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

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[+]these authors contributed equally to this work

Commented [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~~ing~~ 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~~G~~alectin (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' o~~Overall patient survival was compared ~~between~~ among ~~different~~ groups ~~of~~ stratified by~~G~~ galectin 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 c~~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 factor~~s~~ 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.

~~**Keywords:**~~
~~Galectin-1; Galectin-3; Galectin-7; ovarian cancer; overall survival~~

Commented [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.

Commented [A3]: **Focus area:**
Highlighting the rationale and novelty of the study.

Recommended actions:
While background information on galectins is provided, the rationale for conducting this study and its novelty are not explicitly stated in the abstract. Include a statement for this, while framing existing information more concisely to adhere to the journal word limit.

Commented [A4]: As the term "immunoreactive" is not used elsewhere in the abstract, the abbreviation "IR" is not needed and has been removed.

Commented [A5]: While protein symbols are capitalized, protein names are only capitalized if they appear at the beginning of a sentence.

Commented [A6]: I have provided a concluding statement for your Abstract here.

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~~1.~~ Introduction

Ovarian cancer is the most lethal gynecological malignancy, ranking fifth in estimated cancer deaths among women in the USA[1] ~~[1]~~. First-line treatment consists of primary debulking surgery followed by platinum and paclitaxel chemotherapy[2] ~~[2]~~. ~~Still~~Despite these treatments, the 5-year relative survival rate for epithelial ovarian cancer patients ~~is~~ remains below~~less than~~ 50%[3] ~~[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[4] ~~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[5,6] ~~[5,6]~~. Although ~~for these and various other molecules~~ the prognostic value independent~~ly~~ 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[4] ~~[4]~~. Further specification of anti-cancer therapies~~y~~ necessitates~~arily~~ ~~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 β-galactosides and ~~a~~ significant similarity in the carbohydrate-recognition domain (CRD)[7] ~~[7]~~. The first member of this family to be described was g~~G~~alectin (Gal)-1, which ~~is~~ can be isolated as a homodimer~~s~~ ~~composed of~~comprising two identical CRD subunits[8] ~~[8]~~. Since then, a growing number of ~~the~~ g~~G~~alectin family members have~~s~~ ~~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[9] ~~[9]~~. Similar to Gal-1, Gal-7 typically occurs ~~in~~ as a homodimer~~s~~, while Gal-3 is the only g~~G~~alectin characterized as a chimeric protein that is known to form higher order oligomers[10,11] ~~[10,11]~~. In several types of cancer ~~types~~, g~~G~~alectins are known to affect tumour growth, metastasis, angiogenesis, cell migration, ~~as well as tumor~~ invasiveness, and progression, and they are therefore ~~very likely~~good candidates for proteins with ~~to show a~~ prognostic value for patient~~s'~~ survival[9,12] ~~[9,12]~~.

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

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Commented [A9]: Section numbers are not used in this journal and have been removed.

Commented [A10]: In-text citations have been formatted per the journal's guidelines.

Commented [A11]: **Focus area:**
Deletion of irrelevant background information

Recommended actions:
Information that is not relevant to understanding the rationale for the study, such as information on Gal oligomerization or the number of CRD domains, should be removed to make this section succinct.

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to occur in advanced stages of epithelial ovarian cancer and is also ~~connected~~ associated with ~~poorer~~ reduced progression-free survival in univariate analysis[14] ~~[14]~~. However, these results have not yet been reproduced for overall survival or confirmed by multivariate analysis[15] ~~[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~~ed~~.

High cytoplasmic Gal~~ectin~~-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[16] ~~[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[17] ~~[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~~unclear.

Finally, Gal~~ectin~~-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[16,18] ~~[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 g~~G~~alectins are correlated in ovarian cancer, and there is a critical ~~desperate~~ need for a comprehensive study~~ies~~ of various g~~G~~alectins ~~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~~z~~sed 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 Galectin expression.~~

~~2.~~ Results

~~2.1.~~ *Gal-1 ~~T~~tumour and ~~S~~stromal ~~S~~staining ~~I~~is a ~~N~~negative ~~P~~prognostic indicator of ~~for O~~overall ~~S~~survival*

Gal~~ectin~~-1 staining was ~~successfully performed on~~conducted in 150 ovarian cancer specimens. Gal-1 was present in the cytoplasms and the nuclei of ovarian cancer cells, as well as in the peritumoural stromae (~~Figure 1~~Fig. 1). In 102 cases (68.0%), the ~~cytoplasms of~~ tumour cell cytoplasms ~~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).

Commented [A13]: Focus area:
Insufficient background information and literature review

Recommended actions:
The Introduction does not provide a sufficient background of the problem studied. 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 along with suggestions for additional papers that can be cited in the ***High Impact Peer Review Report*.** These points should be discussed in the manuscript.

Commented [A14]: Focus area:
Restructuring of information in the Results section

Recommended actions:
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.

Commented [A15]: The journal specifies that the abbreviation “Fig.” should be used except for at the beginning of sentences, where “Figure” should be used.

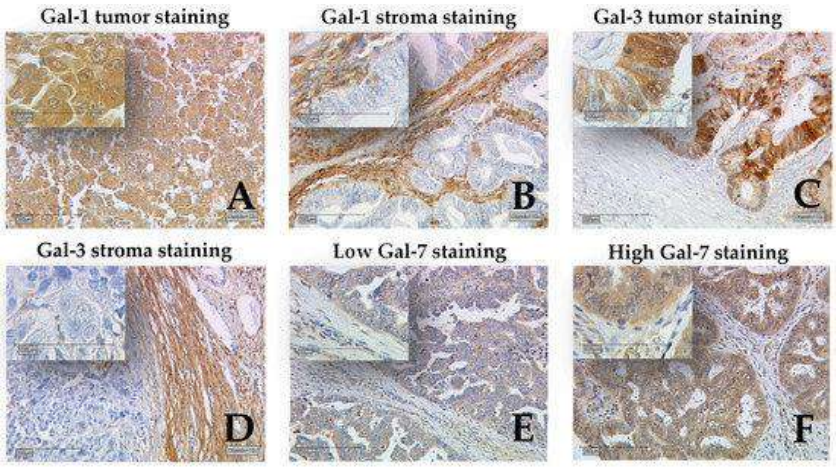

Figure 1. Detection of gGalectins by immunohistochemistry. Representative photomicrographs are shown. ~~Galectin (~~Gal~~)~~-1 was present in the cytoplasm and the nucleus~~i~~ of ovarian cancer cells (**A**) as well as the peritumoural stroma (**B**).~~:~~ Gal-3 staining was observed in the nucleus~~i~~, cytoplasm (**C**), and stroma (**D**).~~:~~ Staining for Gal~~ectin~~-7 was mainly observed in the cytoplasm (**E**), with~~;~~ only a few individual cases showinged 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.

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

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 = 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; FIGO = Fédération Internationale de Gynécologie et d'Obstétrique; NS = Not significant ($p > 0.05$)

Gal-1 staining in the cytoplasm and nucleus ~~showed~~ differednces ~~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 for~~with serous and clear cell subtypes exhibited Gal-1-positive nuclei, while the endometrioid and mucinous subtypes ~~had~~ exhibited weaker nuclear Gal-1 staining~~s~~. Furthermore, Gal-1 staining in the nucleus, cytoplasm, and stroma ~~were~~ was significantly higher in cases with advanced tumour 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 ~~cases~~ those with advanced FIGO stage ($p = 0.013$).

The ~~S~~survival times of ~~different~~ groups characterized by their~~of~~ 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 cases~~than those with low Gal-1 expression in the stroma ($p = 0.045$). A ~~C~~comparison of

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

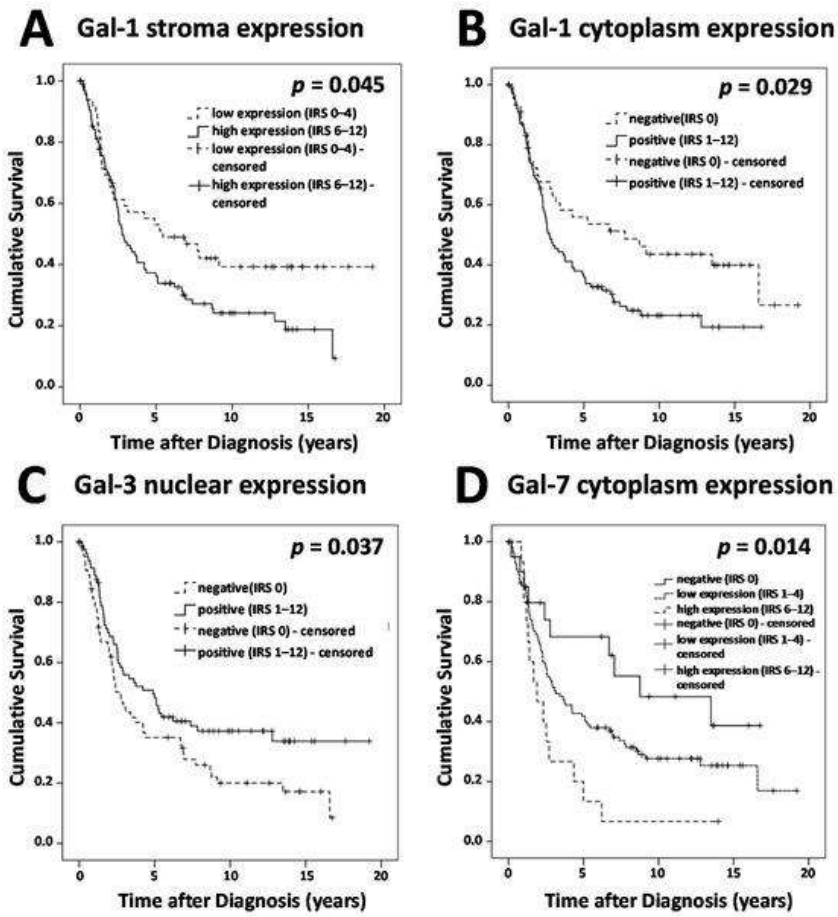

Figure 2. Survival times were plotted as Kaplan-Meier graphs. Percentage of living patients (vertical axis) was plotted in dependence ofagainst time (horizontal axis). Patients without an observed event (death) who exited the study before the observation period ended have been censored, as indicated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

Commented [A18]: Please verify that this edit maintains your intended meaning.

~~2.2.~~ *Presence of Gal-3 in the ~~N~~nucleus~~i~~ ~~I~~s a~~A~~ p~~P~~ositive p~~P~~rognostic indicator~~ator~~ in o~~O~~varian c~~C~~ancer*

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 peritumoural stromae (~~Figure 1~~Fig. 1). Median IR scores for Gal-3 in the nucleus~~i~~, cytoplasm, and stroma were 1, 2, and 1, respectively. Gal-3 staining ~~showed~~ was correlated~~ions~~ with clinical and pathological ~~data~~ variables (~~Table 3~~Table 3). Gal-3 expression in the stroma and nucleus ~~was~~ differed among~~nt for several~~ different histological subtypes (p = 0.008 and~~,~~ p = 0.013, respectively). Gal-3 stromal 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. Tumours 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~~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 patient~~'~~s 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 e~~E~~xpression ~~L~~levels p~~P~~redicts ~~Shortened O~~overall s~~S~~urvival in o~~O~~varian c~~C~~ancer*

Staining for Gal~~ecin~~-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 amongences ~~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 ~~cases~~ those ~~displaying a~~with high Gal-7 expression were compared to ~~cases~~ those with low Gal-7 expression (~~Figure 2~~Fig. 2). We observed ~~a~~ significantly reduced overall survival for cases with high Gal-7 expression and ~~a better~~improved 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 2~~Table 2).

Table 4. Correlations between Gal-7 staining ~~correlated with~~and clinical and pathological ~~data~~factors.

Clinical and Pathological Variables	Gal-7 Expression Cytoplasm			*p*
	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~~*

Commented [A19]: This title makes the subject of this section more clear.

Results of the ~~A~~analysis of the correlations among galectin expression patterns~~analysis is~~ are shown in ~~Table 5~~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 ~~results~~ of Gal-3 in the cytoplasm, nucleus, and stroma were positively correlated ~~among~~ with each other. Furthermore, we ~~found~~ observed correlations between Gal~~ectin~~-1 and -3 staining in the nucleus, cytoplasm, and stroma. Gal-7 staining ~~showed~~ was positively correlated~~ions~~ 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 ~~share a negative prognostic impact on~~ overall survival in ovarian cancer. ~~In accordance~~Consistent with this, *in vitro* experiments have shown~~ed~~ that the overexpression of Gal~~ectin~~-1 significantly increases migration~~ve~~ and invasion behaviours in~~ve behavior of~~ ovarian cancer cells[19] ~~[19]~~. Furthermore, Gal-1 knockdown experiments in ovarian cancer cells ~~displayed~~ result in ~~a~~ reductions in cell growth, migration, and invasion. Possible mechanisms for this include the interaction of Gal-1 ~~interaction~~ with H-Ras ~~and~~ to activat~~eion of~~ the Raf/extracellular signal-regulated kinase (ERK) pathway, as well as ~~the~~ downregulat~~eion 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~~ies~~ of ovarian cancer cells to cisplatin, ~~illustrating~~ reflecting a possible explanation for the ~~decreased~~ reduced survival of ovarian cancer patients with increased Gal-1 expression[14] ~~[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[20] ~~[20]~~. OTX008, for instance, is a new compound able to bind~~ing~~ non-covalently to Gal-1 on the side back face, ~~was able to~~ inhibiting the proliferation and invasion of various cancer cells lines[21] ~~[21]~~. The ~~A~~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*[22] ~~[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 ~~A~~accumulation of Gal-1 in the peritumoural stroma has been described for various other tumour entities[23-25] ~~[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 ~~s~~cells~~,~~ adjacent to malignant cells~~,~~ express *GAL~~al~~-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 G~~g~~al-1 expression in fibroblast ~~s~~cells. ~~Both~~ These experiments suggest that ~~primarily the~~ ovarian cancer cells ~~might~~ may be primarily responsible for stromal Gal-1 expression[26] ~~[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[17,27] ~~[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

Commented [A20]: Focus area:
Focus on clinical implications of the study findings

Recommended actions:
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 lacking in the text.

Commented [A21]: 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.

Commented [A22]: This description is a bit vague. Please be more specific if possible.

Commented [A23]: Typically, gene symbols--and not gene names--are italicized to distinguish them from proteins.

Commented [A24]: 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 ~~A~~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[28,29] ~~[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[30] ~~[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 poor~~er~~ prognosis has only been observed in gastric cancer ~~so~~ thus far[31] ~~[31]~~. In cholangiocarcinoma, Gal-3 expression ~~was~~ is associated with a poorly ~~-~~differentiated type, while *in vitro* experiments show~~ed~~ significantly increased cell migration and invasion after suppression of Gal-3 expression[32] ~~[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[33] ~~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[34] ~~[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 ~~n~~or ~~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 analyse~~i~~s. ~~Further c~~Cell culture experiments ~~were able to prove~~have demonstrated that Gal-7 expression is induced by a mutant form of p53. ~~Also~~In addition, G~~g~~al-7 was shown to increase the proliferation[16] ~~[16]~~, invasiveness, and motility of ovarian cancer cells, while ~~inter~~acting as an immunosuppressant~~ive~~ by killing Jurkat T ~~-~~cells and human peripheral T ~~-~~cells[18] ~~[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 g~~G~~alectins share binding affinities and ~~have~~ exhibit similarities in protein structure, suggests ~~the assumption~~ that ~~G~~galectins might also share common functions in ovarian cancer molecular biology. However, ~~since~~ as these~~is~~ observations are~~is~~ rather descriptive, further investigations into~~s are required to explore~~ the biological characteristics and functions of different g~~G~~alectins are required to determine their ~~manner(s) in which they are~~ similarities and ~~or~~ differences,~~t~~ ~~in~~ specifically in regards to their role(s) in ovarian cancer.

Commented [A25]: Focus area:
Statement not well supported by findings

Recommended actions:
This statement is premature and does not correspond to the facts. Other studies obtained the opposite results both in terms of Gal-3 localization and cancer prognosis. This statement should be deleted; instead, the reason for this discrepancy between the present and earlier studies should be discussed.

Commented [A26]: Please verify that this edit maintains your intended meaning.

5. Conclusions

In this study, We were able towe showed that Galectin expression of galectins and theiritsits impacts on overall survival ofin ovarian cancer patients isare strongly dependent onf itstheir cellular localization, whether it is in the nucleus or cytoplasm of tumour cells or the peritumoural stroma. We elaboratedfound 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 couldmay 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.

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 s--University (LMU) of Munich, Germany between 1990 and 2002 were analyszed 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 ±12 years. Mean overall survival was 3.2 ± 3.0 years, and 104 deaths were observed in total. The mean follow--up time period was 5.1 ± 4.8 years.

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 xXylol 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 gGalectin staining. In particular, fFor Galectin-1 (Gal-1) staining, slides were blocked using Ppower Bblock

Commented [A27]: 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?

Commented [A28]: **Focus areas:**
Number of patients and lack of control samples

Recommended actions:
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.

Commented [A29]: This heading is recommended by the journal.

(BioGenex, San Ramon, CA, USA) for 3 min at room temperature and incubated with a~~A~~nti-Gal~~ectin~~-1 primary antibody (goat, polyclonal; R&D Systems, Minneapolis, MN, USA) at a final concentration of 0.033 µg/mL in P~~p~~ower ~~b~~Block ~~(BioGenex, San Ramon, CA, USA)~~ for 16 h at 4 °C. ~~Galectin-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 a~~A~~nti-Gal~~ectin-~~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 ~~Galectin-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 a~~A~~nti-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-matched~~ing~~ 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 P~~p~~ost-B~~b~~lock 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 were~~as~~ 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. The~~A~~ 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) are~~has 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 score~~S~~ = 0 was considered ~~as~~ negative and an IR score~~S~~ ≥ 1 as positive. For stromal staining, Gal-1 groups ~~of~~ with low expression (IR score~~S~~ < 5) and

Commented [A30]: '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.

Commented [A31]: City and country information is generally not provided after the first reference for each manufacturer.

high expression (IR scoreS ≥ 5) were compared. For analysis of Gal-3 staining, negative cases with an IR scoreS = 0 were compared to positive cases with an IR scoreS ≥ 1. Gal-7 expression was grouped as negative (IRS = 0), low (1 ≤ IRS ≤ 4), and high (IRS ≥ 6).

4.3. Statistical aAnalysis

Statistical data was obtainedanalyses were performed using SPSS 23.0 (v23, IBM, Armonk, NY, USA). statistic software. DDistributions of clinicopathological variables was were tested with Cchi-Ssquare Statisticstests. Mann-Whitney *U*-tests was were used to compare the IR scores of gGalectins between among different clinical and pathological subgroups. Correlations between among immunohistochemical staining results were calculated using Spearman's correlation analysis. Kaplan-Meier curves and lLog-rank tests (Mantel-Cox) were used to compare survival times between among different groups. Data are presented with as the mean ± standard deviation. Values of $p < 0.05$ were considered as significant.

4.4. Ethics Sstatement

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 hadwere 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 beenwere 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 Ddeclaration of Helsinki, 1975.

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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.

~~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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Author Contributions

Udo Jeschke conceived and designed the experiments; Christina Kuhn and Simone Hofmann performed the experiments; Heiko Schulz analyszed 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~~. A~~ll authors read and approved the final version of the manuscript.

~~Conflicts of Interest~~Competing Financial Interests

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The authors declare no conflict of interest.