion (AIC); therefore, the best genetic models were those with the lowest AIC values. Differences were considered statistically significant when $p < 0.05$. Survival curves were estimated by the Kaplan-Meier method and median survival time was reported with a 95% confidence interval (95% CI). The log-rank test was used to determine if there was a difference in survival curves between different groups of patients. Univariable and multivariable analyses were performed using the Cox proportional hazards model.

3. Results

The distributions of the analyzed SNPs (*SIRT1* rs3818292, rs3758391, and rs7895833) in the control

Table 2
Analysis of Hardy-Weinberg equilibrium in the control group

SNP	Allele frequencies		Genotype distribution	p value
SIRT1 rs3818292	0.93 A	0.07 G	3/49/357	0.364
SIRT1 rs3758391	0.75 C	0.25 T	18/169/222	0.043
SIRT1 rs7895833	0.86 A	0.14 G	5/106/298	0.190

Table 3
Frequencies of genotypes and alleles of SIRT1 rs3818292, rs3758391 and rs7895833 in patients with LSCC and the control group

Polymorphism	LSCC n (%)	Control group n (%)	p value
rs3818292			
A/A	255 (84.4)	357 (87.3)	0.133
A/G	47 (15.6)	49 (12.0)	
G/G	0 (0)	3 (7.0)	
Total	302 (100)	409 (100)	
Allele			
A	557 (92.2)	763 (93.3)	0.445
G	47 (7.8)	55 (6.7)	
rs3758391			
C/C	151 (50.0)	222 (54.3)	0.115
C/T	127 (42.1)	169 (41.3)	
T/T	24 (7.9)	18 (4.4)	
Total	302 (100)	409 (100)	
Allele			
C	429 (71.0)	613 (75.3)	0.099
T	175 (28.0)	205 (24.7)	
rs7895833			
A/A	215 (71.2)	298 (72.9)	0.819
A/G	82 (27.2)	106 (25.9)	
G/G	5 (1.7)	5 (1.2)	
Total	302 (100)	409 (100)	
Allele			
A	512 (84.8)	702 (85.8)	0.579
G	92 (15.2)	116 (14.18)	

group matched the Hardy-Weinberg equilibrium (HWE) ($p > 0.001$) (Table 2).

We analyzed the distributions of frequencies of the following genotypes and alleles of *SIRT1* rs3818292, rs3758391, and rs7895833 in the LSCC and control groups. There was no statistically significant difference between these two groups (Table 3). Binomial logistic regression was performed to evaluate the impact of *SIRT1* rs3818292, rs3758391, and rs7895833 on LSCC development. Our results revealed that *SIRT1* rs3758391T/T genotype carriers had statistically significantly increased LSCC development odds under the codominant model (OR = 1.960 95% CI = 1.028–3.737; $p = 0.041$) (Table 4).

We analyzed the distributions of *SIRT1* rs3818292, rs3758391, and rs7895833 genotype and allele frequencies between the control and patient with LSCC (in early- and advanced-stages) groups. The patient group

Table 4
Binomial logistic regression analysis of SIRT1 rs3818292, rs3758391, and rs7895833 in the control and patients with LSCC groups

Model	Genotype/allele	OR (95% CI)	p value	AIC
SIRT1 rs3818292				
Codominant	A/G vs. A/A	1.343 (0.872–2.067)	0.180	968.375
	G/G vs. A/A	0.000 (0.000–0.000)	0.999	
Dominant	A/G + G/G vs. A/A	1.265 (0.826–1.937)	0.279	970.323
Recessive	G/G vs. A/A + A/G	0.000 (0.000–0.000)	0.999	968.164
Overdominant	A/G vs. A/A + G/G	1.354 (0.880–2.084)	0.168	969.599
Additive	G	1.173 (0.781–1.763)	0.442	970.903
SIRT1 rs3758391				
Codominant	C/T vs. C/C	1.105 (0.811–1.505)	0.528	969.216
	T/T vs. C/C	1.960 (1.028–3.737)	*0.041*	
Dominant	C/T + T/T vs. C/C	1.187 (0.881–1.599)	0.259	970.216
Recessive	T/T vs. C/C + C/T	1.875 (0.999–3.522)	0.051	967.617
Overdominant	C/T vs. C/C + T/T	1.031 (0.762–1.393)	0.845	971.453
Additive	T	1.237 (0.968–1.581)	0.089	968.600
SIRT1 rs7895833				
Codominant	A/G vs. A/A	1.072 (0.765–1.503)	0.685	973.095
	G/G vs. A/A	1.386 (0.396–4.847)	0.609	
Dominant	A/G + G/G vs. A/A	1.086 (0.780–1.513)	0.624	971.251
Recessive	G/G vs. A/A + A/G	1.360 (0.390–4.741)	0.629	971.259
Overdominant	A/G vs. A/A + G/G	1.065 (0.761–1.492)	0.712	971.355
Additive	G	1.093 (0.805–1.484)	0.568	971.166

OR: odds ratio; CI: confidence interval; p value: significance level (alpha = 0.05); AIC: Akaike Information Criterion.

was divided into subgroups: early (I–II stage) and advanced (III–IV stage) LSCC. The *SIRT1* rs3758391 TT genotype and *SIRT1* rs7895833 allele G were statistically significantly more frequent in the advanced LSCC stage subgroup than in the control group (9.5% vs. 4.4%, $p = 0.029$ and 18.7% vs. 12.8%, $p = 0.048$, respectively) (Table 5). Binomial logistic regression showed that *SIRT1* rs3758391 genotype T/T carriers had statistically significantly increased odds of LSCC development into advanced stages under the codominant and recessive genetic models (OR = 2.387 95% CI = 1.091–5.222; $p = 0.029$ and OR = 2.287 95% CI = 1.070–4.888; $p = 0.033$, respectively) (Table 6).

The LSCC patients' group was divided into four subgroups according to tumor size (T1, T2, T3, and T4). Analysis of *SIRT1* rs3818292, rs3758391, and rs7895833 genotypes and allele frequencies between the control group and patients' subgroups was performed. *SIRT1* rs3758391 TT genotype was more frequent in T4 subgroup than in controls (11.9% vs. 4.4%, $p = 0.012$). In the overall group, rs3758391 genotype (C/C, C/T, and T/T) distributions between the T4 subgroup and controls were statistically significantly different ($p = 0.039$) (Table 7). Also, we found statistically significant differences in *SIRT1* rs7895833 genotype (A/A, A/G, and G/G) distributions between the T4 subgroup and control groups ($p = 0.030$). Further analysis revealed that the *SIRT1* rs7895833 G/G genotype was

more frequent in the T4 subgroup than in controls (6.0% vs. 1.2%, $p = 0.008$) (Table 7). The binomial logistic regression revealed that *SIRT1* rs3818292 genotype A/G and rs7895833 genotype A/G carriers had a statistically significantly increased odds of LSCC development into T3 under the codominant and overdominant genetic models (OR = 2.059 95% CI = 1.039–4.081, $p = 0.039$ and OR = 2.076 95% CI = (1.048–4.115), $p = 0.036$; OR = 1.796 95% CI = (1.018–3.170), $p = 0.043$ and OR = 1.826 95% CI = (1.035–3.223), $p = 0.038$, respectively) (Table 8). The *SIRT1* rs3758391T/T genotype and SIRT1 rs7895833 G/G genotype increased odds of LSCC development into T4 under codominant and recessive genetic models (OR = 3.083 95% CI = 1.239–7.672, $p = 0.015$ and OR = 2.945 95% CI = (1.226–7.077), $p = 0.016$; OR = 5.183 95% CI = (1.342–20.010), $p = 0.017$ and OR = 5.130 95% CI = (1.342–19.618), $p = 0.017$ respectively) (Table 9).

We also performed a comparative analysis of the distribution of the *SIRT1* rs3818292, rs3758391, and rs7895833 genotypes and alleles in the LSCC patient subgroups with metastasis to the neck lymph nodes and without metastases to the neck lymph nodes, and the control group. We found that the rs3758391 TT genotype was statistically significantly more frequent in patients without metastases to the neck lymph nodes than in healthy controls (8.3% vs. 4.4%, $p = 0.037$) (Table 10). However, these differences can be consid-

Table 5
Frequencies of genotypes and alleles of SIRT1 rs3818292, rs3758391, and rs7895833 in the control group and early and advanced stage LSCC subgroups

Genotype/allele	Control group % n = 409	Early-stage (I + II) % n = 176	p value	Advanced stage (III + IV) % n = 126	p value	p value*
rs3818292						
A/A	357 (87.3)	152 (86.4)	0.455	103 (81.7)	0.130	0.275
A/G	49 (12.0)	24 (13.6)		23 (18.3)		
G/G	3 (0.7)	0 (0.0)		0 (0.0)		
Total	409 (100)	176 (100)		126 (100)		
Allele						
A	763 (93.3)	328 (93.2)	0.953	229 (90.9)	0.199	0.296
G	55 (6.7)	24 (8.6)		23 (9.1)		
rs3758391						
C/C	222 (54.3)	89 (50.6)	0.413	62 (49.2)	0.083	0.692
C/T	169 (41.3)	75 (42.6)		52 (41.3)		
T/T	18 (4.4)[1]	12 (6.8)		12 (9.5)[1]		
Total	409 (100)	176 (100)		126 (100)		
Allele						
C	613 (74.9)	253 (71.9)	0.273	176 (69.8)	0.108	0.587
T	205 (25.1)	99 (28.1)		76 (30.2)		
rs7895833						
A/A	298 (72.9)	132 (75.0)	0.706	83 (65.9)	0.154	0.081
A/G	106 (25.9)	43 (24.4)		39 (31.0)		
G/G	5 (1.2)	1 (0.6)		4 (3.2)		
Total	409 (100)	176 (100)		126 (100)		
Allele						
A	702 (85.8)	307 (87.2)	0.525	205 (81.3)	0.084	*0.048*
G	116 (14.2)	45 (12.8)		47 (18.7)		

*Early stage vs. advanced stage; [1]SIRT1 rs3758391 TT genotype was statistically significantly more frequent in the advanced LSCC stage subgroup than the control group, $p = 0.029$.

Table 6
Binomial logistic regression analysis of SIRT1 rs3758391 in healthy controls and patients with LSCC advanced stage

Model	Genotype/allele	OR (95% CI)	p value	AIC
SIRT1 rs3758391				
Codominant	C/T vs. C/C	1.102 (0.724–1.676)	0.651	583.597
	T/T vs. C/C	2.387 (1.091–5.222)	*0.029*	
Dominant	C/T + T/T vs. C/C	1.225 (0.822–1.828)	0.319	585.070
Recessive	T/T vs. C/C + C/T	2.287 (1.070–4.888)	*0.033*	581.802
Overdominant	C/T vs. C/C + T/T	0.998 (0.665–1.497)	0.992	586.063
Additive	T	1.317 (0.951–1.823)	0.097	583.341

OR: odds ratio; CI: confidence interval; p value: significance level (alpha = 0.05); AIC: Akaike Information Criterion.

ered clinically irrelevant because of the low distribution incidence.

The LSCC patient group was divided into three subgroups according to the tumor differentiation grade G1, G2, and G3. Analysis of *SIRT1* rs3818292, rs3758391, and rs7895833 genotypes and allele frequencies between the control group and patient subgroups was performed. The *SIRT1* rs3758391 TT genotype, as well as T allele, were more frequent in the G1 subgroup than in controls (10.0% vs. 4.4%, $p = 0.034$; 32.2% vs. 25.1%, $p = 0.048$, respectively). In the overall group, rs3758391 genotype (C/C, C/T, and T/T) distributions between the G3 subgroup and controls were statisti-

cally significantly different ($p = 0.003$), and the further analysis showed that *SIRT1* rs3758391T/T genotype was more frequent in the G3 subgroup than in controls and the G2 subgroup (20.0% vs. 4.4%, $p < 0.001$; 20.0% vs. 5.3%, $p < 0.001$, respectively) (Table 11). Moreover, we found statistically significant differences in *SIRT1* rs7895833 genotype (A/A, A/G, and G/G) distributions between the G3 subgroup and the control group ($p < 0.001$). Further analysis revealed that the *SIRT1* rs7895833 G/G genotype was more frequent in the G3 subgroup than in controls, and the G1 and G2 subgroups (12.0% vs. 1.2%, $p < 0.001$; 12.0% vs. 1.1%, $p = 0.009$ and 12.0% vs. 0.5%, $p < 0.001$, re-

Table 7

Frequencies of genotypes and alleles of SIRT1 rs3818292, rs3758391, and rs7895833 in the healthy control group and LSCC patients with different tumor size

Genotype/allele	Control group % n = 409	T1 % n = 113	p value	T2 % n = 63	p value	T3 % n = 59	p value	T4 % n = 67	p value
rs3818292									
A/A	357 (87.3)	95 (84.1)	0.366	57 (90.5)	0.668	46 (78.0)	0.087	57 (85.1)	0.629
A/G	49 (12.0)	18 (15.9)		6 (9.5)		13 (22.0)		10 (14.9)	
G/G	3 (0.7)	0 (0.0)		0 (0.0)		0 (0.0)		0 (0.0)	
Total	409 (100)	113 (100)		63 (100)		59 (100)		67 (100)	
Allele									
A	763 (93.3)	208 (92.0)	0.517	120 (95.2)	0.404	105 (89.0)	0.093	124 (92.5)	0.753
G	55 (6.7)	18 (8.0)		6 (4.8)		13 (11.0)		10 (7.5)	
rs3758391									
C/C	222 (54.3)	54 (47.8)	0.217	35 (55.6)	0.967	30 (50.8)	0.688	32 (47.8)	*0.039*
C/T	169 (41.3)	50 (44.2)		25 (39.7)		25 (42.4)		27 (40.3)	
T/T	18 (4.4)[1]	9 (8.0)		3 (4.8)		4 (6.8)		8 (11.9)[1]	
Total	409 (100)	113 (100)		63 (100)		59 (100)		67 (100)	
Allele									
C	613 (74.9)	158 (69.9)	0.128	95 (75.4)	0.912	85 (72.0)	0.459	91 (67.9)	0.086
T	205 (25.1)	68 (30.1)		31 (24.6)		33 (28.0)		43 (32.1)	
rs7895833									
A/A	298 (72.9)	84 (74.3)	0.494	49 (77.8)	0.657	36 (61.0)	0.085	46 (68.7)	*0.030*
A/G	106 (25.9)	29 (25.7)		13 (20.6)		23 (39.0)		17 (25.4)	
G/G	5 (1.2)[2]	0 (0.0)		1 (1.6)		0 (0.0)		4 (6.0)[2]	
Total	409 (100)	113 (100)		188 (100)		59 (100)		67 (100)	
Allele									
A	702 (85.8)	197 (87.2)	0.604	111 (88.1)	0.491	95 (80.5)	0.129	109 (81.3)	0.176
G	116 (14.2)	29 (12.8)		15 (11.9)		23 (19.5)		25 (18.7)	

T – tumor size; [1] SIRT1 rs3758391T/T genotype was statistically significantly more frequent in T4 patients' subgroup than in the control group (11.9% vs. 4.4%, $p = 0.012$); [2] SIRT1 rs7895833 G/G genotype was statistically significantly more frequent in T4 patients' subgroup than in the control group (6.0% vs. 1.2%, $p = 0.008$).

Table 8

Binomial logistic regression analysis of SIRT1 rs3818292, rs7895833 in healthy control and patients with LSCC T3 groups

Model	Genotype/allele	OR (95% CI)	P	AIC
SIRT1 rs3818292				
Codominant	A/G vs. A/A	2.059 (1.039–4.081)	*0.039*	353.882
	G/G vs. A/A	0.000 (0.000–0.000)	0.999	
Dominant	A/G + G/G vs. A/A	1.940 (0.982–3.833)	0.056	353.257
Recessive	G/G vs. A/A + A/G	0.000 (0.000–0.000)	0.999	355.787
Overdominant	A/G vs. A/A + G/G	2.076 (1.048–4.115)	*0.036*	352.606
Additive	G	1.703 (0.903–3.214)	0.100	354.123
SIRT1 rs7895833				
Codominant	A/G vs. A/A	1.796 (1.018–3.170)	*0.043*	353.311
	G/G vs. A/A	0.000 (0.000–0.000)	0.999	
Dominant	A/G + G/G vs. A/A	1.715 (0.973–3.023)	0.062	353.234
Recessive	G/G vs. A/A + A/G	0.000 (0.000–0.000)	0.999	355.243
Overdominant	A/G vs. A/A + G/G	1.826 (1.035–3.223)	*0.038*	352.442
Additive	G	1.531 (0.902–2.597)	0.114	354.208

OR: odds ratio; CI: confidence interval; p value: significance level (alpha = 0.05); AIC: Akaike information criterion.

spectively) (Table 11). Also, the G allele at rs7895833 was statistically significantly more frequent in G3 subgroup patients than in controls (26% vs. 14.2%, $p = 0.023$) (Table 11). The binomial logistic regression was then applied to evaluate the impact of *SIRT1* rs3818292, rs3758391, and rs7895833 on the development of LSCC with different tumor differentiation grades. No statistically significant impact was found.

SIRT1 serum levels were measured in 24 samples from the control group and 29 from the LSCC group. We did not find any statistically significant differences in *SIRT1* serum levels between the LSCC and control

Table 9
Binomial logistic regression analysis of SIRT1 rs3758391, rs7895833 in healthy control and patients with LSCC T4 groups

Model	Genotype/allele	OR (95% CI)	P	AIC
SIRT1 rs3758391				
Codominant	C/T vs. C/C	1.108 (0.640–1.921)	0.714	385.607
	T/T vs. C/C	3.083 (1.239–7.672)	*0.015*	
Dominant	C/T + T/T vs. C/C	1.298 (0.774–2.178)	0.322	357.850
Recessive	T/T vs. C/C + C/T	2.945 (1.226–7.077)	*0.016*	383.741
Overdominant	C/T vs. C/C + T/T	0.959 (0.566 1.623)	0.875	388.805
Additive	T	1.454 (0.961–2.201)	0.077	385.760
SIRT1 rs7895833				
Codominant	A/G vs. A/A	1.039 (0.571–1.891)	0.90	385.843
	G/G vs. A/A	5.183 (1.342–20.010)	*0.017*	
Dominant	A/G + G/G vs. A/A	1.226 (0.700–2.146)	0.477	388.333
Recessive	G/G vs. A/A + A/G	5.130 (1.342–19.618)	*0.017*	383.859
Overdominant	A/G vs. A/A + G/G	0.972 (0.537–1.759)	0.925	388.821
Additive	G	1.401 (0.862–2.277)	0.173	387.051

OR: odds ratio; CI: confidence interval; *p* value: significance level (alpha = 0.05); AIC: Akaike information criterion.

Table 10
Frequencies of genotypes and alleles of SIRT1 rs3818292, rs3758391, and rs7895833 in healthy controls and LSCC patients with and without metastases to the neck lymph nodes

Genotype/allele	Control group % n = 409	Not -spread to lymph nodes % n = 252	p value	Spread to lymph nodes % n = 50	p value	p value*
rs3818292						
A/A	357 (87.3)	210 (83.3)	0.099	45 (90)	0.759	0.235
A/G	49 (12.0)	42 (16.7)		5 (10.0)		
G/G	3 (0.7)	0 (0.0)		0 (0.0)		
Total	409 (100)	252 (100)		50 (100)		
Allele						
A	763 (93.3)	462 (91.7)	0.276	95 (95.0)	0.510	0.256
G	55 (6.7)	42 (8.3)		5 (5.0)		
rs3758391						
C/C	222 (54.3)	124 (49.2)	0.086	27 (54.0)	0.874	0.764
C/T	169 (41.3)	107 (42.5)		20 (40.0)		
T/T	18 (4.4)[1]	21 (8.3)[1]		3 (6.0)		
Total	409 (100)	252 (100)		50 (100)		
Allele						
C	613 (74.9)	355 (70.4)	0.073	74 (74.0)	0.838	0.473
T	205 (25.1)	149 (29.6)		26 (26.0)		
rs7895833						
A/A	298 (72.9)	178 (70.6)	0.817	37 (74.0)	0.282	0.267
A/G	106 (25.9)	71 (28.2)		11 (22.0)		
G/G	5 (1.2)	3 (1.2)		2 (4.0)		
Total	409 (100)	252 (100)		57 (100)		
Allele						
A	702 (85.8)	427 (84.7)	0.583	85 (85.0)	0.825	0.944
G	116 (14.2)	77 (15.3)		15 (15.0)		

*Metastases to the neck lymph nodes vs. no metastases; [1] SIRT1 rs3758391 TT genotype was statistically significantly more frequent in patients without metastases to the neck lymph nodes than in the control group, *p* = 0.037.

groups (0.395 (0.714) ng/ml vs. 0.417 (0.596) ng/ml, *p* = 0.957) (Fig. 1). Also, we performed a comparison of the genotype distribution and serum levels of *SIRT1* between the control and LSCC groups. LSCC patients with *SIRT1* rs3818292 AG genotype had statistically significantly lower *SIRT1* serum levels than controls (*p* = 0.034) (Table 12). However, the power setting

of this study sample was rather low and reached 37%. Therefore, the results of *SIRT1* serum level analysis were considered as the tendency.

Finally, the 5-year survival rate of LSCC patients was determined. The 5-year overall survival rate of LSCC patients was 62.7% and the LSCC-specific survival rate of five years was 72.6%. Analyzing LSCC patients' 5-

Table 11
Frequencies of genotypes and alleles of SIRT1 rs3818292, rs3758391, and rs7895833 in healthy controls and LSCC patients with different tumor differentiation grades

Genotype/allele	Control group % $n = 409$	G1 % $n = 90$	p value	G2 % $n = 188$	p value	G3 % $n = 25$	p value
rs3818292							
A/A	357 (87.3)	73 (81.1)	0.161	162 (86.2)	0.417	21 (84.0)	0.769
A/G	49 (12.0)	17 (18.9)		26 (13.8)		4 (16.0)	
G/G	3 (0.7)	0 (0.0)		0 (0.0)		0 (0.0)	
Total	409 (100)	90 (100)		188 (100)		25 (100)	
Allele							
A	763 (93.3)	163 (90.6)	0.201	350 (93.1)	0.903	46 (92.0)	0.728
G	55 (6.7)	17 (9.4)		26 (6.9)		4 (8.0)	
rs3758391							
C/C	222 (54.3)	41 (45.6)	0.064	99 (52.7)	0.857	12 (48.0)	*0.003*
C/T	169 (41.3)	40 (44.4)		79 (42.0)		8 (32.0)	
T/T	18 (4.4)[1,2]	9 (10.0)[1]		10 (5.3)[3]		5 (20.0)[2,3]	
Total	409 (100)	90 (100)		188 (100)		25 (100)	
Allele							
C	613 (74.9)	122 (67.8)	*0.048*	277 (73.7)	0.64	32 (64.0)	0.086
T	205 (25.1)	58 (32.2)		99 (26.3)		18 (36.0)	
rs7895833							
A/A	298 (72.9)	61 (67.8)	0.602	140 (74.5)	0.705	15 (60.0)	< 0.001
A/G	106 (25.9)	28 (31.1)		47 (25.0)		7 (28.0)	
G/G	5 (1.2)[4]	1 (1.1)[5]		1 (0.5)[6]		3 (12.0)[4,5,6]	
Total	409 (100)	90 (100)		188 (100)		3 (100)	
Allele							
A	702 (85.8)	150 (83.3)	0.393	327 (87.0)	0.493	37 (74.0)	*0.023*
G	116 (14.2)	30 (16.7)		49 (13.0)		13 (26.0)	

G-Tumor differentiation grade; [1] SIRT1 rs3758391T/T genotype was statistically significantly more frequent in the G1 subgroup than in the control group, $p = 0.034$; [2] SIRT1 rs3758391T/T genotype was statistically significantly more frequent in the G3 subgroup than in the control group, $p < 0.001$; [3] SIRT1 rs3758391T/T genotype was statistically significantly more frequent in the G3 subgroup than in the G2 subgroup, $p < 0.001$; [4] SIRT1 rs7895833 G/G genotype was statistically significantly more frequent in the G3 subgroup than in the control group, $p < 0.001$; [5] SIRT1 rs7895833 G/G genotype was statistically significantly more frequent in the G3 subgroup than in the G1 subgroup, $p = 0.009$; [6] SIRT1 rs7895833 G/G genotype was statistically significantly more frequent in the G3 subgroup than in the G2 subgroup, $p < 0.001$.
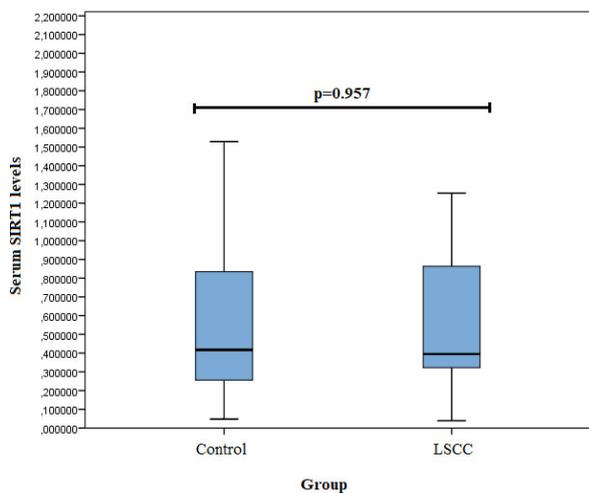
Fig. 1. Serum SIRT1 levels in controls and patients with LSCC.

year survival rate and the genotype distribution of *SIRT1* (rs3818292, rs3758391, and rs7895833), we have not found a statistically significant impact of these SNPs on the patients' 5-year survival rate (Fig. 2).

Table 12
Genotype distribution and serum SIRT1 levels

SNP/genotype	SIRT1 level (pg/mL)		P-value
	LSCC median (IQR)	Controls median (IQR)	
SIRT1 rs3818292			
A/A	0.48 (0.86)	0.42 (0.62)	0.396*
A/G	0.20 (0.30)	0.57 (−)	*0.034*
G/G	−	−	−
SIRT1 rs3758391			
C/C	0.39 (0.57)	0.36 (0.42)	0.661*
C/T	0.56 (1.33)	0.65 (2.01)	0.622*
T/T	−	−	−
SIRT1 rs7895833			
A/A	0.40 (0.76)	0.41 (0.52)	0.587*
A/G	0.39 (4.92)	0.65 (14.18)	0.338*
G/G	−	−	−

* Obtained using the Mann-Whitney U test.

4. Discussion

The *SIRT1* is located on the long arm of chromosome 10 (10q21.3) [36]. Evidence from many studies has revealed that *SIRT1* participates in the regulation of diverse cellular processes, including carcinogene-
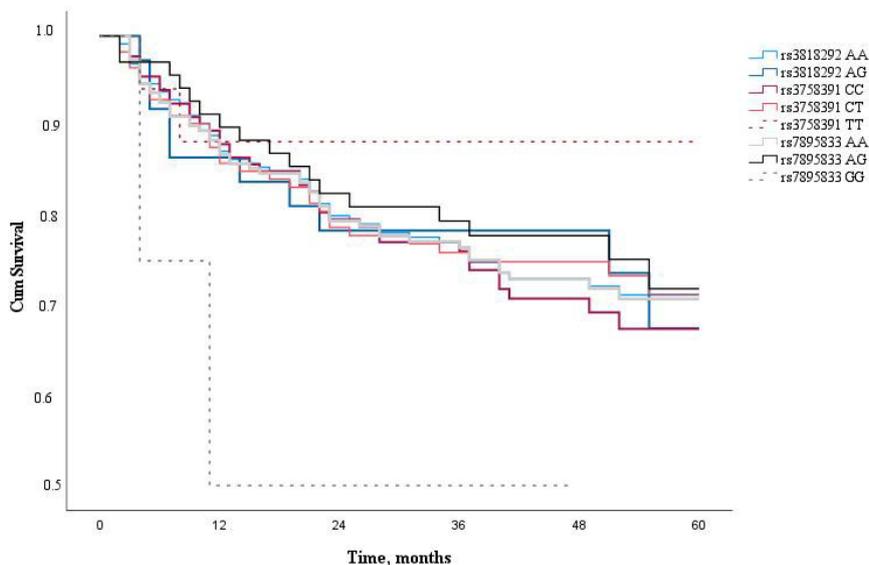
Fig. 2. Five year survival analysis of LSCC patients according to SIRT1 SNPs (rs3818292, rs3758391, and rs7895833).

sis [37]. In cancer development, the role of *SIRT1* is dual because it can function as both a tumor promoter and a tumor suppressor [28,37–39]. The *SIRT1* acts as a tumor suppressor in holding genomic stability and negatively regulates anti-apoptotic genes, resulting in increased anticancer ability [37]. On the other hand, to meet the tumor's high-energy needs and promote its development, *SIRT1* not only induces angiogenesis but also improves HIF1α/HIF2α (regulation of the oxygen-sensitive genes controlling angiogenesis, glycolysis, and cellular apoptosis) and suppresses p53 (interference of p53-dependent apoptosis in response to DNA damage signals) [37].

Epigenetic modifications are critical for LSCC carcinogenesis [8]. Gene variants may account for different *SIRT1* expressions, determining individuals' susceptibility to specific pathologies [19,24,26,40–45]. However, little is known about the effect of polymorphism of the *SIRT1* in both LSCC carcinogenesis and disease progression. Therefore, the focus of the present study was to estimate the associations between *SIRT1* SNPs (rs3818292, rs3758391, rs7895833), *SIRT1* serum levels, and LSCC.

In the present study, we found that *SIRT1* rs3758391 T/T genotype carriers had statistically significantly increased odds of LSCC. Moreover, the current study results revealed that patients with this genotype had 2.387 odds of LSCC development into advanced stages. To the best of our knowledge, this is the first report that associates SNP in the *SIRT1* gene (rs3758391) with LSCC in a pure and homogenous LSCC patients' cohort.

According to the literature, the *SRT1* rs3758391 polymorphism has been associated with healthy aging in the Chinese population and better cognitive function in older individuals [46–48]. Also, several studies have shown the role of this *SIRT1* SNP in several diseases, including type 2 diabetes mellitus [49], cardiovascular diseases [50], systemic *lupus erythematosus* [51], and major depressive disorder [52]. Also, the accumulating evidence has suggested the importance of the *SIRT1* rs3758391 in cancer development [43,45,53,54]. Furthermore, Shafieian et al. found that the *SIRT1* rs3758391 TT genotype increased the risk of urinary bladder cancer in the Iranian population [45]. Shaker et al. study showed that carriers of the *SIRT1* rs3758391 CC genotype were more often in the colorectal cancer group than in the control group [54]. Rizk et al. study revealed that the *SIRT* rs3758391 polymorphism (TT genotype) was associated with the increased breast cancer risk and that the *SIRT1* rs3758391T allele was a potential risk factor for breast cancer [43]. Kan et al. conducted a large-scale case-control study on diffuse large B cell lymphoma and compared results of patients with the healthy control group. They found that the *SIRT1* rs3758391T allele presented an increased risk of diffuse large B cell lymphoma. Also, they noticed *SIRT1* mRNA expression upregulation in diffuse large B cell lymphoma patients with TT genotype. Moreover, a worse survival rate was revealed in patients with TT genotypes. The authors concluded that the *SIRT1* rs3758391 might be used as a biomarker for the prognosis of susceptibility and survival of diffuse large B cell

lymphoma [53]. We did not find a significant impact of *SIRT1* SNPs on LSCC patients' 5-year survival rate in the present study. Lv et al. did not find any association between *SIRT1* gene polymorphisms, including rs3758391, and lung cancer [55].

The results of the present study revealed that two SNPs of *SIRT1* (rs3818292 and rs7895833) are associated with the increased odds of LSCC development into T3 and T4. No associations between the aforementioned *SIRT1* SNPs and any cancer were described in the literature, so far. However, these polymorphisms of *SIRT1* have been investigated mainly in the context of metabolism. They have been associated with the increased risk of visceral (rs3818292) and general (rs7895833) obesity in adults and decreased risk (a protective effect) of obesity in children (rs7895833) [56–58]. Associations of *SIRT1* rs3818292 with diabetic nephropathy and nondiabetic type 1 cardiorenal syndrome have also been found [59,60]. Kilic et al., in their study, found that *SIRT1* rs7895833 might affect longevity. They observed a significant increase in serum *SIRT1* level in older people and carriers of *SIRT1* rs7895833, suggesting an association between *SIRT1* rs7895833 SNP and longevity [61].

In the present study, we measured the serum concentration of *SIRT1* in patients with LSCC and compared it with the results of the control group. We did not find any statistically significant differences between these groups. However, comparing the genotype distribution and serum levels of *SIRT1* between the control and LSCC groups, we found that only LSCC patients with *SIRT1* rs3818292 AG genotype demonstrated a clear tendency to significantly lower *SIRT1* serum levels than controls. These findings are in some contradiction with the data of the literature. For instance, Shaker et al. in their study found that patients with colorectal cancer had a significant increment in the mean level of serum *SIRT1* as compared to the control group and the mean level of serum *SIRT1* was higher in patients with tumor size $\geqslant 5.0$ cm compared to the size < 5.0 cm [54]. Rizk et al. conducted a study on breast cancer and found significantly higher serum *SIRT1* levels in breast cancer patients than in the control group. However, no significant relationship was observed between the *SIRT1* levels and clinicopathological factors (types of breast cancer, tumor size, lymph node involvement, metastasis, receptor status), except the tumor differentiation grades (higher *SIRT1* level in Grade 2 compared to Grade 1 patients, and in Grade 3 compared to Grade 2 patients) [43]. Thus, it can be presumed that increased *SIRT1* serum levels were associated with some particu-

lar and rather aggressive tumors (colorectal and breast cancers) and tumor differentiation grades. The LSCC, on the contrary, is a relatively less aggressive tumor considering rather low local spreading and low metastatic rate [62,63]. Therefore, LSCC possibly does not cause *SIRT1* serum level increment. Consequently, the role of *SIRT1* serum levels as an indicator of the prognosis of LSCC remains doubtful.

On the other hand, Noguchi et al. assessed the *SIRT1* expression immunohistochemically using surgical samples from 437 consecutive head and neck squamous cell carcinoma (HNSCC) patients. Results of the study showed that the *SIRT1* expression acted as a tumor suppressor and served as an indicator for a good prognosis [33]. Yu et al. investigated *SIRT1* expression in laryngeal and hypopharyngeal squamous cell carcinoma (HSCC) tissue samples using immunohistochemical staining and its correlations with clinical parameters. They found a significant correlation between the decreased *SIRT1* expression and clinical tumor stage, metastasis to the neck lymph nodes, and the patient's survival rate. The authors concluded that *SIRT1* acts as a tumor suppressor in LSCCs and HSCC [64]. However, those authors pulled their LSCC and HNSCC patients into one group; therefore, these results cannot be directly compared to the cohort of pure LSCC patients used in the present study. On the other hand, the results of the current study may suggest that in cases of LSCC, the *SIRT1* serum levels remain unchanged despite the increment of *SIRT1* expression directly in the tumor tissue. This phenomenon may be caused by a relatively low tumor suppressor activity of *SIRT1* related to the less aggressive nature of LSCC.

The strength of the present study is a relatively large and homogenous group of pure LSCC patients (302 subjects) and age and sex-matched controls (409 subjects). This specific feature ensured a comprehensive analysis of associations between *SIRT1* serums levels and selected *SIRT1* SNPs and the development of a particular tumor in one anatomical region of the head and neck, i.e., LSCC.

Several limitations of the present study must be considered. The sample size for analysis of serum concentration levels of *SIRT1* was limited and too small to reach the desired power setting. Therefore, the results obtained in the present study regarding the associations between the *SIRT1* serum concentration levels and LSCC should be considered as the tendency. Further investigation with a large enough sample size is foreseen to confirm the possible role of serum concentration levels of *SIRT1* in LSCC development. The data about

smoking and alcohol consumption was available and analyzed in a limited cohort of study participants that did not match the patient and control groups. Therefore, the impact of smoking and alcohol consumption habits was not completely investigated. However, this is the target task for future investigation.

5. Conclusion

The present study showed a statistically significant association between *SIRT1* rs3758391T/T genotype and increased odds of LSCC development, as well as increased LSCC development into the advanced stages and T4 odds. Also, *SIRT1* rs3818292 and rs7895833 SNPs revealed increased odds of LSCC development into T3 and T4. LSCC patients with *SIRT1* rs3818292 AG genotype showed a tendency to manifest with lower *SIRT1* serum levels. No associations between *SIRT1* (rs3818292, rs3758391, and rs7895833) SNPs and the 5-year survival rate of LSCC patients were found in the present study. Therefore, the role of *SIRT1* in cancer, including LSCC, remains incompletely understood. Further research is needed to investigate possible mechanisms of the associations found, before selecting candidates for prognostic factors or a future therapeutic target.

Author contributions

Conception: R.L., V.U.
Interpretation or analysis of data: R.L., A.V., G.G., V.U., P.V., V.L.
Preparation of the manuscript: P.V., V.U.
Revision for important intellectual content: R.L., V.U., V.L.
Supervision: R.L., V.U.

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