

**Expression Patterns of Galectins-1, -3, and -7 ~~Are~~ are Prognostic Markers for Overall Survival ~~of~~ in Ovarian Cancer Patients**

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**Comment [A1]:** Please note my changes to the title, and, if these changes are acceptable, be sure to use the revised title in all relevant instances.

Please be sure to provide author names, affiliations, and contact information according to the journal's guidelines (the corresponding author should be identified with an asterisk).

Finally, please make sure you go through the Scientific Editing Report and incorporate the recommendations.

**Abstract:** There is a ~~tremendous~~ considerable need for the development of new useful prognostic factors in ovarian cancer. Galectins are ~~a family of~~ carbohydrate-binding proteins ~~which that~~ have been suggested to serve as prognostic factors for various cancer types. In this study, the ~~presence~~ expression of ~~g~~Galectin (Gal)-1, -3, and -7 was investigated in 156 ovarian cancer specimens ~~by using~~ immunohistochemical staining. ~~Staining was evaluated in the cytoplasm and nucleus of cancer cells as well as the peritumoral stroma using a semi quantitative score (Remmele (IR) score).~~ Patients' ~~Overall~~ patient survival was compared ~~between among different groups of stratified by~~ Galactin expression. ~~Galectin (Gal)-1 and -3~~ staining was observed in the peritumoural stroma as well as the nucleus and cytoplasm of tumour cells, while Gal-7 was only present in the cytoplasm ~~of tumor cells~~. Patients with Gal-1 expression in the cytoplasm or high Gal-1 expression in the peritumoural stroma showed reduced overall survival. Nuclear Gal-3 staining correlated with ~~a better~~ clinical outcomes. ~~We observed a significantly reduced overall survival for e~~Cases with high Gal-7 expression ~~exhibited significantly reduced overall survival, while and a better survival for Gal-7-~~ negative cases ~~exhibited improved survival, when compared to cases with low expression of Gal-7. We were able to show that b~~Our results indicate that ~~oth~~ tumour and stromal staining of Gal-1 ~~and cytoplasmic staining of Gal-7 could~~ serve as negative prognostic factors for ovarian cancer, ~~while nuclear~~. ~~We were able to confirm cytoplasmic Gal-7 as a negative prognostic factor.~~ Gal-3 staining ~~in the nucleus could may represent~~ be a new positive prognosticator for ovarian cancer. ~~These findings suggest that galectins may represent promising new targets for ovarian cancer treatment.~~

**Comment [A2]:** I have edited this section to ensure that it complies with the journal's limit of 200 words. Specifically, less relevant information has been removed and the presentation of the results has been worded more concisely. A good abstract also concludes with 1-2 sentences describing the broader implications of the results. I have therefore added a sentence serving this purpose.

Text justification has been turned off, as requested by the journal.

**Comment [A3]:** As the term "immunoreactive" is not used elsewhere in the abstract, the abbreviation "IR" is not needed and has been removed.

**Comment [A4]:** While protein symbols are capitalized, protein names are only capitalized if they appear at the beginning of a sentence.

**Comment [A5]:** I have provided a concluding statement for your Abstract here.

**Comment [A6]:** Please note that keywords are not requested in the manuscript file by the journal.

**Keywords:**

Galectin 1; Galectin 3; Galectin 7; ovarian cancer; overall survival

## 1. Introduction

Ovarian cancer is the most lethal gynecological malignancy, ranking fifth in estimated cancer deaths among women in the USA<sup>1</sup>[1]. First-line treatment consists of primary debulking surgery followed by platinum and paclitaxel chemotherapy<sup>2</sup>[2]. ~~Still~~Despite these treatments, the 5-year relative survival rate for epithelial ovarian cancer patients ~~is~~remains belowless than 50%<sup>3</sup>[3]. A lack of screening methods and the frequent presentation with advanced stage disease are considered as the main reasons for the poor outcomes of ovarian cancer patients.

Prognosticators in ovarian cancer include ~~D~~disease stage at diagnosis, extent of residual disease after surgery, histological subtype, and a ~~high~~the volume of ascites<sup>4</sup>~~can be used as prognosticators in ovarian cancer~~ [4]. Numerous studies have aimed to ~~introduce~~identify new biological prognostic factors in ovarian cancer. Recently, the carbohydrate stem cell marker TF1 has been proposed as a negative prognostic marker in ovarian cancer displaying wild-type p53, while estrogen receptor promoter methylation ~~could~~predicts overall survival in low-grade ovarian carcinoma patients<sup>5,6</sup> [5,6]. Although ~~for these and various other molecules~~ the prognostic value independently of clinical parameters has been ~~proven~~demonstrated for these and various other molecules, ~~until today to date~~, with the exception of ~~for~~ breast cancer gene (BRCA)-status, no biological marker is commonly accepted<sup>4</sup>[4]. Further specification of anti-cancer therapies ~~necessitates~~requires an improvement of in the biological prognostic markers in for ovarian cancer.

Galectins ~~have been defined as~~belong to a family of proteins sharing two main characteristics: a binding affinity for  $\beta$ -galactosides and a significant similarity in the carbohydrate-recognition domain (CRD)<sup>7</sup>[7]. The first member of this family to be described was gGalectin (Gal)-1, which ~~is~~can be isolated as a homodimers ~~composed of~~comprising two identical CRD subunits<sup>8</sup>[8]. Since then, a growing number of the gGalectin family members ~~have~~had a growing number of members been identified, but only Galectin (Gal)-1–4, Gal-7–10, Gal-12, and Gal-13 are known to be present in humans<sup>9</sup>[9]. Similar to Gal-1, Gal-7 typically occurs in as a homodimers, while Gal-3 is the only gGalectin characterized as a chimeric protein that is known to form higher order oligomers<sup>10,11</sup> [10,11]. In several types of cancer types, gGalectins are known to affect tumor growth, metastasis, angiogenesis, cell migration, as well as tumor invasiveness, and they are therefore very likely good candidates for proteins with to show a prognostic value for patients' survival<sup>9,12</sup> [9,12].

The role of GalectinGal-1 in cancer has been studied by various groups, ~~and several papers already exist on this topic~~. For In patients' sera and ovarian cancer tissues, it has been shown that a combination of CA-125 and Galectin-1 serves as a possible two-marker combination for the preoperative discrimination of benign and malignant ovarian masses [13]<sup>13</sup>. AlsoIn addition, patients suffering from metastatic epithelial ovarian cancer were observed to show exhibit higher serum Gal-1 levels than those with non-metastatic typecancer. Elevated Gal-1 staining of the peritumoral stroma ~~staining of Gal-1~~ was shown

**Comment [A7]:** Per the guidelines of the target journal, the word count of the manuscript, barring the Abstract, Methods, and reference adheres to the requirement of 4500 words.

**Comment [A8]:** Section numbers are not used in this journal and have been removed.

**Comment [A9]:** In-text citations have been formatted per the journal's guidelines.

**Comment [A10]:** This is implied by the beginning of the sentence and has been removed to reduce redundancy.

to occur in advanced stages of epithelial ovarian cancer and is also ~~connected-associated~~ with ~~poorer-reduced~~ progression-free survival in univariate analysis<sup>14</sup> ~~[14]~~. However, these results have not yet been reproduced for overall survival or confirmed by multivariate analysis<sup>15</sup> ~~[15]~~. ~~Due to this~~ Thus, the ~~possibility-potential~~ of Gal-1 as an independent prognostic marker in ovarian cancer ~~still needs to be~~ requires further investigation<sup>ed</sup>.

High cytoplasmic Galeetin-3 expression has been suggested as a negative prognostic factor, as it was shown to correlate with ~~shorter-reduced~~ progression-free survival in ovarian cancer<sup>16</sup> ~~[16]~~. However, in another study, Gal-3 expression did not correlate ~~to-with~~ reduced overall survival, ~~but-though~~ a cytoplasmic staining pattern was associated with poor outcome when compared to patterns including nuclear staining<sup>17</sup> ~~[17]~~. Although Gal-3 staining ~~has been observed in the nucleus and stroma~~ has been observed, ~~their-its~~ influence on overall survival ~~still maintains~~ remains elusive<sup>unclear</sup>.

Finally, Galeetin-7 has been proposed ~~by two independent groups~~ to serve as ~~a~~ negative prognostic factor in ovarian cancer ~~by two independent groups~~. In both studies, its influence on progression-free survival and overall survival ~~has been~~ was confirmed by univariate and multivariate analysis<sup>16,18</sup> ~~[16,18]~~. ~~Yet~~ However, there is further disagreement ~~remains regarding~~ whether Gal-7 staining occurs predominantly in the nucleus or the cytoplasm. ~~In addition~~ Also, it ~~remains-is currently~~ unknown ~~if-whether~~ there is a correlation ~~between-the~~ expressions of different gGalectins ~~are correlated~~ in ovarian cancer, and there is a ~~critical desperate~~ need for ~~a~~ comprehensive studies of various gGalectins ~~on-in~~ a representative ovarian cancer panel. Therefore, in this study, we investigated the prognostic ~~influence-value~~ of Gal-1, -3, and -7 in patients with epithelial ovarian cancer and analy<sup>zed</sup> correlations ~~to each other~~ among the expression patterns of the three proteins ~~and-as well as to-with~~ clinical and pathological parameters. ~~We hypothesize~~ Our results suggest that Gal-1, -3, and -7 are ~~localization-dependent~~ prognostic ~~factors~~ for overall survival in ovarian cancer patients, ~~dependent of the localization of the Galeectin expression~~.

## 2. Results

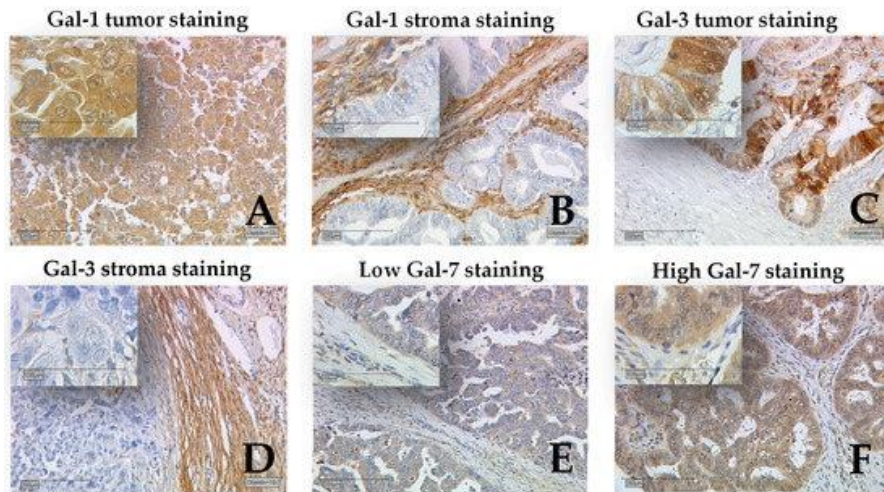
### 2.1. Gal-1 (Tumour and stromal) staining is a Negative Prognostic indicator of for Overall Survival

Galeetin-1 staining was ~~successfully performed on~~ conducted in 150 ovarian cancer specimens. Gal-1 was present in the cytoplasm<sup>s</sup> and the nuclei of ovarian cancer cells, as well as ~~in~~ the peritumoural stromae (Figure 1 ~~Fig. 1~~). In 102 cases (68.0%), the ~~eytoplasm~~s of tumour cell ~~cytoplasm~~s ~~were-was~~ positive for Gal-1, with a median Remmele immunoreactive (IR) score ~~(IRS)~~ of 3. The ~~P~~peritumoural stroma was positive for Gal-1 in 148 cases (98.0%), with a median IR ~~score~~s of 8. Gal-1 expression ~~was~~ significantly correlated with several clinical and pathological ~~data-factors~~ (Table 1 ~~Table 1~~).

**Comment [A11]:** The Introduction does not provide a sufficient background of the problem studied. Thus, the biological functions of galectins related to tumorigenesis, including malignant transformation, invasion, and metastasis, are not described, and it is unclear how galectins are involved in all these processes. I have provided recommendations in this regard in the *Scientific Editing Report*. These points should be discussed in the manuscript.

**Comment [A12]:** The results section requires greater re-organization, because the presentation of the data does not correspond to their placement in your illustrations. I have elaborated on this in my assessment of your manuscript.

**Comment [A13]:** The journal specifies that the abbreviation "Fig." should be used except for at the beginning of sentences, where "Figure" should be used.



**Figure 1.** Detection of ~~g~~Galectins by immunohistochemistry. Representative photomicrographs are shown. ~~Galeetin~~(Gal)-1 was present in the cytoplasm and the nucleus of ovarian cancer cells (A) as well as the peritumoral stroma (B). Gal-3 staining was observed in the nucleus, cytoplasm (C), and stroma (D). Staining for Galeetin-7 was mainly observed in the cytoplasm (E), with only a few individual cases showing nuclear staining (F). ~~10× magnification was used for the outer pictures and 50× magnification for the inserts. The s~~Scale bars, ~~in in the outer pictures equal 200 μm (10× magnification) in main images, and the scale bars in the inserts equal 100 μm (50× magnification) in insets.~~

**Comment [A14]:** This is stated again in the next sentence and has therefore been removed to reduce redundancy.

**Table 1.** Correlations between Gal-1 staining ~~correlated with~~and clinical and pathological ~~data~~factors.

**Comment [A15]:** Since the figures and tables were not included in your editing request, they have not been edited. However, note that the journal states that tables should be submitted as editable text, rather than as images.

Clinical and Pathological Variables	Gal-1 Expression Cytoplasm		P	Gal-1 Expression Stroma		P	Gal-1 Expression Nucleus		P
	negative	positive		low	high		negative	positive	
Histology									
Serous	26	79	0.008	34	71	NS	27	78	0.002
Clear cell	5	7		6	6		3	9	
Endometrioid	8	12		7	13		11	9	
Mucinous	9	4		3	10		9	4	
Tumor Stage									
pT1	22	17	<0.001	20	19	0.006	19	20	0.020
pT2+	26	84		30	80		31	79	
Lymph node									
pN0/pNX	36	65	NS	34	67	NS	43	58	0.001
pN1	12	37		16	33		7	42	
Distant Metastasis									
pM0/pMX	47	97	NS	49	95	NS	49	95	NS
pM1	1	5		1	5		1	5	
Grading									
G1	20	16	<0.001	13	23	NS	14	22	NS
G2+	22	80		31	71		31	71	
FIGO									
I/II	22	21	0.001	17	26	NS	21	22	0.013
III/IV	24	78		31	71		28	74	
Age									
≤60 years	27	52	NS	28	51	NS	24	55	NS
>60 years	21	50		22	49		26	45	

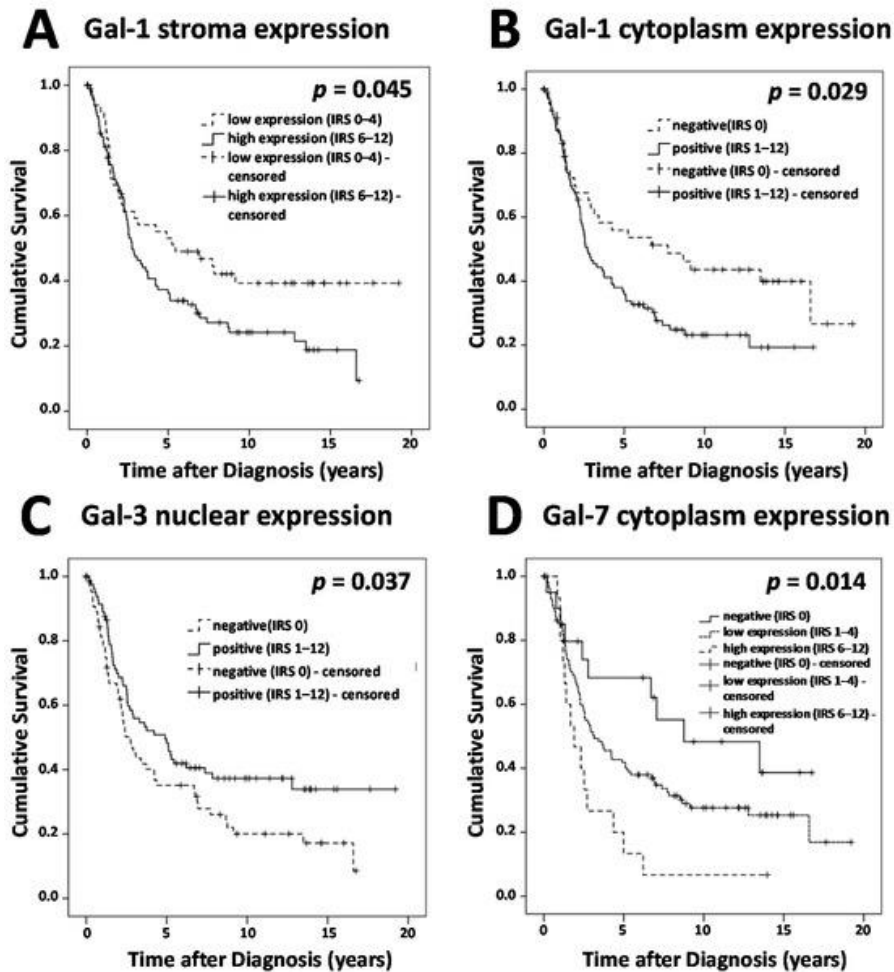
TNM staging was ~~accomplished-performed~~ according to ~~the actual~~ standards of ~~the~~ Union for International Cancer Control (UICC); pT1 = tumor stage 1; pT2+ = tumor stage 2 or higher; pN0 = lymph node stage 0; pNX = lymph node stage not evaluated; pN1 = lymph node stage 1; pM0 = distant metastasis stage 0; pMX = distant metastasis not evaluated; pM1 = distant metastasis stage 1; G1 = grade 1; G2+ = grade 2 or higher; FIGO = Fédération Internationale de Gynécologie et d'Obstétrique; NS = Not significant ( $p > 0.05$ )

Gal-1 staining in ~~the~~ cytoplasm and nucleus ~~showed-differed~~~~nces for-among~~ several histological subtypes ( $p = 0.008$  ~~and~~  $p = 0.002$ , respectively). Cytoplasmic Gal-1 staining was significantly stronger in serous, clear cell, or endometrioid subtypes, while for ~~the~~ mucinous subtype, we ~~found-observed~~ more negative cases. ~~Also~~~~In addition~~, more cases ~~showed-Gal-1-positive-nuclei-forwith~~ serous and clear cell subtypes ~~exhibited-Gal-1-positive-nuclei~~, while ~~the~~ endometrioid and mucinous subtypes ~~had-exhibited~~ weaker nuclear Gal-1 stainings. Furthermore, Gal-1 staining in ~~the~~ nucleus, cytoplasm, and stroma ~~were-was~~ significantly higher in cases with advanced tumor stage ( $p < 0.001$ ,  $p = 0.006$ , ~~and~~  $p = 0.02$ , respectively). Gal-1 expression in the cytoplasm was significantly higher in cases with higher grading ( $p < 0.001$ ) and advanced FIGO (Fédération Internationale de Gynécologie et d'Obstétrique) stage ( $p = 0.001$ ). ~~The IR scores of nuclear Gal-1 staining in the nucleus showed-were~~ higher ~~IR scores~~ in lymph node-positive cases ( $p = 0.001$ ) and ~~eases-those~~ with advanced FIGO stage ( $p = 0.013$ ).

~~The~~ Survival times of ~~different~~ groups ~~characterized by their~~ Gal-1 expression in ~~the~~ nucleus, cytoplasm, and stroma ~~have-been-were~~ compared (Figure 2~~Fig. 2~~). Cases with Gal-1 expression in the cytoplasm showed significantly reduced overall survival compared to cases without any Gal-1 expression in the cytoplasm ( $p = 0.029$ ). Moreover, cases displaying high Gal-1 expression in the stroma showed ~~a-significantly-reduced-poorer~~ outcomes ~~compared-to-eases~~~~than those~~ with low Gal-1 expression in the stroma ( $p = 0.045$ ). ~~A~~ ~~C-comparison of~~



cases negative versus and positive cases for Gal-1 expression in the nucleus did not show reveal any differences with regard in terms of overall survival. However, based on considering a multivariate analysis, only Gal-1 stroma staining would serve as an independent prognostic factor (Table 2 Table 2).



**Figure 2.** Survival times were plotted as Kaplan-Meier graphs. Percentage of living patients (vertical axis) was plotted in dependence of against time (horizontal axis). Patients without an observed event (death) who exited the study before the observation period ended have been censored, as indicated. Censoring has been marked in the graphs. Survival times of different groups of stratified by Galectin expression have been are compared. Galectin expression was

determined in the cytoplasm, nucleus, and stroma using Remmele immunoreactive (IR) scores. (A) Cases displaying high Gal-1 expression in the stroma showed a significantly reduced outcome survival compared to cases with low Gal-1 expression in the stroma. (B) (A) Cases with Gal-1 expression in the cytoplasm showed significantly reduced overall survival compared to cases without any Gal-1 expression in the cytoplasm. (C) (B) Cases without Gal-3 expression in the nucleus showed significantly reduced overall survival compared to cases with nuclear Gal-3 expression. (D) (C) Cases with high Gal-7 expression showed a significantly reduced overall survival and Gal-7-negative cases showed better overall survival when compared to cases with low expression of Gal-7. (D) Galactin expression was determined in cytoplasm, nucleus, and stroma using Remmele (IR) scores.

**Table 2.** Multivariate analysis of prognostic factors for overall survival in ovarian cancer.

Covariate	Coefficient (b <sub>1</sub> )	HR Exp (b <sub>1</sub> )	95% CI		p-Value
			Lower	Upper	
Histology (serous vs. other)	0.211	1.235	0.658	2.317	0.511
Grade (G1 vs. G2, G3)	0.942	2.565	1.290	5.100	0.007
FIGO (I, II vs. III, IV)	1.140	3.126	1.537	6.357	0.002
Patients' age (≤60 vs. >60 years)	0.312	1.367	0.861	2.169	0.185
Gal-1 stroma (low vs. high)	0.571	1.770	1.044	2.999	0.034
Gal-1 cytoplasm (neg. vs. pos.)	-0.187	0.830	0.423	1.626	0.586
Gal-3 nucleus (neg. vs. pos.)	-0.265	0.767	0.480	1.227	0.269
Gal-7 cytoplasm (neg. vs. pos.)	0.636	1.889	1.160	3.077	0.011

HR = hazard ratio; CI = confidence interval

**Comment [A16]:** Please verify that this edit maintains your intended meaning.

## 2.2- Presence of Gal-3 in the Nucleus is a Positive Prognostic Indicator in Ovarian Cancer

Gal-3-positive nuclei were observed in 83 (55%) out of 151 cases, while 96 cases (63.6%) showed cytoplasmic Gal-3 staining and 85 cases (56.3%) presented with Gal-3-positive peritumoral stroma (Figure 1). Median IR scores for Gal-3 in the nucleus, cytoplasm, and stroma were 1, 2, and 1, respectively. Gal-3 staining showed correlations with clinical and pathological data variables (Table 3). Gal-3 expression in the stroma and nucleus was differed amongst for several different histological subtypes ( $p = 0.008$  and  $p = 0.013$ , respectively). Gal-3 stroma staining was stronger in the serous and clear cell subtypes but weaker in the endometrioid and mucinous subtypes, while nuclear Gal-3 staining was stronger in the serous, clear cell, and mucinous subtypes but weaker in the endometrioid subtype. Tumors rated as pT1 presented with significantly stronger nuclear Gal-3 staining than those rated pT2 or higher staged cases ( $p = 0.042$ ). We observed a correlations of between Gal-3 staining in the nucleus and cytoplasm with patients' age ( $p = 0.022$  and  $p = 0.013$ , respectively), with observing higher IR scores for patients younger than 60 years. For



In our study panel, Gal-3 overexpression in the cytoplasm was not correlated with poorer outcomes ~~of in~~ ovarian cancer patients. ~~Also Similarly~~, Gal-3 staining in the peritumoural stroma ~~could not serve as~~ was not observed to be as a prognostic factor. ~~However~~ In contrast, nuclear Gal-3 expression could serve as a positive prognostic factor (Figure 2 Fig. 2). Cases without Gal-3 expression in the nucleus showed significantly reduced overall survival compared to cases with nuclear Gal-3 expression ( $p = 0.034$ ). According to the results of a multivariate analysis, ~~however~~, nuclear Gal-3 staining ~~could not serve as~~ was not an independent prognostic factor, probably due to its strong correlations with patients' age, tumour stage, and histology (Table 2 Table 2).

**Table 3.** Correlations between Gal-3 staining ~~correlated with and~~ clinical and pathological data factors.

Clinical and Pathological Variables	Gal-3 Expression Cytoplasm		P	Gal-3 Expression Stroma		P	Gal-3 Expression Nucleus		P
	neg.	pos.		neg.	pos.		neg.	pos.	
Histology									
Serous	37	69	NS	42	64	0.008	44	62	0.013
Clear cell	3	9		2	10		3	9	
Endometrioid	12	9		13	8		16	5	
Mucinous	3	9		9	3		5	7	
Tumor Stage									
pT1	12	27	NS	21	18	NS	12	27	0.042
pT2+	43	68		44	67		55	56	
Lymph node									
pN0/pNX	39	62	NS	47	54	NS	48	53	NS
pN1	16	34		19	31		20	30	
Distant Metastasis									
pM0/pMX	53	92	NS	64	81	NS	65	80	NS
pM1	2	4		2	4		3	3	
Grading									
G1	9	28	NS	16	21	NS	13	24	NS
G2+	40	62		44	58		51	51	
FIGO									
I/II	13	30	NS	21	22	NS	15	28	NS
III/IV	41	62		43	60		51	52	
Age									
≤60 years	22	57	0.022	33	46	NS	28	51	0.013
>60 years	33	39		33	39		40	32	

TNM staging was ~~accomplished-performed~~ according to ~~actual the~~ standards of the UICC; pT1 = tumour stage 1; pT2+ = tumour stage 2 or higher; pN0 = lymph node stage 0; pNX = lymph node stage not evaluated; pN1 = lymph node stage 1; pM0 = distant metastasis stage 0; pMX = distant metastasis not evaluated; pM1 = distant metastasis stage 1; G1 = grade 1; G2+ = grade 2 or higher; NS = Not significant ( $p > 0.05$ ).

### ~~2.3. Gal-7 eExpression Levels pPredicts Shortened Overall sSurvival in oOvarian cCancer~~

Staining for Gale~~ein~~-7 was mainly observed in the cytoplasm; only a few individual cases showed nuclear staining (Figure 1 Fig. 1). Cytoplasmic Gal-7 staining was present in 129 (86.6%) out of 149 specimens, with a median IR score of 3. In total, 20 cases ~~presented-were~~ negative for Gal-7, while 114 cases showed low and 15 cases showed high expression of Gal-7. Gal-7 expression appeared to ~~show-differ~~ among~~ences for several different~~ histological subtypes ( $p = 0.026$ ). The strongest Gal-7 staining was found in the serous subtype, and the weakest ~~was in the~~ endometrioid subtype (Table 4 Table 4). No other correlations ~~of-between~~

Gal-7 staining and with pathological data was were found. Survival times of Gal-7-negative cases and ~~eases those displaying a~~ with high Gal-7 expression were compared to ~~eases those~~ with low Gal-7 expression (Figure 2Fig. 2). We observed a-significantly reduced overall survival for cases with high Gal-7 expression and a-betterimproved survival for Gal-7-negative cases, ~~when~~ compared to ~~that of~~ cases with low expression of Gal-7 ( $p = 0.014$ ). ~~Also~~In addition, according to the results of a-multivariate analysis, ~~higher~~Gal-7 expression can be confirmed as an independent prognostic factor for overall survival in ovarian cancer (Table 2Table 2).

**Table 4.** ~~Correlations between~~ Gal-7 staining ~~correlated withand~~ clinical and pathological ~~datafactors~~.

Clinical and Pathological Variables	Gal-7 Expression Cytoplasm			<i>p</i>
	neg.	low	high	
Histology				
Serous	10	83	12	0.026
Clear cell	0	10	2	
Endometrioid	7	13	0	
Mucinous	3	8	1	
Tumor Stage				
pT1	4	29	5	NS
pT2+	15	85	10	
Lymph node				
pN0/pNX	15	75	8	NS
pN1	5	39	7	
Distant Metastasis				
pM0/pMX	19	110	14	NS
pM1	1	4	1	
Grading				
G1	6	25	3	NS
G2+	12	80	11	
FIGO				
I/II	8	29	4	NS
III/IV	11	81	11	
Age				
≤60 years	12	59	8	NS
>60 years	8	55	7	

TNM staging was ~~accomplished-performed~~ according to ~~actual the~~ standards of ~~the~~ UICC; pT1 = tumour stage 1; pT2+ = tumour stage 2 or higher; pN0 = lymph node stage 0; pNX = lymph node stage not evaluated; pN1 = lymph node stage 1; pM0 = distant metastasis stage 0; pMX = distant metastasis not evaluated; pM1 = distant metastasis stage 1; G1 = grade 1; G2+ = grade 2 or higher; NS = Not significant ( $p > 0.05$ ).

#### ~~2.4. Correlations among galectin expression patterns~~Analysis

**Comment [A17]:** This title makes the subject of this section more clear.

Results of the Analysis of the correlations among galectin expression patterns analysis is shown in Table 5. For Gal-1 staining, we observed positive correlations between among staining results in the cytoplasm, nucleus, and stroma. Also Similarly, the staining results of Gal-3 in the cytoplasm, nucleus, and stroma were positively correlated among with each other. Furthermore, we found-observed correlations between Galectin-1 and -3 staining in the nucleus, cytoplasm, and stroma. Gal-7 staining showed was positively correlated with Gal-1 staining in the cytoplasm and nucleus and all types of Gal-3 staining.

**Table 5.** Correlation analysis of galectin expression patterns.

Staining	Gal-1 Cytoplasm	Gal-1 Stroma	Gal-1 Nucleus	Gal-3 Cytoplasm	Gal-3 Stroma	Gal-3 Nucleus	Gal-7 Cytoplasm
Gal-1 cytoplasm							
cc	1.000	0.382	0.748	0.356	0.263	0.282	0.272
p	.	<0.001	<0.001	<0.001	0.001	<0.001	0.001
n	150	150	150	149	149	149	146
Gal-1 stroma							
cc	0.382	1.000	0.231	0.123	0.280	-0.006	-0.040
p	<0.001	.	0.004	0.135	0.001	0.937	0.633
n	150	150	150	149	149	149	146
Gal-1 nucleus							
cc	0.748	0.231	1.000	0.302	0.315	0.329	0.249
p	<0.001	0.004	.	<0.001	<0.001	<0.001	0.002
n	150	150	150	149	149	149	146
Gal-3 cytoplasm							
cc	0.356	0.123	0.302	1.000	0.293	0.839	0.276
p	<0.001	0.135	<0.001	.	<0.001	<0.001	0.001
n	149	149	149	151	151	151	146
Gal-3 stroma							
cc	0.263	0.280	0.315	0.293	1.000	0.267	0.231
p	0.001	0.001	<0.001	<0.001	.	0.001	0.005
n	149	149	149	151	151	151	146
Gal-3 nucleus							
cc	0.282	-0.006	0.329	0.839	0.267	1.000	0.335
p	<0.001	0.937	<0.001	<0.001	0.001	.	<0.001
n	149	149	149	151	151	151	146
Gal-7 cytoplasm							
cc	0.272	-0.040	0.249	0.276	0.231	0.335	1.000
p	0.001	0.633	0.002	0.001	0.005	<0.001	.
n	146	146	146	146	146	146	149

Correlations among IR scores of Gal-1, -3, and -7 staining in different compartments were correlated-assessed with each other using Spearman's correlation analysis. cc = correlation coefficient, p = two-tailed significance, n = number of patients.

### 3. Discussion

In this study, we assessed the prognostic value of Gal-1, -3, and -7 expression on overall survival in ovarian cancer patients. According to our data, Gal-1 staining in the cytoplasm and stroma predicts poor survival. Consistent with this, *in vitro* experiments have shown that the overexpression of Galectin-1 significantly increases migration and invasion behaviours in ovarian cancer cells<sup>19</sup> [19]. Furthermore, Gal-1 knockdown experiments in ovarian cancer cells displayed a reduction in cell growth, migration, and invasion. Possible mechanisms for this include the interaction of Gal-1 with H-Ras and to activation of the Raf/extracellular signal-regulated kinase (ERK) pathway, as well as the downregulation of matrix metalloproteinase-9 (MMP-9) and c-Jun could have been explored as possible mechanisms. Moreover, Gal-1 overexpression could may significantly decrease the sensitivity of ovarian cancer cells to cisplatin, illustrating reflecting a possible explanation for the decreased survival of ovarian cancer patients with increased Gal-1 expression<sup>14</sup> [14]. Thus, Gal-1 is represents a promising new target for ovarian cancer therapy. For this purpose, and several compounds targeting Gal-1 have been introduced<sup>20</sup> [20]. OTX008, for instance, is a new compound able to binding non-covalently to Gal-1 on the side back face, was able to inhibiting the proliferation and invasion of various cancer cells lines<sup>21</sup> [21]. The Anti-proliferative effects of OTX008 correlated with Gal-1 expression across a large panel of cell lines. Moreover, OTX008 efficiently inhibited the growth of ovarian cancer xenografts *in vivo*<sup>22</sup> [22].

According to the results of a multivariate analysis in this study, only Gal-1 stromal staining could serves as an independent prognostic factor for overall survival. The Accumulation of Gal-1 in the peritumoural stroma has been described for various other tumour entities<sup>23-25</sup> [23,24,25]. Some groups tried have to investigated the mechanisms responsible for this phenomenon. *In situ* hybridization experiments were able to showed that fibroblast-seells, adjacent to malignant cells, express *GAL-1* mRNA, illustrating suggesting a possible explanation for peritumoural Gal-1 accumulation. Also In addition, it was demonstrated that ovarian cancer cells produce Gal-1 and release it into the medium. Furthermore, conditioned medium obtained from ovarian carcinoma cells is able to induces increased-elevated Gal-1 expression in fibroblast-seells. Both These experiments suggest that primarily the ovarian cancer cells might may be primarily responsible for stromal Gal-1 expression<sup>26</sup> [26]. Our exploration findings regarding of the positive correlation between Gal-1 staining in the peritumoural stroma and malignant cells is consistent with this hypothesis. However, it requires further investigations to are required to explain cases of without Gal-1 expression in the stroma but not in cancer cells, and -but in the stroma or vice versa.

Several groups have suggested that higher Gal-3 expression is associated with reduced progression-free survival in ovarian cancer<sup>17,27</sup> [17,27]. However, in these studies, observation detection of Gal-3 expression was limited to the cytoplasm, while and the prognostic value of nuclear Gal-3 staining has not been further studied. We could not confirm a negative

**Comment [A18]:** I recommend that your Discussion better emphasize the contribution of this study to the prognostic potential of galectins in ovarian cancer. This is something that is sorely lacking in the text.

**Comment [A19]:** A good Discussion section usually begins with a brief summary of the aims or overall results of the study. I have added a sentence describing this here.

**Comment [A20]:** This description is a bit vague. Please be more specific if possible.

**Comment [A21]:** Typically, gene symbols-- and not gene names--are italicized to distinguish them from proteins.

**Comment [A22]:** Please provide in-text citations for the studies mentioned here.

influence of cytoplasmic Gal-3 overexpression on overall survival ~~for in~~ our study panel. On the contrary, nuclear Gal-3 staining served as a positive prognostic factor, although ~~it was~~ not independent of the influence of clinical and pathological parameters. ~~Thus, it is~~ Apparently, ~~it is the~~ nuclear and not cytoplasmic Gal-3 expression that has a major influence on patients' outcomes. In line with this, Gal-3 has been observed to play an important role in nuclear ~~cell~~ physiology, as it is involved in the ~~mechanisms-processes~~ of pre-mRNA ~~splicing or and~~ mRNA transport<sup>28,29</sup> ~~[28,29]~~. Furthermore, cell culture experiments using human cervix adenocarcinoma (HeLa) ~~-cells~~ ~~showed-demonstrated a~~ delayed activation of the DNA damage repair response ~~activation~~ and a decrease in ~~the~~ G2/M cell cycle checkpoint arrest in the absence of Gal-3<sup>30</sup> ~~[30]~~. A similar mechanism ~~could be~~ is conceivable in ovarian cancer, predisposing cells for further mutations in the absence of nuclear Gal-3. To our knowledge, reduced Gal-3 expression as an indicator of poorer prognosis has only been observed in gastric cancer ~~so thus far~~<sup>31</sup> ~~[31]~~. In cholangiocarcinoma, Gal-3 expression ~~was is~~ associated with a poorly ~~-~~differentiated type, while *in vitro* experiments ~~showed~~ significantly increased cell migration and invasion after suppression of Gal-3 expression<sup>32</sup> ~~[32]~~. However, for ovarian cancer, *in vitro* experiments ~~showed~~ have shown that knockdown of Gal-3 inhibits migration and invasion of cancer cells, while increasing apoptosis and sensitivity to carboplatin<sup>33</sup> ~~increases~~ ~~[33]~~. Moreover, paclitaxel and additional treatment with a Gal-3 inhibitor ~~treatment~~ ~~showed~~ resulted in synergistic cytotoxic effects and increased apoptosis in an ~~on~~ ovarian cancer cell line<sup>34</sup> ~~[34]~~. ~~Since there are disagreements~~ Due to the discrepancies in previous research and to the fact that our data ~~is are not neither~~ consistent with either previous studies on progression-free survival ~~nor with recent results of in vitro research~~, further investigation ~~on into~~ the prognostic role of Gal-3 in ovarian cancer is definitely required.

As recently proposed by other groups, we were able to confirm Gal-7 as a negative prognosticator for overall survival in ovarian cancer ~~in according to both~~ uni- and multivariate analyses. ~~Further~~ Cell culture experiments ~~were able to prove~~ have demonstrated that Gal-7 expression is induced by a mutant form of p53. ~~Also~~ In addition, Gal-7 was shown to increase the proliferation<sup>16</sup> ~~[16]~~, invasiveness, and motility of ovarian cancer cells, while interacting as an immunosuppressive ~~antive~~ by killing Jurkat T<sub>h</sub>-cells and human peripheral T<sub>h</sub>-cells<sup>18</sup> ~~[18]~~. ~~All in all~~ Together, these investigations confirm Gal-7 as a ~~new~~ promising new target for specific therapeutic ~~option-treatment of in~~ epithelial ovarian cancer.

We observed ~~various a variety of~~ positive correlations ~~between-among the expression patterns of~~ Gal-1, -3, and -7. This observation, ~~and along with~~ the fact that gGalectins share binding affinities and have exhibit similarities in protein structure, suggests ~~the assumption~~ that Ggalectins might also share common functions in ovarian cancer molecular biology. However, ~~since as these is~~ are is rather descriptive, further investigations ~~into are~~ are required to explore the biological characteristics and functions of different gGalectins are required to determine their ir-manner(s) in which they are similarities ~~and or~~ differences, ~~in~~ specifically in regards to their role(s) in ovarian cancer.

**Comment [A23]:** Please verify that this edit maintains your intended meaning.

## 5. Conclusions

In this study, ~~We were able to~~ we showed that ~~Galectin~~ expression of galectins and ~~their~~ impacts on overall survival ~~of~~ in ovarian cancer patients ~~is~~ are strongly dependent on ~~its~~ their cellular localization, ~~whether it is~~ in the nucleus or cytoplasm of tumour cells or the peritumoural stroma. We ~~elaborated~~ found that Gal-1 tumour and stromal staining, and Gal-7 staining in the cytoplasm serves as a negative prognostic factors for overall survival in ovarian cancer, while nuclear Gal-3 staining ~~could~~ may serve as a positive prognostic factor. According to the results of a multivariate analysis, Gal-1 stromal staining and Gal-7 staining are prognostic factors that are independent of clinical and pathological parameters.

**Comment [A24]:** This section was moved to before the Materials and Methods section to improve readability and flow.

This section could be further enhanced by the addition of a discussion of any limitations of the study, as well as the broader implications of your study. As *Scientific Reports* caters to a broad audience, are there other fields of research that may be impacted by your study?

## 4. Materials and Methods

### 4.1. Patients

Formalin-fixed, paraffin-embedded (FFPE) ovarian cancer samples from 156 female patients who underwent surgery at the Department of Obstetrics and Gynecology, Ludwig-Maximilians-University (LMU) of Munich, Germany between 1990 and 2002 were analyzed in this study. Women diagnosed ~~for with~~ benign or ~~for~~ borderline tumours of the ovary were excluded, and no patient had received neo-adjuvant chemotherapy. Tumour grading [~~G1~~ (n = 38), G2 (n = 53), G3 (n = 53)]~~},~~ and histological characterization [~~serous~~ (n = 110), endometrioid (n = 21), clear cell (n = 12), mucinous (n = 13)]~~},~~ were performed by a gynecological pathologist. Tumour staging was ~~accomplished~~ performed using FIGO classifications [~~I~~ (n = 35), II (n = 10), III (n = 103), IV (n = 3)]~~},~~ TNM classification was performed according to the UICC. Data on the extension of the primary tumour ~~was were~~ available in 155 cases [~~T1~~ (n = 40), T2 (n = 18), T3 (n = 93), T4 (n = 4)]~~},~~ data on lymph node involvement ~~was were~~ available in 95 cases [~~N0~~ (n = 43), N1 (n = 52)]~~},~~ and data on the presence of distant metastasis ~~was were~~ available in 9 cases [~~M0~~ (n = 3), M1 (n = 6)]~~},~~ Clinical data ~~was were~~ retrieved from patients' charts, and follow-up data ~~was were~~ requested from the Munich Cancer Registry. Patients' age at surgery ranged ~~between from~~ 31 ~~and to~~ 88 years, with a median age of  $62 \pm 12$  years. Mean overall survival was  $3.2 \pm 3.0$  years, and 104 deaths were observed in total. The mean follow-up ~~time period~~ was  $5.1 \pm 4.8$  years.

**Comment [A25]:** The number of patients was not justified by power analysis, and it is unclear whether the sample size was sufficient to achieve statistical significance.

There appear to be no control samples, i.e., those from cancer-free individuals.

You need to address these two points prior to submission.

**Comment [A26]:** This heading is recommended by the journal.

### 4.2. Immunohistochemistry

Resected ovarian cancer tissue samples were fixed in formalin and embedded in paraffin after surgery. For histopathological investigations, sections were dewaxed in ~~x~~Xylol for 20 ~~minutes min~~ and immersed in 3% hydrogen peroxide (Merck, Darmstadt, Germany) to quench endogenous peroxidase. Then, slides were rehydrated in a descending series of alcohol (100%, 75%, and 50%), and cooked in a pressure cooker for 5 ~~minutes min~~ in sodium citrate buffer (0.1 mol/L citric acid, 0.1 mol/L sodium citrate, pH 6.0) ~~in a pressure cooker~~ to ensure epitope retrieval. Afterwards, slides were washed in distilled water and phosphate-buffered saline (PBS), followed by a specific procedure for staining each ~~Galectin~~ staining. ~~In particular, for Galectin 1 (Gal-1) staining, slides were blocked using Ppower Bblock~~



(BioGenex, San Ramon, CA, USA) for 3 min at room temperature and incubated with aAnti-Gal-eetin-1 primary antibody (goat, polyclonal; R&D Systems, Minneapolis, MN, USA) at a final concentration of 0.033 µg/mL in Ppower bBlock (BioGenex, San Ramon, CA, USA) for 16 h at 4 °C. Galeectin-3 (Gal-3) staining was performed by blocking specimens with 1.5% horse serum (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature and incubating with aAnti-Galeectin-3 primary antibody (mouse, monoclonal; Novocastra Reagents, Leica Biosystems, Wetzlar, Germany) at a final concentration of 4.6 µg/mL in PBS for 16 h at 4 °C. For Galeectin-7 (Gal-7) staining, specimens were blocked with Blocking Solution [(Reagent 1; ZytoChem Plus HRP Polymer System (Mouse/Rabbit); Zytomed Systems GmbH, Berlin, Germany)] for 5 minutes-min at room temperature. Slides were then incubated with aAnti-Gal-7 (rabbit, polyclonal; Abcam, Cambridge, UK) at a final concentration of 2.5 µg/mL in PBS for 16 h at 4 °C. Afterwards, for Gal-1 and -3 staining, slides were incubated with isotype-matcheding anti-goat/mouse-IgG secondary antibody and avidin-biotin-peroxidase complex, both for 30 min at room temperature, according to the instructions of the ABC Vectastain kit (Vector Laboratories, Burlingame, CA, USA). For Gal-7 staining, specimens were incubated in Ppost-Bblock reagent (Reagent 2; Zytomed Systems GmbH, Berlin, Germany) and HRP-Polymer (Reagent 3; Zytomed Systems GmbH, Berlin, Germany) for 30 min at room temperature, according to the manufacturer's protocol for the ZytoChem Plus HRP Polymer System (Mouse/Rabbit) (Zytomed Systems GmbH). All slides were washed twice in PBS for 2 min after every incubation step. For visualization reaction, every-specimens wereas stained with 3,3'-diaminobenzidine chromogen (DAB; Dako, Glostrup, Denmark). The reaction was; stopped after 30 s-to-2 min with tap water, and specimens were counterstained in Mayer acidic hematoxylin, dehydrated in an ascending series of alcohol followed by xylol, and covered with Consul Mount (Thermo Shandon, Pittsburgh, PA, USA). Tissue sections that had been previously incubated with isotype-matched rabbit-/mouse-/goat- IgG (Dako, Hamburg, Germany) instead of the primary antibody served as negative controls. For positive controls, tissue slides of placental tissue (Gal-1, -3) or breast cancer (Gal-7) tissues were used. Primary antibodies were chosen due to the high expected staining specificities according to the results of positive-control staining, as well as descriptions; and example pictures on the manufacturers's homepages. TheA semi-quantitative method (IR score; Remmele IR score) was performed-determined by two independent observers in consensus to obtain staining results. For this purpose, the predominant staining intensity (0 = negative, 1 = low, 2 = moderate, and 3 = strong) and the percentage of stained cells (0 = 0%, 1 = 1–10%, 2 = 11–50%, 3 = 51–80%, and 4 = 81–100% stained cells) arehas-to-be multiplied, resulting in values from 0 to 12. Staining intensity was measured in the cytoplasm and the nucleus of the-cancer cells; and in the peritumoural stroma. Cut-off points for IR scores were chosen specifically for each staining with regard to the distribution pattern of IR scores in the collective sample. For Gal-1 staining in the cytoplasm and nucleus of cancer cells, an IR scoreS = 0 was considered as-negative and an IR scoreS ≥ 1 as positive. For stromal staining, Gal-1 groups of-with low expression (IR scoreS < 5) and

**Comment [A27]:** 'Room temperature' can vary widely with climate, season, and time of day. In order to clearly state the conditions of the study in a manner that facilitates replication by other researchers, please consider using an exact temperature or an approximate temperature range instead.

**Comment [A28]:** City and country information is generally not provided after the first reference for each manufacturer.

high expression (IR ~~score~~<sup>S</sup>  $\geq 5$ ) were compared. For analysis of Gal-3 staining, negative cases with an IR ~~score~~<sup>S</sup> = 0 were compared to positive cases with an IR ~~score~~<sup>S</sup>  $\geq 1$ . Gal-7 expression was grouped as negative (IRS = 0), low ( $1 \leq \text{IRS} \leq 4$ ), and high (IRS  $\geq 6$ ).

#### 4.3. ~~Statistical~~ <sup>a</sup>~~Analysis~~

Statistical ~~data was obtained~~<sup>analyses were performed</sup> using SPSS 23.0 (~~v23~~, IBM, Armonk, NY, USA)-~~statistic software~~. ~~D~~Distributions of clinicopathological variables ~~was~~ <sup>were</sup> tested with ~~C~~chi-Square ~~Statistical tests~~. Mann-Whitney *U*-tests ~~was~~ <sup>were</sup> used to compare ~~the~~ IR scores of ~~g~~Galectins ~~between~~ <sup>among</sup> different clinical and pathological subgroups. Correlations ~~between~~ <sup>among</sup> immuno~~histo~~chemical staining results were calculated using Spearman's correlation analysis. Kaplan-Meier curves and ~~I~~Log-rank tests (Mantel-Cox) were used to compare survival times ~~between~~ <sup>among</sup> different groups. Data are presented ~~with~~ <sup>as</sup> the mean  $\pm$  standard deviation. Values of  $p < 0.05$  were considered ~~as~~ significant.

#### 4.4. ~~Ethics~~ <sup>S</sup>~~Statement~~

All tissue samples used for this study were left-over material from the archives of ~~the~~ LMU Munich; Department ~~of~~ Gynecology and Obstetrics, ~~which Ludwig Maximilians University, Munich, Germany, that had~~<sup>were</sup> initially ~~been~~ collected for histopathological diagnostics. All diagnostic procedures had already been fully completed at the time the histopathological investigations for the current study were performed. Patients' data ~~have been~~<sup>were</sup> fully anonymized. The study was approved by the Ethics Committee of LMU Munich. All experiments were performed according to the standards set ~~forth~~ in the ~~D~~declaration of Helsinki, [1975].

#### 5. ~~Conclusions~~

~~We were able to show that Galectin expression and its impact on overall survival of ovarian cancer patients is strongly dependent of its localization, whether it is in the nucleus or cytoplasm of tumor cells or the peritumoral stroma. We elaborated that Gal-1 tumor and stroma staining, and Gal-7 staining in the cytoplasm serves as a negative prognostic factor for overall survival in ovarian cancer, while nuclear Gal-3 staining could serve as a positive prognostic factor. According to the results of a multivariate analysis, Gal-1 stroma staining and Gal-7 staining are prognostic factors, independent of clinical and pathological parameters.~~

#### ~~Acknowledgments~~

~~This study was funded by the FöFoLe program of the Ludwig Maximilians University of Munich for Heiko Schulz.~~

#### ~~Author Contributions~~

~~Udo Jeschke conceived and designed the experiments; Christina Kuhn and Simone Hofmann performed the experiments; Heiko Schulz analyzed the data and wrote the paper.~~

**Comment [A29]:** Scientific Reports requires a Data Availability Statement to be included in the Methods section of submitted manuscripts (see '[Availability of materials and data](#)' section for more information).

~~Elisa Schmoeckel and Doris Mayr revised the manuscript for important intellectual content. Sven Mahner and Udo Jeschke initiated and supervised the study. All authors read and approved the final version of the manuscript.~~

#### ~~Conflicts of Interest~~

~~The authors declare no conflict of interest.~~

#### **References**

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Udo Jeschke conceived and designed the experiments; Christina Kuhn and Simone Hofmann performed the experiments; Heiko Schulz analyzed the data and wrote the paper.; Elisa Schmoeckel and Doris Mayr revised the manuscript for important intellectual content.; Sven Mahner and Udo Jeschke initiated and supervised the study; a- All authors read and approved the final version of the manuscript.

#### **~~Conflicts of Interest~~Competing Financial Interests**

The authors declare no conflict of interest.

**Comment [A30]:** Please make sure you provide a complete list of references in keeping with the journal.

**Comment [A31]:** The below sections have been placed after the references in keeping with the journal's guidelines.

**Comment [A32]:** This heading is recommended by the journal.

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