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HSP70 In triple negative breast cancer: Prognostic value and clinical significance

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ABSTRACT

Background: Triple-negative breast cancer (TNBC) has the worst prognosis and the highest immunogenic potential of all breast cancer subtypes. It is characterized by a lack of estrogen and progesterone receptors as well as HER2. A major component of the tumor microenvironment (TME) of TNBC is tumor-infiltrating lymphocytes (TILs). A chaperone heat shock protein 70 (HSP70) is involved in several pathways that enable tumour growth and progression, as well as in immune modulation.

Methods: Immunohistochemical analysis of HSP70 expression in immune cells, as well as expression of immunosuppressive markers CTLA4 and PD-L1 and major TILs components: CD8, CD4 and Tregs were analyzed in the superficial and deep tumor layer of primary TNBC and compared with established clinicopathological parameters. Clinical data and surgical tissue samples from 68 TNBC patients who underwent initial surgery were included in the analysis and 36 control samples from benign breast tissue biopsies.

Results: A higher expression of TILs, CD4, CD8 and PD-L1 was found in the invasive tumor front (ITF), as compared to the tumor center (TC) ($p < 0001$). HSP70 positive immune cells (HSP70(+) IC) in TC were associated with adverse clinical and pathological markers: higher stage of disease ($P = 0.013$), higher grade ($P = 0.05$) and a higher pN status ($P < 0.001$). In addition, higher expression of HSP70(+) IC from TC was correlated with the higher expression of FOXP3(+)T cells both in ITF ($N = 61$, rho = 0.42, $p < 0.001$) and in metastatic tissue from the draining lymph nodes ($N = 13$, rho = 0.61, $P = 0.026$).

Conclusion: Correlations between HSP70 immune cells expression and individual TILs components support the hypothesis of its active role in inducing immunosuppression and tumor progression. Routine determination of HSP70 expression, in immune cells of TC, may be of added value in the clinical decision-making process concerning axillary surgery.

1. Introduction

Triple-negative breast cancer (TNBC) accounts for 15-20 % of all breast cancer (BC) subtypes, and is related to rapid, aggressive growth, more frequent relapse and shorter survival time following disease progression. It is characterized by a lack of prognostic and predictive biomarkers, as well as molecular targets for specific anticancer therapeutics. The development of TNBC is driven by other signaling pathways like TP53, AKT3 and PTEN [1–3]. Its high mutation burden and genomic instability result in the neoantigen enriched tumor microenvironment (TME) abundant with tumor infiltrating lymphocytes (TILs) [4–6].

In the early stage of the disease, TILs are involved in host immunity against tumor cells through the activation of tumor-specific CD8+ cy-

ITF, invasive tumor front; TC, tumor center; HSP70(+)IC, HSP70 positive immune cells; TILs, tumor- infiltrating lymphocytes; TME, tumor microenvironment; DCs, myeloid- derived dentritic cells

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totoxic T cells. However, the ability of cancer cells to modulate the immune response enables not only its escape from the immune system but also its progression and metastatic spread in the later course of the disease [7–10]. Although many mechanisms underlying immune tolerance have been revealed in recent years, the role of the immune system, as well as its association with other prognostic factors in TNBC has not yet been fully understood.

Several proteins and immune cells have already been implicated in the process of immunoediting and cancer progression; programmed cell death receptor 1 and its ligand (PD1-PDL-1), cytotoxic T lymphocyte-associated protein 4 (CTLA-4), regulatory T lymphocytes (Tregs), tumor associated macrophages (TAMs), myeloid-derived dendritic cells (DCs) and heat shock proteins (HSP) [11–17].

Heat shock protein 70 (HSP70), a member of the HSP family, is an evolutionarily highly conserved molecule and is expressed in all cells of various organisms and organ systems. There are several isoforms of HSP70 constitutively expressed in intracellular compartments of all human cells. Intracellular HSP70 has a central role in proteostasis; it is required for protein folding and trafficking, as well as the repair of misfolded proteins. During the reparation process, HSP70 inhibit all cellular apoptotic pathways thus allowing the survival of the non-lethally damaged cell. Any cellular stress, including genomic instability, oncogenic signalling and intracellular accumulation of oncoproteins, as well as chemotherapy and irradiation cell damage, activate the heat shock transcription factor 1, ultimately leading to the overexpression of the stress-induced isoforms of HSP70 in the cancer cells [18–20]

According to literature data, the upregulated expression of chaperone HSP70 supports the formation of early-stage BC by prevention of apoptosis and degradation of oncoproteins. HSP70 expression in tumor cells correlates with cell proliferation, differentiation, migration, invasion and apoptosis. Its increased expression is associated with a higher stage of the disease, worse survival and development of resistance to chemotherapy [21–23]. Further on, severely damaged and cancer cells can actively release HSP70 in the extracellular compartment. This extracellular and membrane-bound HSP70 act as a signal molecule (chaperokine) and is implicated in immunological pathways [24]. However, both proinflammatory and antiinflammatory roles have been reported

The aim of this study was to determine the expression of HSP70 in tumor infiltrating immune cells and their relationship with other immune cell markers: CD8, CD4, FOXP3, CTLA4, PD-L1 as well as its associations with the established clinicopathologic prognostic factors in TNBC.

2. Methods

2.1. Patients

The study included 68 consecutive samples of TNBC patients, submitted to surgery in Clinical Hospital Center Rijeka, Croatia, in the period from 2008. to 2016. Clinicopathological characteristics are displayed in Table 1.

Metastatic tissue from the regional lymph nodes of twelve patients was analyzed as well and compared with primary tumor tissue.

Patients with incomplete clinical data, stage IV disease, patients receiving neoadjuvant systemic treatments or those with recurrent disease, as well as those diagnosed with any other malignancy, were excluded from the present analysis. All patients have received adjuvant treatments according to ESMO guidelines for the treatment period.

The control tissue consisted of 36 vacuum-assisted breast biopsy tissue specimens, histologically categorized as benign breast change (B2).

2.2. Tissue microarrays construction

Tissue microarrays (TMA) corresponded to the examined material, in which each tumor biopsy was represented with two cylinders of primary tumor tissue, 2 mm in diameter. An additional cylinder was taken in cases where there was metastasis in the locoregional lymph node. For each tumor case, one area corresponded to the superficial portion of the tumor from the area of the invasive tumor front (ITF) and the other area corresponded to the deep tumor layer i.e. tumor center (TC). ITF was defined as a narrow band-like area at the tumor-host interface with a width of approximately 1 mm between the invasive margin of cancer tissue and the adjacent non-tumorous stroma. TC was defined as an intra-tumoral area consisting of tumor parenchyma and stroma with no direct contact with the peri-tumoral breast tissue [26]. Benign breast change was represented with one cylinder per biopsy. Paraffin-embedded TMA was serially sliced into 4 μ thick slices. As some cores were lost in the process, the number of examined samples sometimes differs between the variables.

Table 1
Clinicopathological characteristics of the study cohort (N = 68).

Clinicopathological characteristics	N (%)
Age	
≤50	13 (24)
>50	55 (76)
Pathological stage (pT)	
1	22 (32)
2	35 (51)
3	5 (9)
4	6 (8)
Histological type	
NST*	37 (54)
Metaplastic	13 (19)
Medullary pattern	15 (22)
Apocrine	3 (5)
Histological grade	
1	2 (2)
2	33 (49)
3	33(49)
Anatomical stage	
1	19 (28)
2	31 (46)
3	18 (26)
Lymph node status (pN)	
0	45 (66)
1	10 (15)
2	6 (4,5)
3	6 (4,5)
Pathological prognostic stage	
1	20 (30)
2	27 (40)
3	21 (30)
Recurrence/metastasis	
No	47 (70)
Local recurrence	9 (11)
Distant metastasis	13 (19)
Months to recurrence	
Median (range)	39 (2-64)
Follow up (months)	
Median (range)	64 (2-124)
Died of disease N (%)	28(41)

**AJCC Cancer Staging Manual [25].
* NST: no special type.

2.3. Immunohistochemistry (IHC)

TMA slices were deparaffinized in a xylene substitute and rehydrated. Heat-induced pretreatment was used to retrieve antigenic epitopes. Tissue slices for detection of primary antibodies of HSP70, CD8, CD4, CTLA4 and FOXP3 were immersed in Target retrieval High pH solution (3 in 1) in a PT link Pre-Treatment Module for 20 minutes at temperatures of 97º C. The "EnVision" immunohistochemical method with the Real Envision Detection K8000 system on the automated immunostainer (DakoCytomation, Autostainer Plus, Glostrup, Denmark®) was

used as a detection system, according to the manufacturer's instructions. According to the manufacturer's protocols, Specifications for immunohistochemical stainings are displayed in Table 2.

2.4. Determination of TILs and immunohistochemical staining readings

TILs were determined as a continuous variable on full HE stained slides according to recommendations of the International TILs Working Group 2014 [27]. The immunophenotype of inflammatory cells was determined on TMA as follows in the description. The percentage of cells positive for CD4 and CD8 was visually determined over the whole tissue area (2 mm in diameter), as a reading of a continuous variable, at a light microscope magnification of 200x. Determination of HSP70, FOXP3 and CTLA4 expression was performed as a reading of a continuous variable, by counting the total number of positive lymphocytes over the whole tissue area, at a light microscope magnification of 400x. PD-L1 was determined according to the manufacturer protocol for PD-L1 reading in TNBC. Immunohistochemical stainings of the control group were determined by estimating the number of positively stained immune cells per whole tissue area in the stromal areas of benign changes. All immunohistochemical stainings of immune cells were cytoplasmic and membranous except for the nuclear anti-FOXP3 staining. All immunohistochemical readings were performed manually, blinded to the patient's clinical characteristics and the tumor's histological characteristics.

2.5. Statistical analysis

Statistical analysis was performed by the Statistica software package for Windows 10, 14 (StatSoft, Inc., Tulsa, OK[R]). Percentages of positive IHC stains were presented as mean and median values. Differences between the two independent samples were determined using the Student t-test for independent samples and the Wilcoxon rank-sum test. Differences between the two dependent samples were determined by the Student t-test for dependent samples and the Wilcoxon signed-rank test. Cut-off values of percentages and numerical readings were determined by Receiver Operating Characteristic (ROC) analysis (MedCalc Version 20.013 and X-tile analysis version 3.6.1[R]). Diagnostic and prognostic values of markers were determined with ROC analysis. All statistical values were considered significant at a P-level of <0.05. Spearman's rank correlation coefficient determined a correlation between the stainings. The degree of correlation was expressed as negligible (less than 0.30); low positive (0.30-0.50); moderately positive (0.50-0.70); high positive (0.70 and 0.90); very highly positive (more than 0.90) [28].

The study protocol was approved by the Ethics Committee of the Clinical Hospital Center Rijeka, Croatia.

Table 2
Immunohistochemical stainings and procedure.

Monoclonal antibody	Manufacturer	Clone	Dilution	Diluent	Incubation (minute)
Hsp70 ab2787	Abcam, Cambridge, UK	5A5	1: 200	DAKO S0809	30
CD4 104R-16	Cell Marque, Rocklin, CA, USA	SP35	1:75	DAKO S0809	60
CD8 IR623	DakoCytomation, Glostrup, Denmark	C8 /144B	factory diluted	DAKO S0809	30
CTLA4 sc-376016	Santa Cruz Biotechnology, Dallas, Texas, USA	F-8 from	1: 100	DAKO S0809	60
FOXP3 ab450	Abcam, Cambridge, UK	mAbcam450	1:200	DAKO S0809	60
PDL-1	Roche Diagnostics GMbH, Mannheim, Germany	SP-142	RTU*, Ventana		

* RTU- ready to use.

3. Results

3.1. Immunohistochemical expression of HSP70 and TILs components

The expression of the examined markers in different parts of the tumorous tissue and in control tissue is displayed in Table 3.

As expected, a statistically significantly higher number of all TILs components and HSP70 positive immune cells (HSP70(+) IC), both in ITF and TC, was found in the TNBC group, as compared to unaltered breast tissue samples. There were no statistically significant differences between the expression of HSP70(+) IC and TILs components in primary tumor and metastasis in the locoregional lymph node. The expression of HSP70(+) IC, CTLA4 and FOXP3 did not differ significantly between superficial and deep tumor layers while statistically significant ($p<0.001$) higher expression of TILs, CD4, CD8 and PD-L1 was found in ITF as compared to the TC, Table 3. Representative photomicrographs of immunohistochemical stainings are shown in Fig. 1.

3.2. Associations of HSP70 with the clinicopathological prognostic factors

Statistically significant associations were detected concerning the expression of HSP70 in immune cells of TC (HSP70(+) IC TC); a higher proportion of HSP70(+) IC TC was associated with the higher anatomical ($P=0.027$) and prognostic ($P=0.013$) stage of the disease, higher histological grade ($P=0.05$) and a higher pathological nodal (pN) status ($P<0.001$, Chi-square test) (Table 4). The results of the ROC analysis have further confirmed the strong association of HSP70(+) IC TC with the axillary lymph node involvement (AUC = 0.78, $P<0.001$) as well as with the additional nodal involvement in cases with only one lymph node involved (AUC = 0.68, $P=0.024$). Moreover, HSP70(+) IC TC has been associated with lymph node capsular penetration in the present analysis (AUC = 0.78, $P<0.001$) (Fig. 2).

3.3. Correlations of HSP70 and TILs components

Several positive correlations of HSP70(+) IC TC were detected concerning the percentage of TILs components. Low correlation toward CTLA4 expression in a TC (N = 64, rho = 0.34, $P=0.006$) and FOXP3 expression in the ITF (N = 61, rho = 0.42, $p<0.001$) as well as moderate correlation toward FOXP3 in the lymph node metastasis (N = 13, rho = 0.61, $P=0.026$).

Table 3
Immunohistochemical expression of TILs components and HSP 70 in primary TNBC, metastasis and control tissue.

Variables	Control tissue (median)	1° tumor (average expression, median)	Metastasis (median)	1° tumor	
				ITF (median)	TC (median)
HSP70	2.0	15.0	70.0	15.5	10.0
P	$<$**0.001***	0.114.		.0.057	
TILs		17.500		20.000	10.000
P				$<$**0.001**	
CD8 ˜	0.647	21.705	21.313	25.833	17.000
P	$<$**0.001****	0.916[1]		$<$**0.001**[1]	
CD4	1.000	17.500	21.000	25.000	10.000
P	$<$**0.001***	0.074ˇ		$<$**0.001**	
PDL-1	0.000	0.40	1.000	0.300	0.100
P	$<$**0.001***	1.000ˇ		$<$**0.001**	
CTLA4	0.000	22.250	80.500	23.500	14.500
P	$<$**0.001***	0.18ˇ		0.237	
FOXP3	0.000	0.750	0.000	1.000	0.000
P	$<$**0.001***	0.724ˇ		0.164	

ITF: invasive tumor front; TC: tumor center; ˜mean; *Wilcoxon rank-sum test; **Student t test for independent samples; [1]t test for dependent samples; ˇWilcoxon signed-rank test.

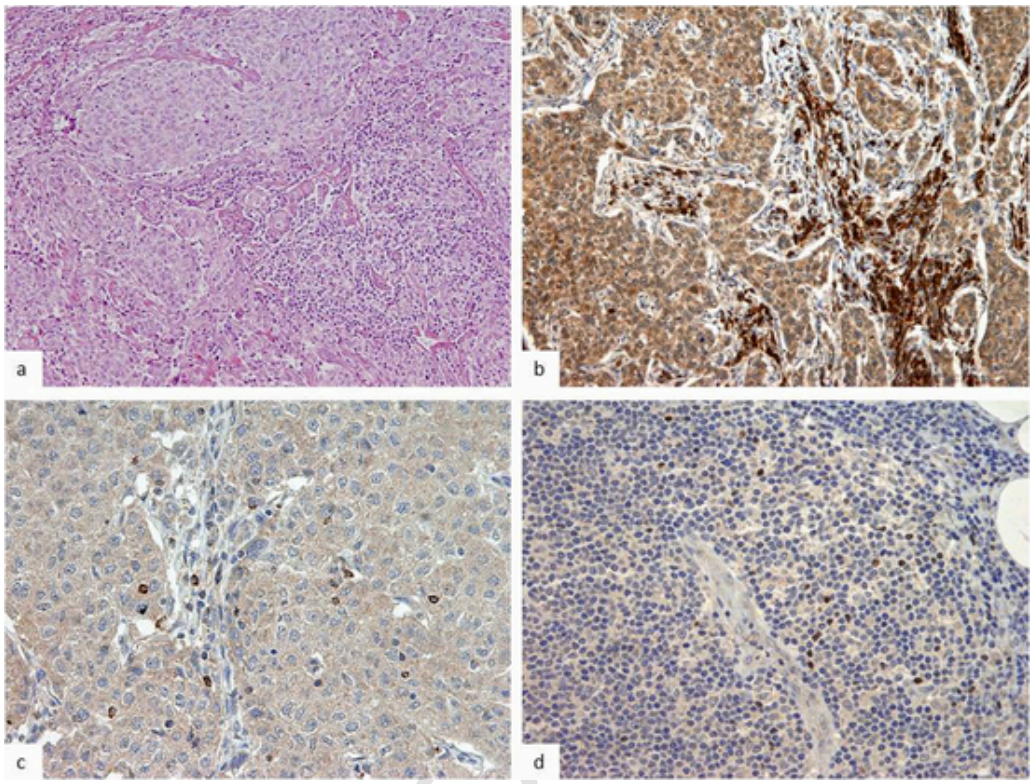

Fig. 1. Immunohistochemical stainings of TILs and TILs components that positively correlated and were most strongly expressed in the area of central part of TNBC: a) high percentage of TILs, b) strong cytoplasmic staining of HSP70 in stromal immune cells and strong cytoplasmic staining of tumor cells, c) cytoplasmic staining of CTLA4 positive single T lymphocytes scattered over the tumor area, d) nuclear staining of FOXP3 positive single T lymphocytes scattered over the tumor area. Magnification 200x.

Table 4
Association of HSP70 in tumor center with the clinicopathological prognostic factors.

	HSP70 low*	HSP70 high*	P (X^2)
Pathological status (pT)			
1	18	4	
2	17	17	0.76
3	2	3	
4	4	1	
Lymph node status (pN)			
0	34	9	**>0,001**
1-3	6	16	
Histological grade			
1,2	23	12	**0.05**
3	13	18	
Pathological prognostic stage			
1	3	17	
2	10	16	**0,013**
3	12	8	
Anatomical stage			
1	3	16	
2	12	18	**0,027**
3	10	7	

* ROC analysis cut-off value 16 %.

4. Discussion

As expected, higher expression of all examined immunomarkers and the HSP70 protein was found in tumor tissue compared to benign breast changes [29–33].

It is considered that molecular interactions between tumor cells, extracellular matrix components and stromal immune cells are reflected predominantly by the changes in the ITF [26,34]. However, the present analysis also refers to changes in the TC and the changes in the metastatic tissue from the draining lymph nodes. A divergent spatial arrangement of TILs with higher values of CD8+ T cells at ITF but CD4+ T cell predominance at the TC has already been described in the literature [34–37].

Although the absolute value of TILs, as well as both CD8+ and CD4+ T cell proportions and the expression of PDL-1, were statistically significantly higher in our ITF specimens, as compared to TC, the expression of HSP70(+)T ly, CTLA4 and FOXP3 were not different between these two layers.

HSP70 is expressed in over 80 % of breast cancer [21,22] and is associated with high-grade tumors, axillary nodal involvement, invasion and dissemination of cancer cells in animal and in vitro models, as well as with poor prognosis and worse patient survival [22,23,38–40]. As a chaperone, it is induced by oncogenic signaling and may suppress senescence and apoptosis, enabling the division of damaged tumor cells and contributing to tumor progression [41,42]. Although we have noticed a strong tumor expression of the HSP70 protein in almost all TNBC cases, in this paper we have remained focused on the significance of its expression in the immune cells. In our previous work [43] we have associated the HSP70(+) IC TC and immunosuppressive tumor microenvironment. In addition to its chaperone function, HSP70 may modulate the immune response [18–20,44].

The proinflammatory role of HSP70 is reported for both innate and acquired immune responses. As an intracellular chaperone molecule HSP70 is involved in antigen processing and presentation leading to antigen-specific T cell response. Extracellular and membrane-bound may represent a danger signal that can activate the innate immune cells by direct bounding to its scavenger receptors leading to inflammation and cytotoxic killing of targeted cells [20,41,42].

However, activation of the anti-inflammatory pathway by extracellular HSP70 is reported as well [24,44]. HSP70 may activate the immature DCs, leading to high IL10 production and specific Tregs prolifera-

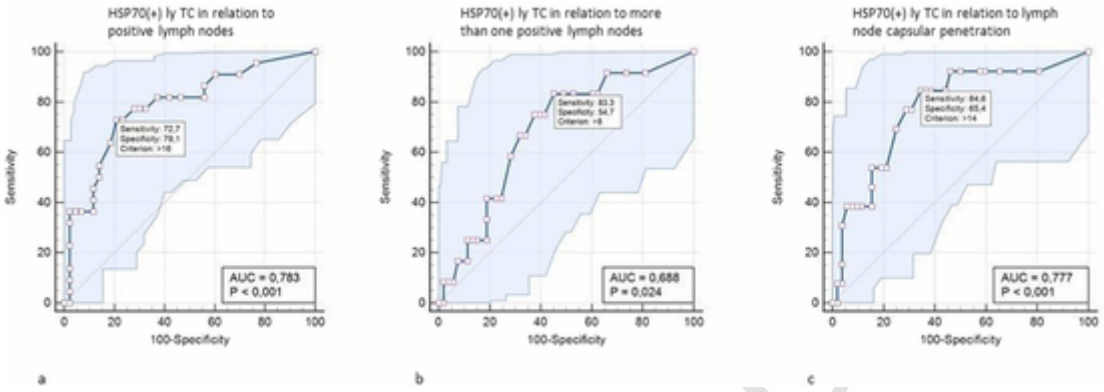

Fig. 2. ROC analysis of HSP70(+) immune cells in central part of tumor(HSP70(+) IC TC) in the assessment of regional lymph nodes and capsule penetration. a) A relation of HSP70(+) IC TC and positive axilla, at a cut off value >16 (AUC = 0.78, P < 0.001). b) A relation of HSP70(+) IC TC and more than one affected axillary lymph node, at a cut off value >8 (AUC = 0.68, P = 0.024). c) A relation of HSP70(+) IC TC and lymph node capsule penetration, at a cut off value >14 (AUC = 0.78, P < 0.001).

tion, both in drainage lymph nodes and in TME, leading to the termination of the specific antitumor immune response. In addition, the increased number of Tregs in the draining lymph nodes may further support immune surveillance and regional tumor spread .

Herein, we have presented positive correlations of HSP70(+) IC TC with the expression of CTLA-4, as well as with the expression of FOXP3+ Tregs, both implying a suppressive role of HSP70 in the context of immuno-oncology. Moreover, the strongest statistical correlation was observed between HSP70(+) IC TC and the expression of FOXP3+Tregs in the metastatic tissue from the draining lymph nodes, further supporting the above-mentioned hypothesis.

Although HSP70(+) IC were equally distributed in both ITF and TC, the statistically significant correlation with TILs density was found only in a deep tumor layer, as well as the association of HSP70(+) IC TC with the adverse clinical and pathological biomarkers; higher anatomical and prognostic stage of the disease, higher tumor grade and higher nodal status.

The strength of the latest association was confirmed with ROC analysis, highlighting the prognostic value of HSP70(+) IC TC. Routine evaluation of HSP expression in immune cells of tumor center may improve the clinical decision-making process concerning axillary surgery in TNBC patients.

With this study design, we were unable to determine whether the chaperone or immunosuppressive chaperokine role of HSP70 is a dominant mechanism underlying the observed association between immune cell HSP70 overexpression and nodal metastasis.

5. Conclusion

Herein, we have presented a comprehensive IHC analysis of HSP70 and major TILs components in a superficial and deep tumour layer of TNBC. Although tissue samples were obtained from patients well balanced through all stages of the disease the major flaw of the study is its relatively small sample size.

Correlations between HSP70 immune cell expression and individual TILs components support the hypothesis of its active role in inducing immunosuppression and tumour progression. Although a different study design is required for a better insight into the mechanism underlying the observed correlation between HSP70 IC and the tumor progression, routine determination of HSP70(+)IC (TC) may be of added value in the clinical decision-making process considering axillary surgery. Possible predictive value of HSP70 immune cell expression will be explored in a new neoadjuvant study.

Ethics approval and consent to participate

The study protocol was approved by The Ethics committee at the Clinical Hospital Center Rijeka, Croatia (2170-29-02/1-19-2). The study was performed in accordance with the Declaration of Helsinki.

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CRediT authorship contribution statement

Ana Car Peterko : Conceptualization, Data curation, Writing – original draft. **Koraljka Rajković-Molek :** Validation, Visualization, Writing – review & editing. **Tamara Gulić :** Investigation, Methodology. **Danijela Veljković Vujaklija :** Investigation, Methodology. **Ingrid Belac Lovasić :** Supervision, Writing – review & editing. **Franjo Lovasić :** Supervision. **Elvira Mustać :** Investigation, Methodology. **Manuela Avirović :** Conceptualization, Data curation, Formal analysis, Writing – original draft.

Conflict of interest

The authors declare no competing interests.

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