

Article

Prognostic Impact of LAG-3 Expression in Early Breast Cancer

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Abstract: Background: Monoclonal antibodies against PD-1 or PD-L1 are established in clinical practice for the treatment of both early and advanced/metastatic triple-negative breast cancer. Beyond the established immune checkpoints (ICPs) (PD-1 and CTLA-4) additional ICPs such as lymphocyte activation gene-3 (LAG-3) are subject of current research. In the present retrospective gene expression analysis, we evaluated the prognostic significance of LAG-3 in 461 patients with early breast cancer. In addition, we examined whether there was a correlation between the different ICPs and CD8 expression; **Methods:** Using microarray-based gene expression analysis, we examined the prognostic significance for metastasis-free survival (MFS) of LAG-3 mRNA expression in the whole cohort of 461 breast cancer patients and among different molecular subtypes. Correlations were analyzed using Spearman-Rho correlation coefficient; **Results:** In the whole cohort, LAG-3 expression had no significant impact on MFS ($p = 0.712$, Log Rank). In the subgroup analyses, there was a trend, that higher LAG-3 expression was associated with favorable outcome in the luminal B ($p = 0.217$), basal-like ($p = 0.370$) and HER2 ($p = 0.089$) subtypes, although significance was not reached. In contrast, in multivariate Cox regression analysis adjusted for age, tumor size, axillary nodal status, histological grade of differentiation and the proliferation marker Ki-67, LAG-3 showed a significant influence on MFS (HR 0.574; 95% CI 0.369–0.894; $p = 0.014$). High LAG-3 significantly correlated with CD8 ($\rho = 0.571$; $p < 0.001$); **Conclusion:** LAG-3 expression had an independent impact on MFS. In addition to PD-1 and PD-L1, further immune checkpoints such as LAG-3 could serve as therapeutic targets in breast cancer.

Keywords: LAG-3; immun checkpoints; immunotherapy; breast cancer

1. Introduction

Immunotherapies such as immune checkpoint inhibitors (ICPi) or antibody drug conjugates (ADCs) [1] are of increasing importance in the development of targeted therapy strategies for various solid tumors, including breast cancer [2, 3]. Breast cancer can be classified into several molecular subtypes (luminal A, luminal B, HER2-positive and triple-negative), which differ in their prognosis, as well as different systemic therapy options (e.g. chemotherapy, endocrine therapy, anti-HER2 therapy) [4]. Triple-negative breast cancer (TNBC), which is defined by the lack of expression of the estrogen -, progesterone - and HER2 (human epidermal growth factor receptor 2) receptors, accounts for 10-15% of all breast cancers and is characterized by more aggressive tumor growth, a poorer grade of differentiation, and a higher proliferation index (Ki-67), as well as a correspondingly poorer prognosis [5]. Due to a higher mutational load and a higher number of tumor-associated, immunogenic neoantigens, immunotherapeutic approaches play an important role in the development of targeted therapy strategies in triple-negative breast cancer. In addition to ADCs, T-cell based approaches, in particular ICPi, are used in the treatment of TNBC [6]. ICPi block the interaction of specific cell surface proteins of

activated T-cells, which serve as negative regulators of T-cell-based immune responses, thereby enhancing the anti-tumor immune response. Currently, the most clinically relevant immune checkpoints (ICPs) include CTLA-4, and in breast cancer, the PD-1 / PD-L1 axis. The rationale for the use of ICPi is to release a brake of the immune system and thereby increase the antitumor activity. Based on the convincing data of numerous phase III studies, immune checkpoint inhibitors such as atezolizumab or pembrolizumab are used in clinical practice both in early TNBC in combination with neoadjuvant cytotoxic therapy (NACT) [7, 8], as well as in metastatic TNBC in combination with taxane-based chemotherapy [9-11]. Nevertheless, the clinical efficacy of ICPi (such as atezolizumab or pembrolizumab) as monotherapy has been modest in phase I trials in advanced and extensively pretreated TNBC [12]. Cases of non-response and primary or secondary resistance when using ICPi as monotherapy have also been observed in other tumor entities such as high-grade serous ovarian cancer [13]. A possible explanation for the development of resistance could be the compensatory upregulation of other immune checkpoints once a single immune checkpoint is blocked by a monoclonal antibody. For example, Huang and colleagues demonstrated in an ovarian cancer mouse model that inhibition of PD-1 by a monoclonal antibody led to compensatory upregulation of other immune checkpoints, such as LAG-3 or CTLA-4 [14]. Another work by Huang et al. demonstrated in a preclinical murine ovarian cancer model that dual blockade of LAG-3 and PD-1 had a synergistic effect in terms of anti-tumor immune response [15]. These data suggest a potential benefit of combinatorial use of ICPi blocking multiple immune checkpoints. For other entities, such as melanoma or non-small cell lung cancer, the clinical efficacy of combining different immune checkpoint inhibitors, in particular the combination of ipilimumab, a monoclonal antibody against CTLA-4 and nivolumab, which blocks PD-1, has been demonstrated in phase 3 studies [16, 17]. Furthermore, the important role of "next generation" immune checkpoints such as LAG-3 is obvious. LAG-3 is expressed on activated tumor-infiltrating lymphocytes (TILs) (for example CD4 and CD8 positive T-cells [18], T-regs [19, 20], and B-lymphocytes) and regulates T-cell function as an inhibitory ICP. To better understand the importance of LAG-3 in breast cancer and its interaction and correlation with other immune checkpoints such as CTLA-4 and PD-1, we evaluated in the present study the prognostic significance of LAG-3 by gene expression analysis in a cohort of 461 breast cancer patients. In addition, we analyzed the prognostic impact of other immune checkpoints (CTLA-4, PD-1) and CD8 expression as a marker for activated cytotoxic T-cells. Furthermore, we examined whether there was a correlation between the different immune checkpoints and CD8 expression.

2. Materials and Methods

2.1. Patient's characteristics

The study cohort included 461 patients with early breast cancer who underwent surgery at the Department of Gynecology and Obstetrics at the University Medical Center Mainz between 1986 and 2000 and from whom sufficient tumor tissue (fresh frozen) was available for successful Affymetrix microarray analysis. The whole cohort consisted of three subgroups with different systemic treatment:

- (i) "N0 cohort": 200 node-negative patients with early breast cancer who received no further adjuvant therapy after surgery and radiation.
- (ii) "Tamoxifen cohort": 165 patients treated with tamoxifen as a single adjuvant therapy.
- (iii) "Chemotherapy cohort": 96 patients treated with either cyclophosphamide, methotrexate, fluorouracil (CMF; n = 34) or epirubicin, cyclophosphamide (EC; n = 62) in the adjuvant setting.

Detailed patient's characteristics are summarized in Table 1. Established prognostic factors (histologic grade of differentiation, tumor size, nodal status, age at initial diagnosis, ER, PR, HER2, and Ki-67) were obtained from pathology reports and the breast cancer database of our department. The median age of patients at initial diagnosis was 62

years. The median follow-up time was 12.75 years; 133 patients (28.9%) developed distant metastases. The study was approved by the Ethics Committee of the Rhineland-Palatinate Medical Association.

Table 1. Patient's characteristics (n=461).

	Number of patients (n = 461)	Percentage (%)
Age at diagnosis		
<=50	104	22.6
>50	357	77.4
Tumor size		
T1	188	40.8
T2	214	46.4
T3	19	4.1
T4	39	8.5
Missing value	1	0.2
Tumor grade		
GI	63	13.7
GII	287	62.3
GIII	111	24
Lymph node status		
N0	254	55.1
N1	140	30.4
N2	49	10.6
Nx	18	3.9
Tumor type		
Invasive ductal (NST)	291	63.1
Invasive lobular	79	17.1
Others	91	19.7
ER		
Positive	381	82.6
Negative	79	17.1
Missing value	1	0.2
PR		
Positive	346	75.1
Negative	114	24.7
Missing value	1	0.2
HER2		
Positive	46	10
Negative	358	77.7
Missing value	57	12.3
Ki-67		
>20 %	138	29.9
≤ 20 %	250	54.2
Missing value	73	15.8
Molecular subtypes		
Luminal A-like	189	41
Luminal B-like	182	39.5
Basal-like	51	11.1
HER2-positive	39	8.5
Distant metastasis		
Yes	133	28.9
No	328	71.1
Treatment collective		
N0, untreated	200	43.4

Tamoxifen	165	35.8
Chemotherapy:	96:	20.8:
• CMF	• 34	• 7.4
• EC	• 62	• 13.4

2.2. mRNA isolation and gene expression analysis

The mRNA isolation and gene expression analysis were performed as previously described [19]. Briefly, tumor samples were frozen and stored at -80 °C. About 50 mg of the frozen breast tumor tissue was fragmented in liquid nitrogen. RLT buffer was applied, and the resulting homogenate was centrifuged through a QIAshredder column (Qiagen). Total RNA was further isolated from the eluate using the RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA yield was determined by UV absorbance, and RNA quality was assessed by rRNA band integrity analysis using an Agilent 2100 Bioanalyzer RNA 6000 LabChip Kit (Agilent Technologies) as previously described [19, 20].

For mRNA expression analysis, fresh frozen tumor tissue from a total of 461 breast cancer samples from the Department of Gynecology and Obstetrics at the University Medical Center Mainz was used to generate HG-U133A arrays (Affymetrix, Santa Clara, CA, USA) and measure the relative transcript frequencies of the genes listed below in the tumor tissue. Based on 5 µg of total RNA, labeled cRNA was synthesized utilizing the Roche Microarray cDNA Synthesis, Microarray RNA Target Synthesis (T7), and Microarray Target Purification Kits (Roche Applied Science, Mannheim, Germany) following the manufacturer's instructions. Raw expression data (CEL files) were standardized by multiarray analysis (fRMA). Most samples were previously deposited (2008 and 2011) with the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) under accession numbers GSE11121 and GSE26971. In addition, the complete data set of the 461 samples used with updated follow-up was previously deposited at NCBI in the GEO database under accession number GSE158309.

For gene expression analysis, the following single genes were considered with the corresponding probe sets:

- LAG-3: 206486_at
- CTLA-4: 221331_x_at
- PD-1: 207634_at
- CD8: 205758_at

In addition, an immune checkpoint signature (CPS) was averaged from the gene expression values of the single genes LAG-3, CTLA-4, and PD-1. For dichotomization, scores above the median of the CPS signature were defined as high expression, whereas scores below the median were defined as low expression.

2.3. Molecular subtypes

Intrinsic subtypes were determined according to Haibe-Kains et al [21], who postulated a three-gene model including estrogen receptor gene (ESR1), HER2, and Aurora kinase A (AURKA). Briefly, ER and HER2 status was determined from the bimodally distributed mRNA levels of the corresponding genes (probe sets: ESR1 205225_at and ERBB2 216836_s_at) based on fRMA-normalized expression values. The cut-off value for ESR1 was determined by model-based clustering. The cut-off value for ERBB2 was determined using the upper quartile plus interquartile range of the mRNA level. For AURKA, the median mRNA expression of the corresponding probe set (208079_s_at) was used as the cut-off value, as described previously [22].

- This procedure resulted in the following molecular subtypes:
- ESR1-positive, HER2-negative, low proliferation (AURKA low) luminal A-like.
 - ESR1-positive, HER2-negative, high proliferation (AURKA high) luminal B-like
 - HER2-positive

- ESR1 negative HER2 negative basal-like

Table 1 shows the absolute and relative frequencies of molecular subtypes determined by gene expression data within the patient cohort studied.

2.4. Statistical analysis

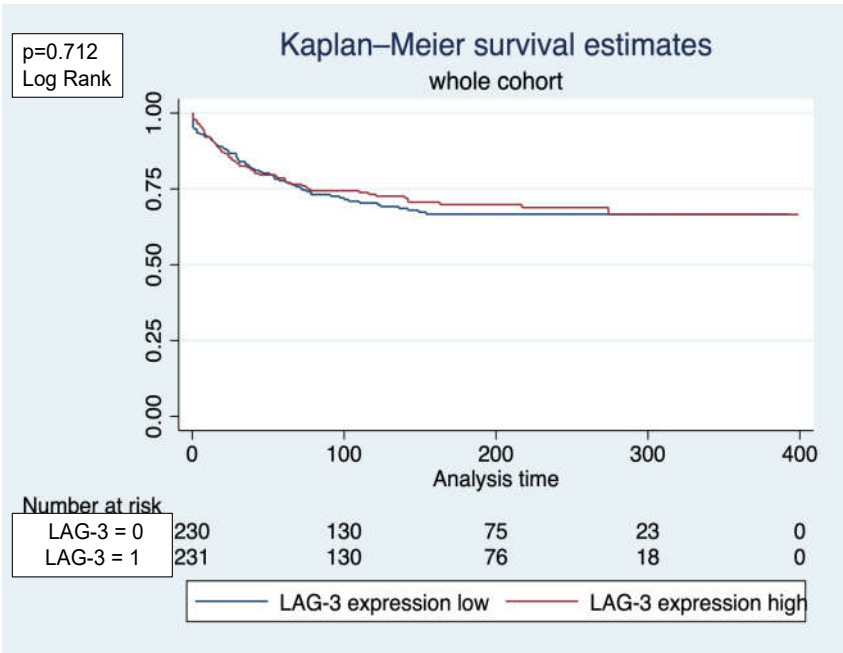
Statistical analyses were performed using the SPSS statistical software program, version 23.0 (SPSS Inc, Chicago, IL, USA) and Stata (Version 17). The prognostic significance of LAG-3, PD-1, CTLA-4 and CD8 expression for MFS was examined by Kaplan–Meier survival analysis (\leq median vs. $>$ median) as well as univariate and multivariate Cox regression analysis. Multivariate Cox regression analysis was adjusted for pT stage (T1 vs. T2, T3, 4), histological grade (GI +GII vs. GIII), Ki-67 (\leq 20% vs. $>$ 20%) and lymph node state (negative vs. positive). Significance of Kaplan–Meier survival analysis was assessed by the p-value of the Log-Rank test. Correlations were analyzed using the Spearman-Rho correlation coefficient.

3. Results

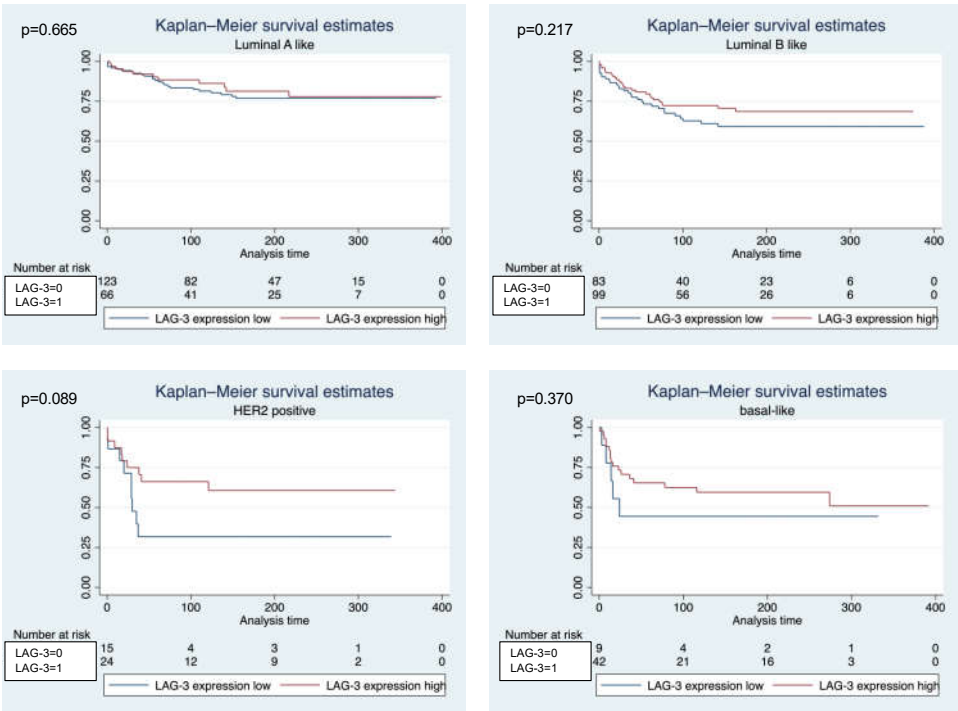
3.1. Prognostic impact of LAG-3, CTLA-4, PD-1 and CD8 mRNA expression

Kaplan–Meier analysis showed that within the whole cohort, consisting of the N0, tamoxifen and chemotherapy subcohorts, LAG-3 expression had no significant impact on MFS ($p = 0.712$, Log Rank) (Figure 1a). Within the subgroup analyses, there was a trend that higher LAG-3 expression was associated with a longer MFS in Luminal B ($p = 0.217$, Log Rank), basal-like ($p = 0.370$ Log Rank) and HER2 ($p = 0.089$ Log Rank) subtypes, although significance was not reached (Figure 1b).

In contrast, in multivariate Cox regression analysis adjusted for age, tumor size, axillary nodal status, histological grade of differentiation and the proliferation marker Ki-67, LAG-3 showed a significant influence on MFS (HR 0.574; 95% CI 0.369–0.894; $p = 0.014$) (Table 2), a higher LAG-3 expression was associated with a longer MFS. In addition to LAG-3 expression, tumor grading (HR 2.583; 95% CI 1.591–4.192; $p < 0.001$) and tumor size (HR 1.626; 95% CI 1.020–2.591; $p = 0.041$) were also identified as independent prognostic factors in multivariate Cox regression analysis (Table 2). Additionally uni – and multivariate Cox regression analyses adjusted for age at diagnosis, tumor size, nodal status, histological grade of differentiation and the proliferation marker Ki-67 were performed among the molecular subtypes showing a significant association between LAG-3 expression and MFS in the Luminal B like subtype. (HR 0.504; 95% CI 0.258–0.985; $p = 0.045$) (Table 3).



a)



b)

Figure 1. a) Kaplan-Meier plot shows no significant effect of LAG-3 expression on MFS in the whole cohort b) Within the subgroup analyses, a trend is apparent that a higher LAG-3 expression was associated with a more favorable outcome (longer MFS) in the Luminal B ($p = 0.217$, Log Rank), basal-like ($p = 0.370$ Log Rank) and HER2 ($p = 0.089$ Log Rank) subtypes, although significance was not reached.

Table 2. Multivariate Cox Regression analysis of LAG-3 for MFS adjusted for age, tumor size, lymph node status, grade of differentiation and the proliferation marker Ki-67.

		HR	95% CI		p-value
			lower	upper	
LAG3	High vs. low	0.574	0.369	0.894	<u>0.014</u>
Age	<50 vs. ≥50	1.176	0.681	2.031	0.561
Tumor size	T2-4 vs. T1	1.626	1.020	2.591	<u>0.041</u>
Lymph node status	N1,2,3 vs. N0	1.494	0.965	2.313	0.071
Grade	GIII vs. GI/II	2.583	1.591	4.192	<u><0.001</u>
Ki-67	>20% vs. <20%	1.350	0.856	2.130	0.197

Table 3. Association between LAG-3 and MFS in molecular subtypes using univariate and multivariate Cox regression analysis adjusted for age at diagnosis, tumor size, nodal status, grading and Ki-67.

Subtype	Univariate Model		Multivariate Model	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Luminal A like	0.855 (0.421-1.739)	0.666	0.614 (0.252-1.496)	0.283
Luminal B like	0.723 (0.430-1.216)	0.221	0.504 (0.258-0.985)	<u>0.045</u>
HER2-positive	0.455 (0.179-1.160)	0.099	0.276 (0.074-1.032)	0.056
Basal-like	0.638 (0.235-1.731)	0.377	0.459 (0.097-2.159)	0.324

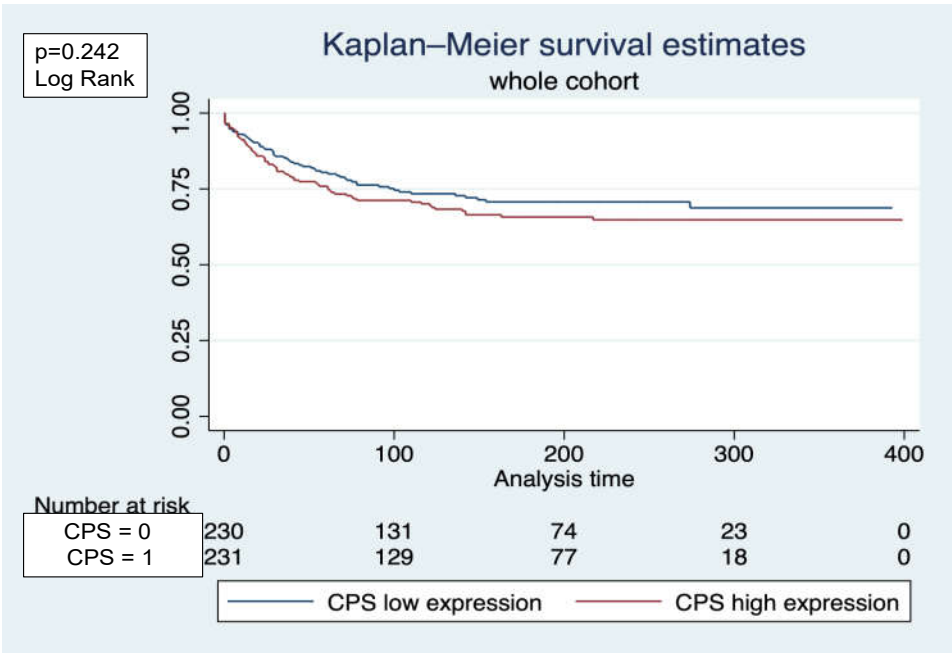
Within the whole cohort, no significant effect of CTLA-4 expression regarding MFS could be demonstrated ($p=0.664$ Log Rank) (Figure A1a). Among the molecular subtypes, there was a trend toward better outcome associated with higher CTLA-4 expression in the basal like subtype, although the significance level was not reached ($p = 0.072$ Log Rank) (Figure A1b). CTLA-4 expression failed to be proven as a significant prognostic factor in univariate and multivariate Cox regression (Table A1). Regarding PD-1 expression, there was no significant effect on MFS within the whole cohort (Figure A2a) or within the molecular subtypes (Figure A2b). With respect to CD8, Kaplan-Meier curves showed that CD8 expression had no significant influence on MFS either in the whole cohort (Figure A3a) or within subgroup analyses (Figure A3b). In contrast, CD8 was a significant prognostic factor in the multivariate Cox regression analysis (HR 0.642; 95% CI 0.421-0.979; $p = 0.040$), in which higher CD8 expression was associated with a better outcome (Table 4).

Table 4. Multivariate Cox Regression analysis of CD8 for MFS adjusted for age, tumor size, lymph node status, grade of differentiation and the proliferation marker Ki-67.

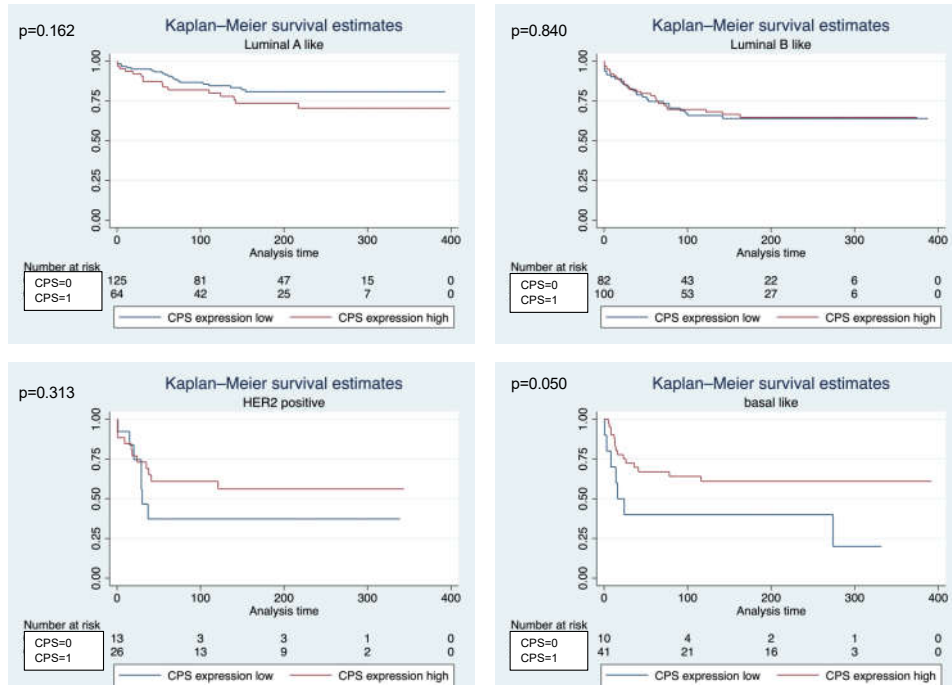
		HR	95% CI		p-value
			lower	upper	
CD8	High vs. Low	0.642	0.421	0.979	<u>0.040</u>
Age	<50 vs. ≥50	1.102	0.635	1.910	0.730
Tumor size	T2-4 vs. T1	1.529	0.959	2.436	0.074
Lymph node status	N1,2,3 vs. N0	1.486	0.958	2.395	0.077
Grade	GIII vs. GI/II	2.245	1.413	3.568	<u>0.001</u>
Ki-67	>20% vs. <20%	1.393	0.880	2.203	0.157

3.2. Prognostic impact of an immune checkpoint associated signature (CPS)

CPS calculated by averaging the gene expression values of LAG-3, CTLA-4 and PD-1 showed no significant effect for MFS in the whole cohort ($p = 0.242$ Log Rank) (Figure 2a). However, subgroup analysis showed a significant effect in the basal like subtype, with higher expression of CPS associated with longer MFS ($p = 0.050$ Log Rank) (Figure 2b). Multivariate Cox regression analysis among the molecular subtypes identified CPS as an independent prognostic factor in the basal like subtype (HR 0.195; 95% CI 0.043-0.88; $p = 0.034$) (Table 5).



a)



b)

Figure 2. a) CPS could not achieve significance for MFS in the whole cohort. b) In contrast, there was a significant effect in the basal like subtype ($p = 0.050$ Log Rank).

Table 5. Association between CPS and MFS in molecular subtypes using univariate and multivariate Cox regression analysis adjusted for age at diagnosis, tumor size, nodal status, grading and Ki-67.

Subtype	Univariate Model	p-value	Multivariate Model	p-value
	HR (95% CI)		HR (95% CI)	
Luminal A like	1.591 (0.824-3.071)	0.166	0.984 (0.426-2.273)	0.984
Luminal B like	0.948 (0.564-1.595)	0.841	0.536 (0.277-1.036)	0.064
HER2-positive	0.616 (0.237-1.604)	0.321	0.705 (0.219-2.274)	0.558
Basal-like	0.420 (0.171-1.034)	0.059	0.195 (0.043-0.881)	<u>0.034</u>

3.3. Correlation effects:

Spearman Rho correlation coefficient showed a strong, significant correlation between LAG-3 and CD8: higher CD8 expression was associated with higher LAG-3 expression ($\rho = 0.571$; $p < 0.001$). This significant correlation between CD8 and LAG-3 expression is illustrated by the scatter plot (Figure 3).

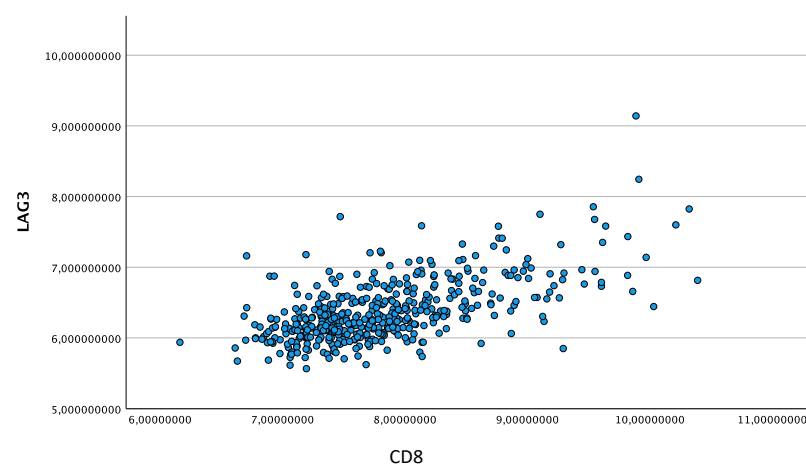


Figure 3. Scatter plot visualizing the correlation between CD8 and LAG-3 expression ($\rho = 0.571$; $p < 0.001$).

A less strong, although still significant, correlation was found between CTLA-4 expression and CD8 expression ($\rho = 0.447$; $p < 0.001$), which is shown in Figure 4.

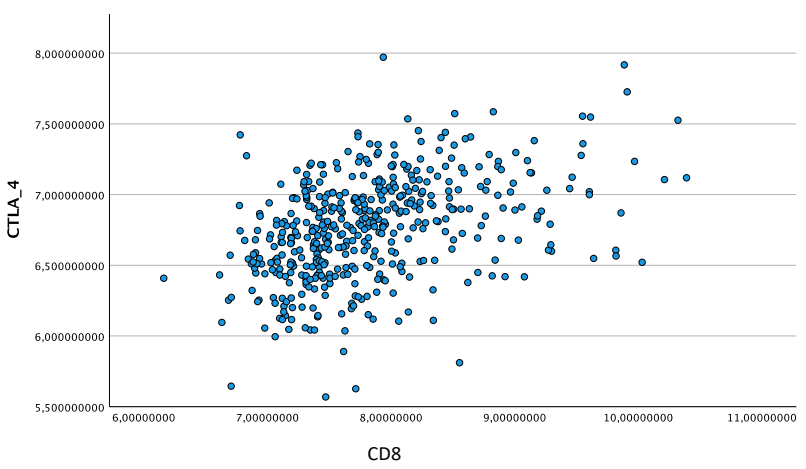


Figure 4. Scatter plot demonstrating the correlation between CD8 and CTLA-4 expression ($\rho = 0.447$; $p < 0.001$).

In contrast, no significant correlation relationship was demonstrated between PD-1 expression and CD8. In addition, the correlation between the immune checkpoints was analyzed. In this context, a strong correlation between LAG-3 and CTLA-4 expression could be shown ($\rho = 0.453$; $p < 0.001$) (Figure 5).

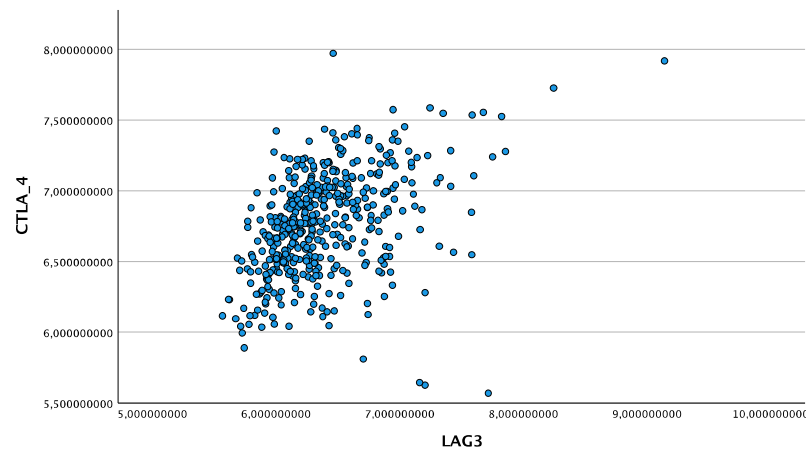


Figure 5. Scatter plot of correlation between CTLA-4 and LAG-3 expression ($\rho = 0.453$; $p < 0.001$).

A less strong but still significant correlation between LAG-3 and PD-1 was also demonstrated ($\rho = 0.243$; $p < 0.001$). In contrast, an inverse, but significant correlation was found between CTLA-4 and PD-1 expression ($\rho = -0.213$; $p < 0.001$).

4. Discussion

In the present retrospective gene expression study, we demonstrated that LAG-3, as a next generation ICP, had independent prognostic significance in terms of MFS in a cohort of 461 breast cancer patients with long-term follow up. Furthermore, we could show a significant correlation between LAG-3 expression and CD8 expression.

Immune checkpoint inhibitors are well established as targeted immunotherapies for the treatment of various solid tumors [21]. In breast cancer, the PD-1/PD-L1 axis is particularly important, so that, for example, atezolizumab, a monoclonal antibody against PD-L1 in combination with nab-paclitaxel is an approved therapeutic option in advanced triple-negative breast cancer [9]. In addition to atezolizumab, the monoclonal anti PD-1 antibody pembrolizumab in combination with taxane-containing chemotherapy is an approved treatment option for metastatic triple negative breast cancer [11]. In early triple-negative breast cancer, recent phase III data have also demonstrated the efficacy of pembrolizumab in combination with neoadjuvant chemotherapy [22, 23].

In contrast, ICPi monotherapy has been shown to be less effective in advanced TNBC, also primary or secondary resistance make it necessary to improve the clinical efficacy of ICPi. In this context, "next generation" immune checkpoints like LAG-3 play an increasing role. LAG-3 is frequently co-expressed with PD-1 on activated CD4- and CD8-positive tumor infiltrating T-cells and is known to be a negative regulator of T-cell function [24]. This is in line with our findings showing a significant correlation between LAG-3 and PD-1 mRNA expression ($\rho = 0.243$; $p < 0.001$) in a cohort of 461 patients with early breast cancer. Furthermore, multivariate Cox regression identified LAG-3 as an independent prognostic factor in the whole cohort, showing that higher LAG-3 expression was associated with better MFS (HR 0.574; 95% CI 0.369–0.894; $p = 0.014$). One possible interpretation is that higher mRNA expression of immune checkpoint regulators implies the presence of TILs associated with better survival. Indeed, we demonstrated a strong correlation between CD8 expression and LAG-3 expression. These findings are in line with the results of a study published by Denkert et al. which analyzed immune-associated mRNA markers in tumor samples from patients of the Gepar-Sixto trial, demonstrating that immunosuppressive markers such as PD-1, PD-L1, CTLA-4 and FoxP3 were positively associated

with the presence of stromal TILs and correlated with treatment response [25]. Among the subgroup of basal like breast cancers, there was a significant effect of higher expression of CPS (calculated from LAG-3, CTLA-4 and PD-1) being associated with a longer MFS ($p = 0.05$, Log Rank). This fits with the results of a previous gene expression study, where we were able to show that a CPS consisting of CTLA-4 and PD-1 had a significant impact in terms of MFS in a subset of triple-negative breast cancers [26]. In the present work, we showed a positive significant correlation between both LAG-3 and PD-1 expression, and LAG-3 and CTLA-4 expression. A possible approach to explain the development of resistance and consequent inadequate response to ICPi therapy is the compensatory upregulation of alternative immune checkpoints once a single immune checkpoint is blocked by a monoclonal antibody. Indeed, Saleh and colleagues demonstrated in a triple-negative cell co-culture model that blocking PD-1 or PD-L1 resulted in compensatory upregulation of LAG-3, TIM-3, and CTLA-4 in CD4-positive T cells [27]. A preclinical mouse model by Woo et al. revealed a synergistic effect of dual blockade of PD-1 and LAG-3, resulting in a good response rate [24]. This promising approach of dual blockade of two immune checkpoints was investigated in a randomized three-arm phase II trial in advanced TNBC using the anti-LAG-3 antibody LAG525 (Leramilimab) either in combination with the anti-PD-1 antibody spartalizumab or with carboplatin or as triplet therapy (NCT03499899). Preliminary results indicated that the most sustained response rates were achieved in the study arm with triplet therapy (ORR 32.4%) at the price of increased side effects [28].

Further studies will show, if dual blockade of different immune checkpoints should also be recommended in breast cancer.

A limitation of the present study is that it was conducted retrospectively and unicentric. A strength is the consecutive inclusion of patients with a sufficient amount of fresh frozen tissue available for successful DNA microarray analysis with long-term follow-up and well-defined adjuvant treatment strategies.

5. Conclusions

In summary, the present study identified LAG-3 as an independent prognostic factor for improved MFS. In addition, we showed a significant correlation between CD8 and LAG-3 expression.

Author Contributions: Anne-Sophie Heimes: Project administration, Conceptualization, Methodology, Validation, Formal analysis, Data Curation, Writing - Original Draft, Visualization, Writing - Review & Editing; Katrin Almstedt: Writing - Review & Editing; Roxana Schwab: Writing - Review & Editing; Anne Runkel: Writing - Review & Editing; Annika Droste: Writing - Review & Editing; Marco J Battista: Writing - Review & Editing; Slavomir Krajnak: Writing - Review & Editing; Antje Lebrecht: Writing - Review & Editing; Mathias Gehrmann: Writing - Review & Editing; Kathrin Stewen: Writing - Review & Editing; Walburgis Brenner: Resources, Writing - Review & Editing; Jan G Hengstler: Methodology, Formal analysis, Data Curation, Writing - Review & Editing; Annette Hasenburg: Resources, Writing - Review & Editing; Marcus Schmidt: Supervision, Conceptualization, Methodology, Validation, Investigation, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing.

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Institutional Review Board Statement: The study was approved by the Ethics Committee of Rhineland-Palatinate, Germany [no. 837.139.05 (4797)]. Written informed consent was obtained from all patients, and all clinical investigations were conducted ethically in accordance with ethical and legal standards and in consideration of the Declarations of Helsinki.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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Appendix:

Figure A1: Kaplan-Meier estimates of CTLA-4 in terms of MFS **a)** Kaplan-Meier estimates of the whole cohort: no significant effect of CTLA-4 expression regarding MFS could be demonstrated ($p=0.664$ Log Rank) **b)** Kaplan-Meier curves among the molecular subtypes showing a trend toward better outcome associated with higher CTLA-4 expression in the basal like subtype, although the significance level was not reached ($p=0.072$ Log Rank)

Figure A2 Kaplan-Meier curves of PD-1 expression in regard of MFS: there was no significant effect on MFS within the whole cohort (A2a) or within the molecular subtypes (A2b).

Figure A3: Kaplan-Meier curves with respect to CD8 in the whole cohort (A3a) and in different molecular subtypes (A3b). Kaplan-Meier curves showed that CD8 expression had no significant influence on MFS in either in the whole cohort (A3a) or within subgroup analyses (A3b).

Table A1: uni- and multivariate Cox regression analyses regarding CTLA-4 expression and MFS: CTLA-4 expression also failed to be proven as a significant prognostic factor in univariate and multivariate Cox regression.

References

1. Hafeez, U., et al., Antibody-Drug Conjugates for Cancer Therapy. *Molecules*, 2020. 25(20).
2. Yi, M., et al., Biomarkers for predicting efficacy of PD-1/PD-L1 inhibitors. *Mol Cancer*, 2018. 17(1): p. 129.
3. La-Beck, N.M., et al., Immune Checkpoint Inhibitors: New Insights and Current Place in Cancer Therapy. *Pharmacotherapy*, 2015. 35(10): p. 963-76.
4. Burstein, H.J., et al., Customizing local and systemic therapies for women with early breast cancer: the St. Gallen International Consensus Guidelines for treatment of early breast cancer 2021. *Ann Oncol*, 2021. 32(10): p. 1216-1235.
5. Metzger-Filho, O., et al., Dissecting the heterogeneity of triple-negative breast cancer. *J Clin Oncol*, 2012. 30(15): p. 1879-87.
6. Schmidt, M. and A.S. Heimes, Immunomodulating Therapies in Breast Cancer-From Prognosis to Clinical Practice. *Cancers (Basel)*, 2021. 13(19).
7. Schmid, P., et al., Pembrolizumab plus chemotherapy as neoadjuvant treatment of high-risk, early-stage triple-negative breast cancer: results from the phase 1b open-label, multicohort KEYNOTE-173 study. *Ann Oncol*, 2020. 31(5): p. 569-581.
8. Mittendorf, E.A., et al., Neoadjuvant atezolizumab in combination with sequential nab-paclitaxel and anthracycline-based chemotherapy versus placebo and chemotherapy in patients with early-stage triple-negative breast cancer (IMpassion031): a randomised, double-blind, phase 3 trial. *Lancet*, 2020. 396(10257): p. 1090-1100.
9. Schmid, P., S.Y. Chui, and L.A. Emens, Atezolizumab and Nab-Paclitaxel in Advanced Triple-Negative Breast Cancer. Reply. *N Engl J Med*, 2019. 380(10): p. 987-988.
10. Miles, D., et al., Primary results from IMpassion131, a double-blind, placebo-controlled, randomised phase III trial of first-line paclitaxel with or without atezolizumab for unresectable locally advanced/metastatic triple-negative breast cancer. *Ann Oncol*, 2021. 32(8): p. 994-1004.
11. Cortes, J., et al., Pembrolizumab plus chemotherapy versus placebo plus chemotherapy for previously untreated locally recurrent inoperable or metastatic triple-negative breast cancer (KEYNOTE-355): a randomised, placebo-controlled, double-blind, phase 3 clinical trial. *Lancet*, 2020. 396(10265): p. 1817-1828.

12. Emens, L.A., Breast Cancer Immunotherapy: Facts and Hopes. *Clin Cancer Res*, 2018. 24(3): p. 511-520.
13. Pujade-Lauraine, E., et al., Avelumab alone or in combination with chemotherapy versus chemotherapy alone in platinum-resistant or platinum-refractory ovarian cancer (JAVELIN Ovarian 200): an open-label, three-arm, randomised, phase 3 study. *Lancet Oncol*, 2021. 22(7): p. 1034-1046.
14. Huang, R.Y., et al., Compensatory upregulation of PD-1, LAG-3, and CTLA-4 limits the efficacy of single-agent checkpoint blockade in metastatic ovarian cancer. *Oncoimmunology*, 2017. 6(1): p. e1249561.
15. Huang, R.Y., et al., LAG3 and PD1 co-inhibitory molecules collaborate to limit CD8+ T cell signaling and dampen antitumor immunity in a murine ovarian cancer model. *Oncotarget*, 2015. 6(29): p. 27359-77.
16. Hellmann, M.D., et al., Nivolumab plus Ipilimumab in Advanced Non-Small-Cell Lung Cancer. *N Engl J Med*, 2019. 381(21): p. 2020-2031.
17. Hodi, F.S., et al., Nivolumab plus ipilimumab or nivolumab alone versus ipilimumab alone in advanced melanoma (CheckMate 067): 4-year outcomes of a multicentre, randomised, phase 3 trial. *Lancet Oncol*, 2018. 19(11): p. 1480-1492.
18. Triebel, F., et al., LAG-3, a novel lymphocyte activation gene closely related to CD4. *J Exp Med*, 1990. 171(5): p. 1393-405.
19. Huang, C.T., et al., Role of LAG-3 in regulatory T cells. *Immunity*, 2004. 21(4): p. 503-13.
20. Kisielow, M., et al., Expression of lymphocyte activation gene 3 (LAG-3) on B cells is induced by T cells. *Eur J Immunol*, 2005. 35(7): p. 2081-8.
21. Qin, S., et al., Novel immune checkpoint targets: moving beyond PD-1 and CTLA-4. *Mol Cancer*, 2019. 18(1): p. 155.
22. Schmid, P., et al., Pembrolizumab for Early Triple-Negative Breast Cancer. *N Engl J Med*, 2020. 382(9): p. 810-821.
23. Schmid, P., et al., Event-free Survival with Pembrolizumab in Early Triple-Negative Breast Cancer. *N Engl J Med*, 2022. 386(6): p. 556-567.
24. Woo, S.R., et al., Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. *Cancer Res*, 2012. 72(4): p. 917-27.
25. Denkert, C., et al., Tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy with or without carboplatin in human epidermal growth factor receptor 2-positive and triple-negative primary breast cancers. *J Clin Oncol*, 2015. 33(9): p. 983-91.
26. Heimes, A.S., et al., Subtype-specific prognostic impact of different immune signatures in node-negative breast cancer. *Breast Cancer Res Treat*, 2017. 165(2): p. 293-300.
27. Saleh, R., et al., Breast Cancer Cells and PD-1/PD-L1 Blockade Upregulate the Expression of PD-1, CTLA-4, TIM-3 and LAG-3 Immune Checkpoints in CD4(+) T Cells. *Vaccines (Basel)*, 2019. 7(4).
28. Carey L.A. et al. A Phase 2 Study of LAG525 in Combination With Spartalizumab (PDR001), PDR001 and Carboplatin (Carbo), or Carbo, as First- or Second-Line Therapy in Patients (Pts) With Advanced (Adv) Triple- Negative Breast Cancer (TNBC). *Annals of Oncology* (2021) 32 (suppl_5): S457-S515. 10.1016/annonc/annonc689, 2021.