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Effects of pregnancy on breast cancer immunology: immune biomarker and TIL quantification

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Breast cancer diagnosed during pregnancy (PrBC) is a rare occurrence but may become more prevalent as women nowadays tend to postpone childbearing until later in life. Further understanding of how pregnancy affects the tumor microenvironment (TME) is essential. We constructed Tissue Microarrays (TMA) of tumor specimens from 126 pregnant breast cancer (BC) patients and examined standard BC markers such as ER, PR, Ki67, HER2, tumor infiltrating lymphocytes (TILs), and immunomarkers HLA class I, HLA-G, PD-L1, TIGIT and Nectin-4. Subsequently, we compared our findings with those from a matched non-pregnant cohort of young BC patients. Pregnant BC patients were younger, had significantly higher proliferation rates and a higher expression of Nectin-4. Higher pregnancy related estrogen levels may boost proliferation und Nectin-4 overexpression, promoting BC progression. No further evidence supporting impaired maternal anti-tumor response in BC was observed in this study.

Breast cancer (BC) stands as the most prevalent female cancer, ranking among the leading causes of cancer-related deaths globally[1]. Additionally, with 2.4–7.3 cases in 100,000 pregnancies, breast cancer is notably prevalent among cancers occurring during pregnancy[2–4]. Previous reports from Western countries indicate growing numbers of pregnancy related breast cancer (PrBC) cases, supporting the hypothesis that delaying the reproductive phase to an older age may contribute to rising incidence[2,4].

Previous studies predominantly referred to Pregnancy-associated breast cancer (PABC), defined as BC diagnosed during pregnancy or within the first postpartum year. Given the distinct prognosis and tumor biology, it is strongly recommended to conduct a separate evaluation for each condition[5].

At the time of diagnosis, PrBC is often diagnosed at more advanced stages, characterized by larger tumors and nodal involvement[6–9]. Nevertheless, overall outcomes align with those of young non-pregnant BC patients when treatment is promptly administered in adherence to the standard of care for young BC patients[7,9].

During pregnancy, elevated serum progesterone levels stimulate T-helper type 2 (Th2)-like-cytokine production in lymphocytes through Progesterone Induced Blocking Factor (PIBF), thereby diminishing cell-mediated and enhancing humoral immunity[10–12]. This induction of feto-maternal immune tolerance is primarily regulated by unique set of molecules expressed in the human placenta. Extravillous trophoblasts (EVT) that invade the decidua, display various of these immune checkpoint molecules on their cell

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Table 1 | Baseline characteristics (categorial) of non-pregnant vs. pregnant BC patients

Parameter	Category	Non-pregnant *N* = 125, *N* (%)	Pregnant *N* = 125, *N* (%)	Overall *N* = 250, *N* (%)	*P* value
Age at diagnosis, years	18–29	5 (4.0)	14 (11.2)	19 (7.6)	<0.001
	30–34	32 (25.6)	56 (44.8)	88 (35.2)	
	35–39	51 (40.8)	44 (35.2)	95 (38.0)	
	≥40	37 (29.6)	11 (8.8)	48 (19.2)	
HR status combined**	ER and PR negative	50 (40.0)	52 (41.6)	102 (40.8)	0.898
	ER and/or PR positive	75 (60.0)	73 (58.4)	148 (59.2)	
HER2 status**	Negative	98 (81.0)	95 (78.5)	193 (79.8)	0.749
	Positive	23 (19.0)	26 (21.5)	49 (20.2)	
	Missing	4	4	8	
Biological subtype**	TNBC	44 (35.2)	45 (36.0)	89 (35.6)	0.989
	HER2 + /HR−	6 (4.8)	7 (5.6)	13 (5.2)	
	HER2 + /HR+	19 (15.2)	19 (15.2)	38 (15.2)	
	HER2-/HR+	56 (44.8)	54 (43.2)	110 (44.0)	
Histological tumor type**	Ductal or ductal-lobular invasive	101 (80.8)	111 (90.2)	212 (85.5)	0.058
	Lobular invasive	9 (7.2)	7 (5.7)	16 (6.5)	
	Other	15 (12.0)	5 (4.1)	20 (8.1)	
	Missing	0	2	2	
Tumor grading**	G1	1 (0.8)	1 (0.8)	2 (0.8)	0.991
	G2	40 (32.0)	39 (31.2)	79 (31.6)	
	G3	84 (67.2)	85 (68.0)	169 (67.6)	
T stage*	T1	38 (30.4)	38 (30.4)	76 (30.4)	0.947
	T2	65 (52.0)	63 (50.4)	128 (51.2)	
	T3	17 (13.6)	17 (13.6)	34 (13.6)	
	T4	5 (4.0)	7 (5.6)	12 (4.8)	
N stage*	N0	0 (0.0)	58 (46.4)	58 (23.2)	<.001
	N1	89 (71.2)	47 (37.6)	136 (54.4)	
	N2	16 (12.8)	14 (11.2)	30 (12.0)	
	N3	20 (16.0)	6 (4.8)	26 (10.4)	
Ki-67**, at diagnosis	≤20%	70 (61.9)	56 (46.7)	126 (54.1)	0.025
	>20%	43 (38.1)	64 (53.3)	107 (45.9)	
	Missing	12	5	17	
Pregnancy trimester	1st trimester	0 (n.a.)	23 (18.5)	23 (18.5)	n.a.
	2nd trimester	0 (n.a.)	43 (34.7)	43 (34.7)	
	3rd trimester	0 (n.a.)	58 (46.8)	58 (46.8)	
	Missing	125	1***	126	

*pT resp. pN, if not available cT and cN; **assessed from stained TMAs (via IHC) by the central pathology, Marburg; *** in 1 patient BC was histologically diagnosed 6 days postpartum; data are *N* (valid %).

surface, including PD-L1 and TIGIT, also found on malignant cells. Moreover, EVT exhibit minimal expression of classical HLA class I or II molecules but abundant non-classical HLA-G[11,12]. HLA-G diminishes uterine natural killer (NK) -cell cytotoxicity by binding to an Immunoreceptor with a tyrosin-based inhibitory motif (ITIM)[11–14] on their cell surface. It further facilitates the differentiation of naïve T-cells into regulatory T-cells (Tregs), which in turn can suppress the activity of cytotoxic CD8 + T-cells (CTLs)[15,16].

Despite this, pregnant women are often believed to be immunocompromised to some extent[17]. This coupled with delayed initial diagnosis in pregnant women[7], may account for commonly observed advanced tumor stages in PrBC.

To characterize descriptively major differences in baseline characteristics as well as immune response markers between pregnant and non-pregnant BC patients, we compared the expression profiles of immune biomarkers in the BCP cohort (BCP registry study; NCT 00196833) with a matched cohort of non-pregnant BC patients.

Results

Comparative analysis—baseline characteristics

Pregnant BC patients were significantly younger than non-pregnant BC patients (median 34 [26–47 years] vs. 37 [27–47 years], $P < 0.001$). The predominant histologic subtype was ductal or ductal-lobular invasive BC (pregnant: 90.2% vs. non-pregnant: 80.8%) with high grading (G3) (pregnant: 68.0% vs. non-pregnant: 67.2%). At the time of diagnosis, the majority BCP tumors were locally advanced (T2; pregnant: 50.4%) and presented with nodal metastases (N+ pregnant: 53.6%). The BCP cohort had a significantly higher proportion of ER negative tumors (57.6% vs. 41.8%; $P = 0.0016$) and a significantly elevated expression of Ki-67 (>20%: 53.3% vs 38.1%, $P = 0.025$).

Categorial baseline characteristics of both cohorts are shown in Table 1.

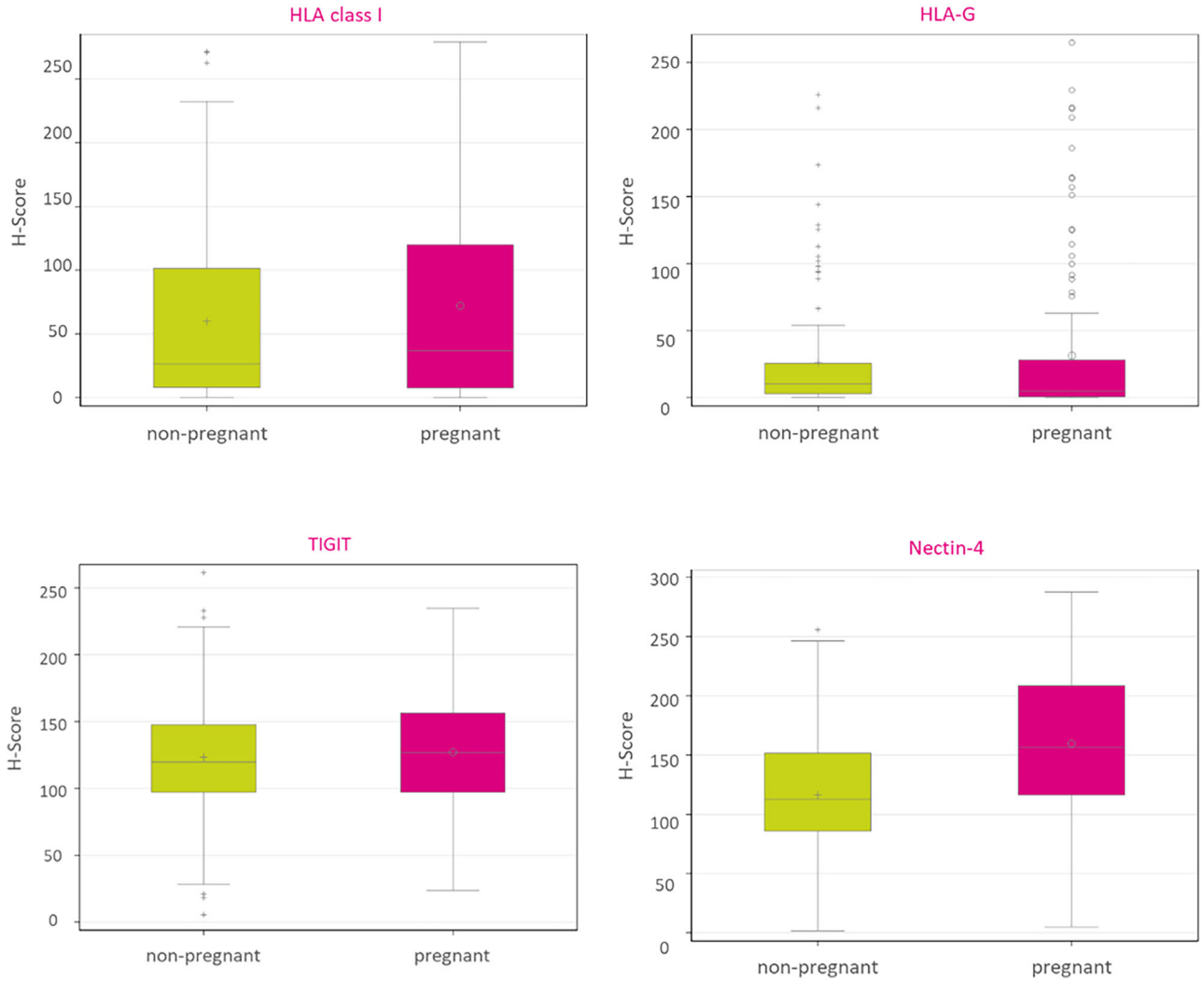


Fig. 1 | Boxplots of Continous biomarkers (HLA, HLA-G, TIGIT, Nectin-4). Boxplots for HLA class I, HLA-G, TIGIT,Nectin-4 in non-pregnant and pregnant cohort.

Expression of immunological markers in both cohorts

The expression of HLA-G demonstrated significant differences in both cohorts, yielding opposing results with each quantification method. While the median HLA-G H-score was higher in the non-pregnant cohort (4.9 vs 10.6, $P = 0.012$), the median percentage of HLA-G expression was higher in the pregnant cohort (3.7% vs 2.0%, $P = 0.007$). A weak correlation between the H-scores of HLA class I and HLA-G was observed in the non-pregnant cohort, and a moderate correlation was noted for both markers in the pregnant cohort (Spearman's $\rho = 0.27$ and 0.45, respectively). Except for Nectin-4, which exhibited significantly higher expression in both quantification methods for the BCP cohort (median 98.9% vs. 94.8%, $P < 0.001$ and H-score 156 vs. 113, $P < 0.001$) compared to non-pregnant cohort, no significant differences were found for TIGIT, PD-L1, the H-score of HLA class I and the number of TILs (Fig. 1; see also exemplary TMA slides in Figs. 2–4).

Given the positive correlation observed between the expression of Nectin-4 and Ki-67 in other studies[18,19], we performed a post-hoc analysis and found that in both cohorts, patients with Ki-67 > 20% exhibited a significantly increased Nectin-4 expression (Table 2).

In a further post-hoc analysis, we compared HR- (defined as ER and PR negative) and HR+ (defined as ER and/ or PR positive) subgroups within both cohorts examining their Nectin-4 expression. Within the non-pregnant cohort, the HR− subgroup exhibited a significant overexpression of Nectin-4 in both quantification methods (% and H-score). In contrast, within the pregnant cohort, only the H-score of Nectin-4 was significantly higher in the HR− subgroup (Table 3).

Subgroup analysis: pregnant cohort and non-pregnant cohort

Comparisons between pregnant patients with T1/2 vs. T3/4, N0 vs. N+ and 1st vs. 2nd vs. 3rd trimester revealed no significant differences in the expression of HLA class I, HLA-G, PD-L1, TIGIT and Nectin-4. Among non-pregnant patients, those with more advanced tumor stages (T3/4) exhibited a significantly higher Nectin-4 H-Score than patients with T1/2 tumors (Median 129 vs. 107, $P = 0.049$).

Pregnant patients with TILs ≤25% exhibited a significantly lower expression of HLA class I compared to pregnant patients with TILs >25% (mean 36.4% vs. 80.9%, $P = 0.003$, H-Score 68.0 vs. 178, $P = 0.002$). Similar results were found in the non-pregnant cohort, where patients with TILs ≤25% (median 12.9% vs. 73.3%, $P = 0.004$, H-score: 24.6 vs. 110.0, $P = 0.010$) demonstrated a significantly reduced expression of HLA class I compared to non-pregnant patients with TILs >25%.

Additionally, the count of PD-L1 negative IC in pregnant BC patients with TILs ≤25% was significantly higher than in pregnant BC patients with TILs >25% (PD-L1 negative IC 84.6% vs. 28.6%, $P = 0.004$). Corresponding results were found in the non-pregnant cohort, where patients with TILs ≤25% had a significantly higher number of PD-L1 negative IC than patients with TILs >25% (PD-L1 negative IC 89.0% vs. 33.3%, $P < 0.001$).

Significant difference can be demonstrated of combined PD-L1 and PD-L1 IC concerning the ER-status in HER2− pregnant cohort. In TNBC the combined and positive PD-L1 IC value was higher than in ER+/HER2− BC (PD-L1 combined 30.4% vs 10.8%, $P > 0.016$ and PD-L1 IC 32.8% vs 10.8%, $P > 0.041$; data not shown).

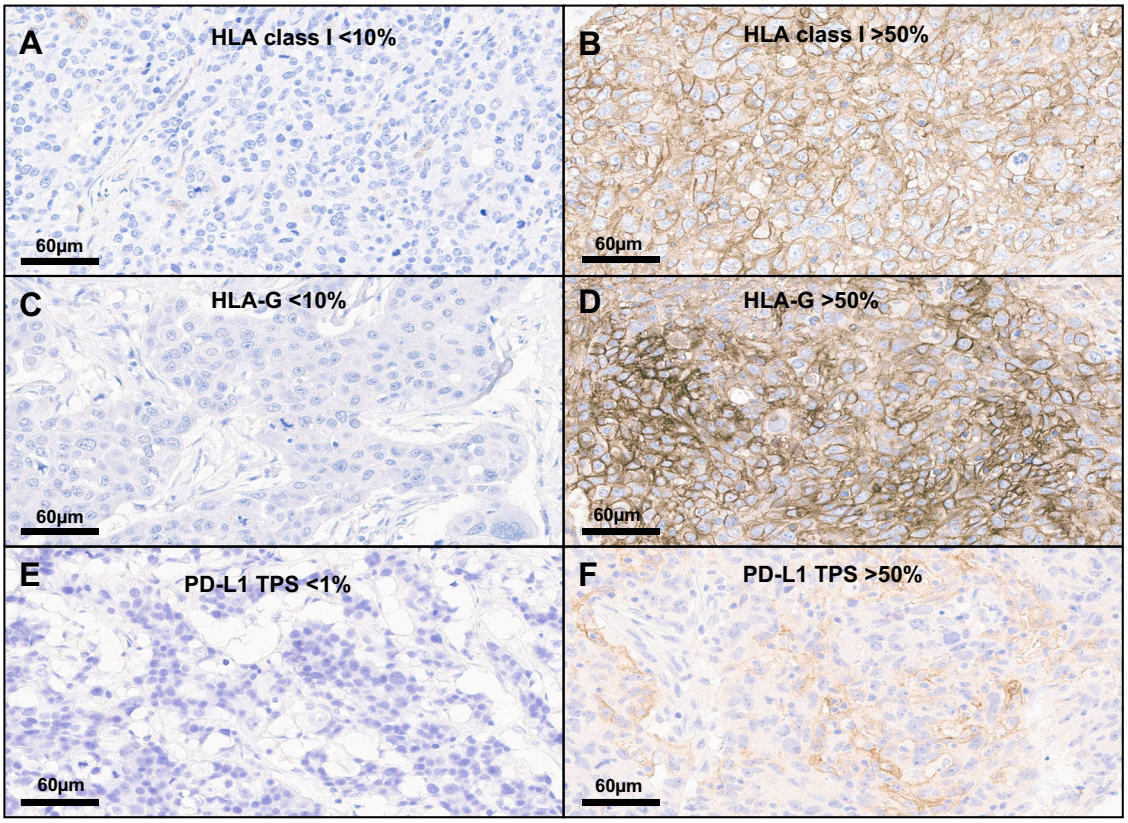
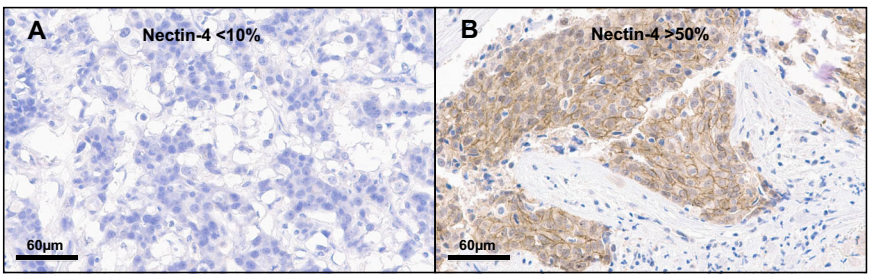


Fig. 2 | TMA slides of biomarkers HLA class I, HLA-G, PD-L1. A–C TMA slide example with <10% (**A**) and > 50% positive (**B**) membranous staining for HLA class I (clon EMR8-5); TMA slide example with <10% (**C**) and >50% positive (**D**) membranous staining for HLA-G (clon 4H84); TMA slide example with TPS <1% (**E**) and with TPS > 50% positive (**F**) membranous staining for PD-L1 (clon 22C3). TPS tumor proportion score.

Fig. 3 | TMA slides of biomarker Nectin-4. Exemplary TMA slide with <10% (**A**) and >50% (**B**) positive membranous staining for Nectin-4 (clon EPR15613-68).



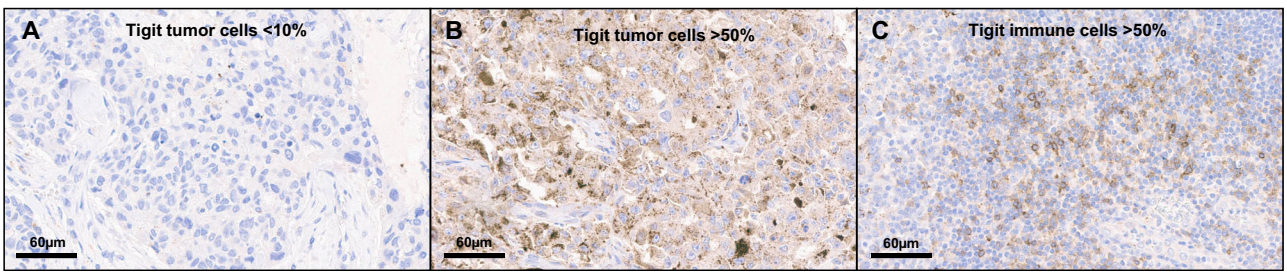


Fig. 4 | TMA slides of biomarker TIGIT. Exemplary TMA slide with <10% (**A**) and >50% positive cytoplasmatic staining for TIGIT (clon BLR047F) on tumor cells (**B**) and TILs (**C**).

Discussion

The primary objective of the analysis was to compare the expression of HLA class I, HLA-G, PD-L1, TIGIT and Nectin-4 exploratively between pregnant and young, non-pregnant BC patients. Our results revealed a significantly higher expression of Nectin-4 in the pregnant cohort.

Differences in proliferation rates between the cohorts could be partially attributed to the age difference between both cohorts, as higher Ki-67 rates

Table 2 | Post-hoc analysis Wilcoxon–Mann–Whitney tests for Nectin-4 (%, H-score) between pregnant and non-pregnant patients with a low (≤20%) vs. high (>20%) Ki-67

		Pregnant				Non-pregnant			
Parameter	**Category**	**Ki-67 ≤20% *N* = 56**	**Ki-67 >20% *N* = 64**	**Overall *N* = 120 *N* (%)**	***P* value**	**Ki-67 ≤20%, *N* = 70**	**Ki-67 >20%, *N* = 43**	**Overall *N* = 113, *N* (%)**	***P* value**
Nectin-4 H-score	Median (range)	122 (4.9, 255)	193 (35.3, 288)	159 (4.9, 288)	<0.001	99.7 (1.7, 246)	135 (21.5, 256)	113 (1.7, 256)	<0.001
	Missing	2	0	2		5	1	6	
Nectin-4 (%)	Median (range)	97.5 (4.8, 100)	99.6 (35.2, 100)	99.0 (4.8, 100)	<0.001	90.4 (1.6, 100)	97.8 (21.0, 100)	94.3 (1.6, 100)	<0.001
	Missing	2	0	2		5	1	6	

Table 3 | Post-hoc analysis Wilcoxon–Mann–Whitney tests for Nectin-4 (%, H-score) between HR- vs. HR+ pregnant and non-pregnant patients

		Pregnant				Non-pregnant			
Parameter	**Category**	**HR− *N* = 52**	**HR + *N* = 73**	**Overall *N* = 125, *N* (%)**	***P* value**	**HR− *N* = 50**	**HR + *N* = 75**	**Overall *N* = 125, *N* (%)**	***P* value**
Nectin-4 H-score	Median (range)	176 (18.6, 283)	144 (4.9, 288)	156 (4.9, 288)	0.045	127 (21.5, 256)	97.8 (1.7, 213)	113 (1.7, 256)	<0.001
	Missing	3	3	6		3	5	8	
Nectin-4 (%)	Median (range)	99.4 (17.8, 100)	98.8 (4.8, 100)	98.9 (4.8, 100)	0.135	98.1 (21.0, 100)	89.9 (1.6, 100)	94.8 (1.6, 100)	<0.001
	Missing	3	3	6		3	5	8	

are more common in younger BC patients[20–22]. However, there are also biological explanations. Elevated Ki-67 rates could result from a general increase in ductal proliferation in mammary tissue during pregnancy due to elevated levels of estrogen in preparation for lactation[23].

Interestingly, tumors in the pregnant cohort exhibited significantly higher proliferation rates according to Ki-67 rates. High proliferation is associated with faster progression[24]. In addition to the recognized challenges of detecting malignancies in the pregnant breast, the notably higher proliferation activity observed in the BCP cohort as measured by the expression of Ki-67 expression may further explain why PrBC tends to present at a more advanced stage at diagnosis[6–9]. Tumors with high proliferation rates, tend to be more aggressive but also demonstrate a better response to neoadjuvant chemotherapy[24], emphasizing the importance of timely treatment for pregnant BC patients.

Another factor could be the high Nectin-4 expression in the pregnant cohort. Nectin-4 overexpression is associated with various adverse conditions in malignancies, including distant metastasis, locally advanced disease, and overall poorer survival[18,19]. Nectins are Ca^{2+} -independent immunoglobulin-like cell adhesion molecules that are involved in regulating diverse cell functions and communications[25]. While Nectin-1, -2 and -3 are widely expressed on healthy adult tissue, Nectin-4 expression is primarily restricted to fetal or placental tissue but is also present in various human cancers[26].

Nectin-4 is recognized for its ability to activate the Pi3K/Akt-signaling pathway, thereby enhancing cell proliferation and promoting endothelial-to-mesenchymal transition (EMT)[27,28]. While, the interactions between Nectin-4 and the Pi3K/Akt-signaling pathway were not specifically investigated this study, their potential connection is intriguing, especially considering their shared expression on trophoblasts. Trophoblastic migration, cell proliferation and invasion are regulated through Pi3K/Akt[29]. Abnormal placentation has been associated with disrupted Pi3K/Akt-signaling[30], and the upregulation of Nectin-4 expression on trophoblasts[31]. Therefore, it is plausible that Nectin-4 also interacts with Pi3K/Akt-pathway on trophoblasts, potentially influenced by pregnancy-specific factors.

One of these pregnancy-specific factors is the elevated level of steroid hormones. As mentioned earlier, estrogen promotes ductal proliferation in the pregnant breast[23]. It is known that in the presence of high estrogen levels estrogen receptors (ERs) are typically downregulated[32]. Consequently, this downregulation could potentially result in an upregulation of estrogen-related receptors (ERR). ERRs are speculated to directly compete with ERs, leading to alterations in other's functions[33]. Unlike ERs, ERRs do not directly bind to natural estrogen but exert influence on the transcription of the same estrogen-responsive elements. For instance, they may enhance the expression of Nectin-4 through the Pi3K/Akt pathway[34].

Previous studies have highlighted an association between Nectin-4 overexpression and a negative HR status[19,26]. Given this, along with the notably increased prevalence of ER negative tumors in the pregnant cohort (57.6% vs. 41.8%; $P = 0.0016$), we were prompted to examine the consistency of our findings with existing literature and performed a post-hoc analysis. It is essential to acknowledge that the results from our post-hoc analysis regarding HR-status and Nectin-4 expression may be subject to distortion. A notable aspect is that 16% of the pregnant patients are ER negative and PR positive, whereas only 1.6% of the non-pregnant patients are ER−/PR + . This imbalance in the ER status between the cohorts raises the possibility that ER negativity might leads to an upregulation of ERRs potentially causing Nectin-4 overexpression. Consequently, the 16% of ER −/PR+ pregnant patients might impact the accuracy of our analysis. This presents a limitation of our study and calls for further investigation.

Within TILs, T-cells are the predominant cell-type, followed by B- and NK-cells[35]. BC is generally considered to be less immunogenic compared to other tumors such as melanoma[36]. This notion is supported by our data where 92.6% of the non-pregnant and 86.9% of the pregnant patients had low TILs (≤25%). The upregulation of HLA class I expression improves T-cell recognition[37]. Accordingly, the subgroup "TILs >25%" in both cohorts exhibited a significantly higher expression of HLA class I. While the overall expression of HLA class I molecules in percentage was significantly higher in the pregnant cohort (27.7% vs. 13.8%, $P = 0.029$), there were no significant differences in categorial TILs between the two cohorts. A recent study by Sajjadi et al. obtained similar results showing no significant differences regarding the number of TILs in PrBC and early-onset BC (EOBC)[20]. These findings contradict previous observations of reduced TILs in pregnant BC patients compared to non-pregnant BC patients, suggesting a pregnancy-induced reduction in maternal immunity[38]. Our data, however, indicates that TILs, although generally low in both cohorts, can vary across biological subtypes. The immune cell infiltration is associated with the biological subtype, typically being highest in TNBC and lowest in HR + / HER2− BC[20,39,40]. Missing differences in our cohort could therefore be explained by our matching process which, in contrast to the other study[38], included "biological subtype" as a matching variable. Nevertheless, it is important to note that these results are limited by the fact that 64 patients in the pregnant cohort have missing data for TILs.

Breast cancer incidence tends to increase with age[41], while fertility experiences a decline[42]. The age group 30–34 is characterized by high birth rates[43] and exhibits a relatively increased incidence of BC compared to younger women[44]. The highest incidence of PrBC was observed in the age group 30–34 years, aligning with previous studies[45,46]. Despite our matching process, pregnant BC patients were younger than non-pregnant BC patients, presenting a limitation of our study as age at diagnosis influences tumor biology[21,22]. The correlation between the age group with the highest birth rate and the most cases of PABC was also been noted by Andersson et al.[2] It is conceivable that if women continue to postpone childbearing into their late thirties, the incidence of PrBC is likely to increase further. Additionally, our matching process using the Mahalanobis distance accommodates this age difference, pairing two matching partners closest to each other while considering all variables[47].

The main limitation of this study results from the 100% nodal positivity observed in the GAIN cohort. It is well-established that the TME varies between primary and metastatic disease[48,49]. While the majority (53.6%) of the pregnant patients in our study exhibited nodal positivity, the inclusion of node negative non-pregnant patients could have enhanced the validity of our results and potentially influenced the expression patterns of immunomarkers. Another limitation is the lack of information on parity status and period between BC diagnosis and latest pregnancy, if applicable, in the GAIN cohort. In addition, the results of this study are subject to some irregularity as BC risk, tumor biology and thereby tumor aggressiveness may vary. Moreover, these three factors are most unfavorable shortly after birth and only improve in the following postpartum years, so the results obtained here should be viewed with caution[5,50]. The cohort was selected primarily due to the availability of extensive pre-existing TMAs and associated data from GAIN patients.

For future BC studies an inclusion of parity status and time of last pregnancy in clinical data collection is advisable.

Our results indicate that BC during and outside of pregnancy exhibit similarities but also notable differences. The PrBC appears to manifest with higher proliferation rates. Furthermore, the overexpression of Nectin-4 and its association with disease progression suggest that the typically more advanced stages observed in PrBC might not solely result from delayed diagnosis. Elevated estrogen levels during pregnancy could potentially influence tumor biology and induce alterations in the TME of PrBC, warranting investigation. Despite the immune alterations associated with, our study found no evidence of a diminished anti-tumor immune response in the pregnant cohort.

Nevertheless, as in any malignant disease timely diagnosis and treatment of PrBC is crucial to optimize the outcome for mother and child. Further information on cancer management during pregnancy can be found on https://esgo.org/network/incip/ [51].

Establishing Nectin-4 as a standard marker in PrBC or any malignancy occurring during pregnancy might improve our understanding of its expression patterns and may represent a potential targeted treatment option to modify standard chemotherapy approaches.

Methods

Patient population

Our pregnant cohort comprised pregnant BC patients enrolled in the Breast Cancer in Pregnancy (BCP) registry study (GBG-29/NCT00196833) by the German Breast Group (GBG)[9]. The trial was conducted according to the Declaration of Helsinki. Between April 2003 and March 2019, a total of 2831 patients were registered. Out of these, tumor samples with representative and untreated tumor regions were available for 127 early breast cancer patients. The study was approved by the ethics committee of Goethe University Frankfurt (Reference No. 254/02), Germany. Written informed consent was obtained prospective from patients who give their willingness to participate in this analysis.

Formalin-fixed paraffin-embedded (FFPE) tissue samples with untreated tumor material from either core biopsies for patients with neoadjuvant treatment ($n = 23$) and unknown therapy setting ($n = 6$) or surgical specimens for patients with adjuvant treatment ($n = 97$) were used to construct tissue microarrays (TMA). Patients receiving palliative treatment ($n = 1$) were excluded. The pregnant cohort was matched with a suitable non-pregnant BC cohort with existing TMAs drawn from the GAIN study ($n = 1305$), which recruited node-positive, high-risk early BC patients randomized to two distinct dose-dense regimens[52]. Matching criteria included age, grading, biological subtype, tumor and nodal stage. Among them, 125 BCP patients and 522 patients from the GAIN study had available information in all matching variables. Additionally, patients from the GAIN study were excluded if postmenopausal and/or older than >47 years (maximum age in the BCP cohort).

Statistical analysis

The nearest neighbour matching in a 1:1 ratio and by using the Mahalanobis distance without replacement[47], was selected as an appropriate method for multivariate cohort matching after a comparison with different other matching methods (propensity score matching, coarsened exact matching (CEM), weighted regression analysis). Although similar results were obtained from other methods, the Mahalanobis distance without replacement proved superior because it allowed the inclusion all 125 pregnant patients and simultaneously provided an acceptable balance of all matching variables between both cohorts. The matching process was executed in R, version 4.1.0, especially employing the R package MatchIt, version 4.1.2[53]. Statistical comparisons between the pregnant and non-pregnant cohorts, as well as between the different subgroups were performed using the Wilcoxon test for two-group comparisons, the Kruskal–Wallis test for comparisons between three groups (for continuous parameters) as well as Fisher's exact test and Pearson's χ^2 test (for binary and categorical parameters). Correlations between markers were analysed by Spearman's rho. All statistical tests were descriptive, and data analysis was performed with SAS® version 9.4 using SAS Enterprise Guide Version 8.3 on Microsoft Windows 10 Enterprise.

Methods

We evaluated classical prognostic breast cancer biomarkers (ER, PR, HER2, Ki-67) and immune response markers (HLA class I, HLA-G, PD-L1, TIGIT and Nectin-4) in a cohort of BC patients. TMAs underwent immunohistochemistry (IHC) staining to evaluate estrogen and progesterone receptor (ER, PR) status and human epidermal growth factor receptor 2 (HER2) status in accordance with the ASCO/ CAP guidelines[54,55]. Additionally, Ki-67 and markers relevant to immune response including HLA class I, HLA-G, PD-L1, TIGIT and Nectin-4, were assessed.

Hematoxylin-eosin-stained slides were analyzed to assess the count of stromal TILs in accordance with the guidelines of the International Immuno-Oncology Biomarker Working Group[56]. The TILs were categorized as low ≤25%, intermediate 26–60% and high >60%.

Positive nuclear staining in tumor cells was employed to assess the expression of Ki-67 as percentage, using the MIB-1 antibody (1:100 dilution, EDTA AG-retrieval, Dako/Agilent). The staining was then categorized as ≤20% vs. >20%. For PD-L1 expression assessment on tumor (TC) and immune cells (IC), the 22C3 antibody (1:50 dilution, EDTA AG-retrieval, Dako/Agilent) was used (see Fig. 1G–I). Positive expression was defined as ≥1% of TC or IC (proportion score) exhibiting membranous staining.

In addition to assessing the percentage of positive membranous staining for HLA class I (MHC I, HLA-ABC) with the EMR8-5 antibody (1:10,000 dilution, Citrat AG-retrieval, Abcam) (see Fig. 1A–C), HLA-G with the 4H84 antibody (1:100 dilution, Citrat AG-retrieval, Abcam) (see Fig. 1D–F), Nectin-4 with the EPR15613-68 antibody (1:2000 dilution, Trilogy AG-retrieval, Abcam) (see Fig. 2A–C) and TIGIT with the BLR047F antibody (1:500 dilution, Trilogy AG-retrieval, Abcam) (see Fig. 3A–D), H-scores for these markers were calculated as continuous variables. The formula used for calculation was: 3× percentage of strongly staining tumor cells + 2× percentage of moderately staining tumor cells + percentage of weakly staining tumor cells, ranging from 0 to 300[57]. Experimental biomarkers, including TIGIT, Nectin-4, HLA-A, and HLA-G were assessed using QuPath's (V0.2.2)[58] digital semi-automatic positive cell detection. Ki-67 on BCP/TMA was assessed using the Ki-67 Quantifier Tool from the Cognition Master Professional Suite (VMScope, Berlin, Germany).

Data availability

All relevant data are within the paper and its supporting information files. The data underlying the results presented in the study are available from GBG. Some restrictions apply due to confidentiality of patient data. Since these data are derived from a prospective clinical trial with ongoing follow-up collection, there are legal and ethical restrictions to sharing sensitive patient-related data publicly. Interested groups may request the "Cooperation Proposal Form" from trafo@gbg.de. Data can be requested in context of a translational research project by sending the form back to trafo@gbg.de. Translational research proposals are approved by the GBG scientific boards.

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References

1. Sung, H. et al. Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* **71**, 209–249 (2021).
2. Andersson, T. M.-L., Johansson, A. L. V., Hsieh, C.-C., Cnattingius, S. & Lambe, M. Increasing incidence of pregnancy-associated breast cancer in Sweden. *Obstet. Gynecol.* **114**, 568–572 (2009).
3. Eibye, S., Kjær, S. K. & Mellemkjær, L. Incidence of pregnancy-associated cancer in Denmark, 1977-2006. *Obstet. Gynecol.* **122**, 608–617 (2013).
4. Lee, Y. Y. et al. Incidence and outcomes of pregnancy-associated cancer in Australia, 1994-2008: a population-based linkage study. *BJOG* **119**, 1572–1582 (2012).
5. Amant, F. et al. The definition of pregnancy-associated breast cancer is outdated and should no longer be used. *Lancet Oncol.* **22**, 753–754 (2021).
6. Sajjadi, E. et al. Immune microenvironment dynamics in breast cancer during pregnancy: impact of gestational age on tumor-infiltrating lymphocytes and prognosis. *Front Oncol.* **13**, 1116569 (2023).
7. Amant, F., Loibl, S., Neven, P. & van Calsteren, K. Breast cancer in pregnancy. *Lancet* **379**, 570–579 (2012).
8. Gooch, J. C. et al. Pregnancy-associated breast cancer in a contemporary cohort of newly diagnosed women. *Breast J.* **26**, 668–671 (2020).
9. Amant, F. et al. Prognosis of women with primary breast cancer diagnosed during pregnancy: results from an international collaborative study. *J. Clin. Oncol.* **31**, 2532–2539 (2013).
10. Szekeres-Bartho, J. & Polgar, B. PIBF: the double edged sword. Pregnancy and tumor. *Am. J. Reprod. Immunol.* **64**, 77–86 (2010).
11. Poole, J. A. & Claman, H. N. Immunology of pregnancy: implications for the mother. *CRIAI* **26**, 161–170 (2004).

12. Gregori, S., Amodio, G., Quattrone, F. & Panina-Bordignon, P. HLA-G orchestrates the early interaction of human trophoblasts with the maternal niche. *Front. Immunol.* **6**, 128 (2015).
13. Koopman, L. A. et al. Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. *J. Exp. Med.* **198**, 1201–1212 (2003).
14. Kopcow, H. D. et al. Human decidual NK cells form immature activating synapses and are not cytotoxic. *Proc. Natl. Acad. Sci. USA* **102**, 15563–15568 (2005).
15. Joller, N. et al. Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. *Immunity* **40**, 569–581 (2014).
16. Al-Khunaizi, N. R., Tabbara, K. S. & Farid, E. M. Is there a role for HLA-G in the induction of regulatory T cells during the maintenance of a healthy pregnancy?. *Am. J. Reprod. Immunol.* **84**, e13259 (2020).
17. Klein, S. L., Passaretti, C., Anker, M., Olukoya, P. & Pekosz, A. The impact of sex, gender and pregnancy on 2009 H1N1 disease. *Biol. Sex Differ.* **1**, 5 (2010).
18. Pavlova, N. N. et al. A role for PVRL4-driven cell-cell interactions in tumorigenesis. *eLife* **2**, e00358 (2013).
19. Fabre-Lafay, S. et al. Nectin-4 is a new histological and serological tumor associated marker for breast cancer. *BMC Cancer* **7**, 73 (2007).
20. Sajjadi, E. et al. Breast cancer during pregnancy as a special type of early-onset breast cancer: analysis of the tumor immune microenvironment and risk profiles. *Cells* **11**, 2286 (2022)
21. Vasseur, F., Baranzelli, M.-C., Fournier, C. & Bonneterre, J. Ki67 chez les patientes jeunes présentant un cancer du sein. *Gynecol. Obstet. Fertil.* **41**, 16–19 (2013).
22. Zouzoulas, D. et al. Breast cancer in women younger than 35 years old. *Arch. Gynecol. Obstet.* **302**, 721–730 (2020).
23. Alex, A., Bhandary, E. & McGuire, K. P. Anatomy and Physiology of the Breast during Pregnancy and Lactation. *Adv. Exp. Med. Biol.***1252**, 3–7 (2020).
24. Denkert, C. et al. Strategies for developing Ki67 as a useful biomarker in breast cancer. *Breast* **24**, S67–S72 (2015).
25. Samanta, D. & Almo, S. C. Nectin family of cell-adhesion molecules: structural and molecular aspects of function and specificity. *Cell Mol. Life Sci.* **72**, 645–658 (2015).
26. M-Rabet, M. et al. Nectin-4: a new prognostic biomarker for efficient therapeutic targeting of primary and metastatic triple-negative breast cancer. *Ann. Oncol.* **28**, 769–776 (2017).
27. Zhang, Y. et al. Nectin-4 promotes gastric cancer progression via the PI3K/AKT signaling pathway. *Hum. Pathol.* **72**, 107–116 (2018).
28. Siddharth, S. et al. Nectin-4 is a breast cancer stem cell marker that induces WNT/β-catenin signaling via PI3K/Akt axis. *Int. J. Biochem. Cell Biol.* **89**, 85–94 (2017).
29. Ding, J. et al. M2 macrophage-derived G-CSF promotes trophoblasts EMT, invasion and migration via activating PI3K/Akt/Erk1/2 pathway to mediate normal pregnancy. *J. Cell Mol. Med.* **25**, 2136–2147 (2021).
30. Shen, H. et al. CD97 Is Decreased in Preeclamptic placentas and promotes human trophoblast invasion through PI3K/Akt/mTOR signaling pathway. *Reprod. Sci.* **27**, 1553–1561 (2020).
31. Ito, M. et al. Potential role for nectin-4 in the pathogenesis of pre-eclampsia: a molecular genetic study. *BMC Med. Genet.* **19**, 166 (2018).
32. Borrás, M. et al. Estradiol-induced down-regulation of estrogen receptor. Effect of various modulators of protein synthesis and expression. *J. Steroid Biochem. Mol. Biol.* **48**, 325–336 (1994).
33. Ariazi, E. A. & Jordan, V. C. Estrogen-related receptors as emerging targets in cancer and metabolic disorders. *Curr. Top. Med. Chem.* **6**, 203–215 (2006).
34. Wang, L. et al. Estrogen-related receptor-α promotes gallbladder cancer development by enhancing the transcription of Nectin-4. *Cancer Sci.* **111**, 1514–1527 (2020).
35. Park, I. H. et al. Prognostic implications of tumor-infiltrating lymphocytes in association with programmed death ligand 1 expression in early-stage breast cancer. *Clin. Breast Cancer* **16**, 51–58 (2016).
36. Vonderheide, R. H., Domchek, S. M. & Clark, A. S. Immunotherapy for breast cancer: what are we missing?. *Clin. Cancer Res.* **23**, 2640–2646 (2017).
37. René, C., Lozano, C. & Eliaou, J.-F. Expression of classical HLA class I molecules: regulation and clinical impacts: Julia Bodmer Award Review 2015. *HLA* **87**, 338–349 (2016).
38. Azim, H. A. et al. Tumour infiltrating lymphocytes (TILs) in breast cancer during pregnancy. *Breast* **24**, 290–293 (2015).
39. Denkert, C. et al. Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. *Lancet Oncol.* **19**, 40–50 (2018).
40. Denkert, C. et al. Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. *J. Clin. Oncol.* **28**, 105–113 (2010).
41. White, M. C. et al. Age and cancer risk: a potentially modifiable relationship. *Am. J. Prev. Med.* **46**, S7–S15 (2014).
42. American College of Obstetricians and Gynecologists Committee on Gynecologic Practice and Practice Committee. Female age-related fertility decline. Committee Opinion No. 589. *Fertil. Steril*. **101**, 633–634 (2014).
43. Statistisches Bundesamt Deutschland - GENESIS-Online. https://www-genesis.destatis.de/genesis/online?operation=tables&levelindex=0&levelid=1664812308377&sortdirection=auf&code=12612-0008&kmaauswahl.x=0&kmaauswahl.y=0#abreadcrumb (2022).
44. Krebs - Brustkrebs. https://www.krebsdaten.de/Krebs/DE/Content/Krebsarten/Brustkrebs/brustkrebs_node.html (2022).
45. Reed, W. et al. Pregnancy and breast cancer: a population-based study. *Virchows Arch.* **443**, 44–50 (2003).
46. Bonnier, P. et al. Influence of pregnancy on the outcome of breast cancer: a case-control study. *Int. J. Cancer* **72**, 720–727 (1997).
47. McLachlan, G. J. Mahalanobis distance. *Reson* **4**, 20–26 (1999).
48. Soysal, S. D., Tzankov, A. & Muenst, S. E. Role of the tumor microenvironment in breast cancer. *Pathobiol. J. Immunopathol. Mol. Cell. Biol.* https://pubmed.ncbi.nlm.nih.gov/26330355/ (2015).
49. Schlam, I. et al. The tumor immune microenvironment of primary and metastatic HER2− positive breast cancers utilizing gene expression and spatial proteomic profiling. *J. Transl. Med.* **19**, 1–14 (2021).
50. Bounous, V. E. et al. Impact of pregnancy on breast cancer features and prognosis. *Curr. Oncol.* **31**, 2305–2315 (2024).
51. Loibl, S. et al. ESMO Expert Consensus Statements on the management of breast cancer during pregnancy (PrBC). *Ann. Oncol.* **34**, 849–866 (2023).
52. Möbus et al. German Adjuvant Intergroup Node-positive Study (GAIN): a phase III trial comparing two dose-dense regimens (iddEPC versus ddEC-PwX) in high-risk early breast cancer patients. *Ann. Oncol.* **28**, 1803–1810 (2017).
53. Ho, D. E., Imai, K., King, G. & Stuart, E. A. MatchIt nonparametric preprocessing for parametric causal inference. *J. Stat. Soft*. **42**, 1–28 (2011).
54. Wolff, A. C. et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. *J. Clin. Oncol.* **31**, 3997–4013 (2013).
55. Allison, K. H. et al. Estrogen and progesterone receptor testing in breast cancer: ASCO/CAP Guideline Update. *J. Clin. Oncol.* **38**, 1346–1366 (2020).
56. Salgado, R. et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. *Ann. Oncol.* **26**, 259–271 (2015).

57. Kraus, J. A., Dabbs, D. J., Beriwal, S. & Bhargava, R. Semi-quantitative immunohistochemical assay versus oncotype DX(®) qRT-PCR assay for estrogen and progesterone receptors: an independent quality assurance study. *Mod. Pathol.* **25**, 869–876 (2012).
58. Bankhead, P. et al. QuPath: open source software for digital pathology image analysis. *Sci. Rep.* **7**, 16878 (2017).

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Author contributions

The study was conducted at the German Breast Group under supervision of S.L. K.G. was responsible for tissue collection from the participating clinics and centres for the BCP cohort. K.G. did the planning of the analyses, selection of immune markers and antibodies with S.L., C.D. and P.J. An introduction to the VMscope was given by P.J. to K.G., which made an exemplary analysis of Ki-67. Immune markers HLA class I, HLA-G, TIGIT and PD-L1 were analysed by M.G., Nectin-4 analysis was done by A.L. K.G. and J.R. planned and constructed the Statistical Analysis Plan. Statistics were performed by J.R. The manuscript was written by K.G. with the aid of J.H. All authors have read and approved the manuscript.

Competing interests

K. Galas has no conflict of interest to declare. The study was funded by the GBG and Philipps-University Marburg, Germany. P. Jank reports research grant and travel costs from Gilead Sciences GmbH (outside of the submitted project). C. Schem declares honoraria from Roche, Lilly, AstraZeneca, MSD Oncology, Exact Sciences and Novartis; operate in a consulting or advisory role for Novartis, AstraZeneca and Roche; reports honoraria for speakers' bureau from Roche, AstraZeneca and Novartis; CS also reports to receive research funding from Roche (Inst.), Daiichi-Sankyo Europe GmbH (Inst.), AstraZeneca (Inst.), GlaxoSmithKline (Inst.), Novartis (Inst.) and Lilly (Inst.); and to receive travel accommodations and expenses from Pfizer, Roche, AstraZeneca, Novartis and Gilead Sciences. J. Rey and V. Nekljudova declare to be GBG Forschungs GmbH employee. GBG Forschungs GmbH received funding for research grants from AbbVie, Amgen, AstraZeneca, BMS, Daiichi-Sankyo, Gilead, Molecular Health, Novartis, Pfizer and Roche (paid to the institution); other (non-financial/medical writing) from Daiichi-Sankyo, Gilead, Novartis, Pfizer, Roche and Seagen (paid to the institution). GBG Forschungs GmbH has licensing fees from VMscope GmbH. In addition, GBG Forschungs GmbH has a patent EP21152186.9 pending, a patent EP19808852.8 pending, and a patent EP14153692.0 pending. C. Denkert reports grants from European Commission H2020, grants from German Cancer Aid Translational Oncology, grants from German Breast Group, during the conduct of the study; personal fees from Novartis, personal fees from Roche, personal fees from MSD Oncology, personal fees from Daiichi-Sankyo, personal fees from AstraZeneca, from Molecular Health, grants from Myriad, personal fees from Merck, other from Sividon diagnostics, outside the submitted work; In addition, Dr. Denkert has a patent VMScope digital pathology software with royalties paid, a patent WO2020109570A1—cancer immunotherapy pending, and a patent WO2015114146A1 and WO2010076322A1—therapy response issued. J. Holtschmidt reports personal fees and non-financial support from Daiichi-Sankyo, non-financial support from Hologic, personal fees from MSD Oncology, Novartis, Palleos Health Care, Pfizer, Roche Pharma and Seagen, outside the submitted work; he also declares to be GBG Forschungs GmbH employee. GBG Forschungs GmbH received funding for research grants from AbbVie, Amgen, AstraZeneca, BMS, Daiichi-Sankyo, Gilead, Molecular Health, Novartis, Pfizer and Roche (paid to the institution); other (non-financial/medical writing) from Daiichi-Sankyo, Gilead, Novartis, Pfizer, Roche and Seagen (paid to the institution). GBG Forschungs GmbH has licensing fees from VMscope GmbH. In addition, GBG Forschungs GmbH has a patent EP21152186.9 pending, a patent EP19808852.8 pending, and a patent EP14153692.0 pending. S. Loibl declares to be GBG Forschungs GmbH employee (CEO); The company receives grants from AbbVie, AstraZeneca, Celgene, Daiichi-Sankyo, Immunomedics/Gilead, Molecular Health, Novartis, Pfizer and Roche; honoraria for Advisory board from Abbvie, Amgen, AstraZeneca, BMS, Celgene, DSI, EirGenix, Gilead, GSK, Lilly, Merck, Novartis, Olema, Pfizer, Pierre Fabre, Relay Therapeutics, Roche, Sanofi and Seagen; honoraria as invented speaker from AstraZeneca, DSI, Gilead, Novartis, Pfizer, Roche, Seage and Medscape. S. Loibl reports non-financial interest as advisory role in AGO Kommission Mamma, PI Aphinity (principal investigator) as member in AGO, ASCO, DKG, ESMO and other non-financial interest from AstraZeneca, Daiichi-Sankyo, Gilead, Novartis, Pfizer, Roche and Seagen. GBG Forschungs GmbH has following/patents pending: EP14153692.0, EP21152186.9, EP19808852.8 and receives licensing fees from VM Scope GmbH.

Additional information

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