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Androgen receptor expression inversely correlates with immune cell infiltration in HER2-positive breast cancer

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ABSTRACT

Introduction: Although targeting human epidermal growth factor receptor 2 (HER2) is a meaningful treatment in HER2-positive breast cancer, ultimately resistance develops. Androgen receptor (AR) expression and immune cell infiltration are thought to be involved in trastuzumab response, and may therefore be of interest as additional targets for therapy in HER2-positive breast cancer.

Aim: To improve insight in the presence of between AR expression, immune cell infiltration and HER2, we analysed HER2-positive breast tumours.

Methods: Primary tumours of 221 patients treated with trastuzumab for metastatic disease were selected. HER2 status was centrally confirmed. AR, T cells (CD3 and CD8), programmed cell death protein 1 (PD-1) and PD-1 ligand 1 (PD-L1) immunohistochemical staining and M2 tumour associated macrophages (TAMs; CD68 and CD163) immunofluorescence were performed. Tumour infiltrating lymphocytes (TILs) were evaluated by hematoxylin and eosin staining.

Results: Sufficient tumour material was available for 150 patients. Oestrogen receptor was expressed in 51.3% of the tumours and AR in 81.3% of the tumours. AR expression was inversely correlated with M2 TAM (*Pearson's* $r = -0.361$, $P < 0.001$), CD3+ ($r = -0.199$, $P < 0.030$) and CD8+ ($r = -0.212$, $P < 0.021$) T cell infiltration. Clustering analysis showed high immune cell infiltration in AR low expressing tumours, and low immune cell infiltration in AR high expressing tumours.

Conclusion: AR expression inversely correlates with immune cell infiltration in HER2-positive breast cancer.

INTRODUCTION

Human epidermal growth factor receptor 2 (HER2) positive breast cancer accounts for 15 - 20% of all invasive breast cancers.[1] Trastuzumab-based anti-HER2 therapy added to chemotherapy improves overall survival (OS) of patients with HER2-positive breast cancer.[2, 3] In the metastatic setting however, eventually resistance to trastuzumab regimens develops. Treatment strategies to counteract this, namely trastuzumab plus pertuzumab, lapatinib or antibody-drug conjugate T-DM1, have improved patients' survival.[4-6] However, eventually resistance to these approaches will develop.[4, 6] Therefore, new targets for rational (combination) therapies are needed. Potentially, androgen receptor (AR)[7, 8] and immune cell composition[9-11] are of relevance in this setting.

AR is a steroid receptor with important functions in cell differentiation and proliferation. In the presence of androgens, the ligand-bound AR binds to hormone response elements. This results in up- or down regulation of specific protein expression.[12] In patients with oestrogen receptor (ER)-negative/HER2-positive non-metastatic breast cancer, AR tumour expression has been associated with a trend for worse prognosis.[13] In preclinical models, targeting both HER2 and AR demonstrated a synergistic antitumour effect.[7, 8] This is supported by preliminary results of an ongoing trial combining the AR signalling inhibitor enzalutamide and trastuzumab in breast cancer patients with HER2-positive/AR-positive tumours (NCT02091960). There was a 24-week clinical benefit observed in 6 of the 18 patients receiving more than four prior lines of therapy.[14]

The immune cell composition of the breast cancer environment has been related to prognosis of patients, depending on the tumour molecular subtype.[15, 16] Moreover, preclinical and clinical evidence suggest that immune cell compositions is able to predict response of in HER2-targeted treatment.[9-11] Presence of CD8+ T cells in tumours predicted a better response to HER2-targeted treatment in preclinical mouse models.[10] However, an immunosuppressive microenvironment, such as presence of tumour associated macrophages (TAMs) or programmed cell death protein 1 (PD-1) expressing immune cells, contributed to HER2-targeted treatment resistance in similar mouse models.[11, 17] These data provided the rationale for combining trastuzumab with the immune checkpoint inhibitor pembrolizumab in 46 patients with HER2-positive tumours, which resulted in a modest 15% objective response rate.[18] This indicates that patient selection for this combination therapy should be improved. Increased knowledge of immune microenvironment composition in HER2-positive breast cancer could possibly be of relevance in this respect.

Therefore, to improve insight in the interaction of AR expression, immune cell infiltration and HER2, we analysed in primary HER2-positive breast tumours the AR expression and infiltration of M2 TAMs, CD3+ and CD8+ T cells, PD-1+, PD-1 ligand 1+ (PD-L1+) cells and tumour-infiltrating lymphocytes (TILs). These findings were related to OS.

MATERIAL AND METHODS

Study population and breast tumour samples

Tissue microarrays (TMAs) containing primary tumour material from a retrospectively collected cohort of 221 patients with HER2-positive metastatic breast cancer were used. Patients treated with trastuzumab and concurrent chemotherapy of physicians' choice, for metastatic breast cancer were identified in the records of 19 hospital pharmacies in the Northern part of the Netherlands. Details of patient selection, patient- and treatment characteristics, follow-up and TMA construction have been described previously.[19, 20] Per tumour, three cores were incorporated in the TMA. Patient, treatment and tumour characteristics, including presence in the primary tumour of expression of oestrogen receptor (ER) and progesterone receptor (PR); with 10% of cells expressing ER or PR used as cut-off for positivity, were collected from the Netherlands Cancer Registry. HER2 status was centrally reviewed as described previously.[19] According to the Dutch Central Committee on Research involving Human Subject, this retrospective non-interventional study did not require approval from an ethical committee in the Netherlands. This study was approved by the Privacy Review Board of the Netherlands Cancer Registry.

AR, CD3, CD8, PD-1 and PD-L1 assessment by immunohistochemistry

AR, CD3, CD8, PD-1 and PD-L1 expression was assessed using freshly cut 4-μm TMA slides. Immunohistochemistry staining was performed in one batch per marker to prevent intensity differences. Positive (with primary antibodies) and negative controls (with immunoglobulin class-matched control sera) were included on normal breast tissue for AR, tonsil for CD3, CD8 and PD-1, and placenta for PD-L1.

For AR, CD3 and CD8 the staining was performed with a Ventana BenchMark Ultra immunostainer (Roche, Ventana Medical Systems, Inc.). The ULTRA CC1 (Roche) for 64 minutes was used for antigen retrieval. Primary antibody (anti-AR: clone SP107, Roche; anti-CD3: clone 2GV6, Roche; anti-CD8: clone SP57, Roche) was pre-diluted by the manufacturer and was performed following manufacturer's protocols. Staining was visualized using the ultraView Universal 3,3 0-diaminobenzidine (DAB) Detection Kit (Roche) following manufacturer's protocols. Heat-mediated antigen retrieval was executed with microwave in a Tris-HCl buffer (pH 9.0) for PD-1 and citrate buffer (10 mM citrate, pH 6.0) for PD-L1. Endogenous peroxidase was blocked with 0.3% H_20_2 in phosphate buffered saline (PBS; pH 7.4). Aspecific binding was blocked with human AB-serum. Primary antibodies (anti-PD-1: clone MRQ-22, Acris; anti-PD-L1: clone SP142, Roche) were diluted in PBS supplemented with 1% bovine serum albumin. Envision Anti-mouse for PD-1 (DAKO) and anti-rabbit for PD-L1 (DAKO) were used as secondary antibodies. Staining was visualized using DAB and haematoxylin counterstaining.

The immunohistochemistry slides were digitized with a Philips Ultra Fast Scanner 1.6 (Philips, Eindhoven, The Netherlands). AR staining was reported as percentage of tumour cells with positive nuclear staining. From each observer, the average percentage of replicate cores was used as the score for each patient. The scores from two observers were averaged and used as the final score for each patient. In case of discrepancy (> 20% difference in score), the two observers discussed

the results to reach a consensus. A cut-off value of 10 % was used for AR positive expression.[13] CD3 and CD8 staining was reported as number of positive cells per mm^2. PD-1 and PD-L1 expression was determined for both immune cells and tumour cells. If any of the cores had positive cellular staining, the sample was considered positive.

M2 TAMs assessment by immunofluorescence

M2 TAMs (expression of both CD68 and CD163) were assessed by multi-colour immunofluorescent staining using freshly cut 4-µm TMA slides as described previously.[21] Briefly, antigen retrieval and endogenous peroxidase blockade were as above mentioned for PD-L1. Thereafter TMA slides were incubated overnight at 4°C with a mixture containing primary antibodies anti-CD68 (clone KP1, Novus Biologicals) and anti-CD163 (clone EPR6539, Abcam). CD68 signal was visualized using DyLight488-conjugated streptavidin (21832, Thermo Fisher Scientific), CD163 using Alex Fluor555-conjugated goat anti-rabbit secondary antibody (ab150078, Abcam). A nuclear counterstain was performed with 4'6-diamidino-2-phenylindole (DAPI). Slides were mounted in Prolong Gold (Life Technologies) and stored in the dark at room temperature. Positive and negative controls with respectively primary antibodies and immunoglobulin class-matched control sera for the staining were included on gallbladder tissue.

The immunofluorescence slides were scanned using a TissueFAXS imaging system and visualized using TissueFAXS Viewer (TissueGnostics, Vienna, Austria). The CD68 and CD163 double-stained cells were considered as M2 TAMs and reported as the number of cells per mm^2. The average number of replicate cores was used as the final score for each tumour.

TILs assessment by haematoxylin and eosin staining

TILs were assessed in the whole tumour slides using the method standardised by the international TILs working group.[22] Results are presented as percentage TILs in the stromal tissue. Only patients who had 2 or more cores containing tumour and stromal cells were included for analysis. The investigators who performed the scoring were blinded for the clinicopathological characteristics.

Statistical analysis

The continuous variables were described by median and interquartile range, and the categorical variables by percentages. The relation between the investigated markers and clinicopathological characteristics was assessed using Mann-Whitney U test for continuous variables and Chi-square or Fisher's exact test for categorical variables. Spearman's correlation coefficient or Chi-square test's Phi coefficient served to assess the correlation between the investigated markers. We created heatmaps using the function "heatmap.2" in "gplots" package in R to have better insight into AR expression and immune cell composition of individual tumours. OS was determined with the Kaplan-Meier method. With a log-rank test the differences in survival between subgroups, generated by the clustering heatmap, were analysed. Hazard ratio (HR) and 95% confidence interval (CI) for comparison were derived from univariate Cox-regression analysis. For all tests, *P*-values less than 0.05 were considered statistically significant and all *P*-values were tested two-sided. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 19.0 (SPSS. Inc.) and R version 3.4.1.

RESULTS

Patient characteristics

From 150 of the 221 identified patients, sufficient primary tumour material was available for analysis. The median age at diagnosis of breast cancer was 51 years (range: 25 - 90). ER expression was observed in 51.3% (77 of 150) of the primary tumours. Patient characteristics of these 150 patients are shown in Table 1. Number of tumours available for assessment of each marker is shown in Supplementary Figure 1.

Table 1 Patient characteristics

Characteristics	**Number (%)**
Total number of patients	**150**
Age* (median, range)	51 (25 - 90)
Age*	
≤ 50	71 (47.3)
> 50	79 (52.7)
Tumour type	
Ductal	133 (88.7)
Others	17 (11.3)
Clinical T stage	
T1	48 (32.0)
T2-4	80 (53.3)
Unknown	22 (14.7)
Tumour ER expression	
Positive	77 (51.3)
Negative	62 (41.3)
Unknown	11 (7.4)
Tumour PR expression	
Positive	58 (38.7)
Negative	75 (50.0)
Unknown	17 (11.3)
Tumour histological grade	
I-II	45 (30.0)
III	76 (50.7)
Unknown	29 (19.3)
Metastatic site	
Visceral	89 (59.3)
Non-visceral	53 (35.3)
Unknown	8 (5.4)

Abbreviation: ER, oestrogen receptor; PR, progesterone receptor; * age at diagnosis of breast cancer.

Expression of the studied markers in the primary tumour

The percentage of AR expression and TILs, and numbers of infiltrating immune cells are shown in Figure 1. The median AR expression per tumour was 60% (interquartile range (IQR): 28 - 88). 81.3% of patients (104 of 128) had an AR positive tumour. The median number of M2 TAMs, CD3+ and CD8+ T cells per tumour was 8 (IQR: 1 - 22), 85 (IQR: 0 - 311) and 35 (IQR: 0 - 149) cells/mm^2, respectively. The median percentage of TILs per tumour was 10% (IQR: 5 - 16). PD-L1 was expressed by intratumoural immune cells in 28% of the tumours (36 of 129) but no PD-L1 expression on tumour cells was seen. Only one tumour expressed PD-1 in the immune cells.

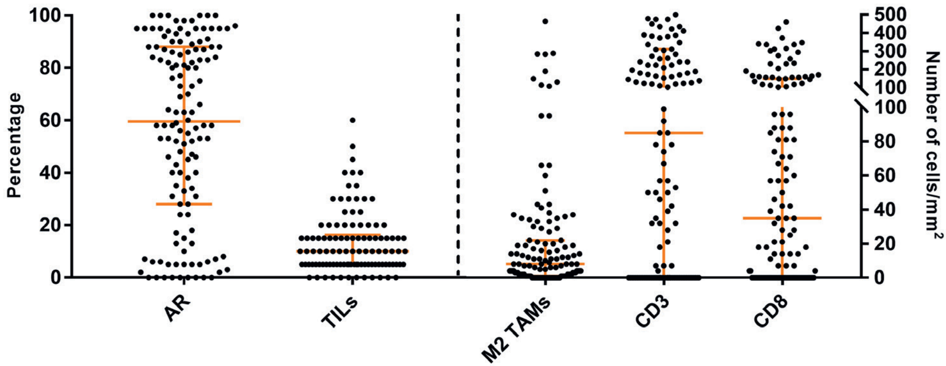

Fig. 1. Expression or infiltration of the studied markers in the human epidermal growth factor receptor 2 (HER2) overexpressing primary breast tumours as assessed by immunohistochemistry or immunofluorescence staining. AR is presented as percentage of cells with positive staining, TILs as percentage stromal TILs in the stromal tissue. M2 TAMs, CD3 and CD8 are presented as number of cells per mm^2. Orange line indicates the median and interquartile range. Abbreviation: AR, androgen receptor; TAMs, M2 tumour-associated macrophages; TILs, tumour-infiltrating lymphocytes.

Relation between clinicopathological characteristics and studied markers

The relation between clinicopathological characteristics and studied markers is shown in Table 2 and Supplementary Table 1. AR positivity was associated with ER positivity, and high M2 TAM infiltration with ER negativity. High CD3+ and CD8+ T cell infiltration were associated with lower clinical T stage at diagnosis of breast cancer. The TIL score did not differ among clinicopathological parameters.

Table 2 Androgen receptor expression and M2 tumour-associated macrophages infiltration in the primary tumour, in relation to clinicopathological characteristics

Characteristics	AR			TAMs		
	N	Percentage (IQR)	*P*-value	N	Median number of cells/mm^2 (IQR)	*P*-value
Age*			0.013			0.956
≤ 50	61	80 (35 - 92)		62	8 (1 - 20)	
> 50	67	53 (7 - 83)		65	7 (2 - 29)	
Tumour type			0.147			0.099
Ductal	116	58 (25 - 87)		115	8 (2 - 27)	
Others	12	81 (35 - 95)		12	4 (0 - 17)	
Clinical T stage			0.344			0.139
T1	44	64 (33 - 88)		42	6 (1 - 18)	
T2-4	63	55 (15 - 87)		65	11 (2 - 36)	
Unknown	21	58 (40 - 88)		20	7 (1 - 19)	
Tumour ER expression			0.011			0.037
Positive	68	74 (41 - 92)		68	4 (1 - 19)	
Negative	52	53 (7 - 80)		51	11 (3 - 29)	
Unknown	8	82 (19 - 90)		8	16 (1 - 237)	
Tumour PR expression			0.172			0.501
Positive	53	66 (35 - 91)		52	8 (2 - 35)	
Negative	61	58 (12 - 86)		62	8 (1 - 20)	
Unknown	14	72 (28 - 93)		13	7 (1 - 58)	
Tumour histological grade			0.934			0.949
I-II	44	58 (32 - 88)		43	8 (2 - 20)	
III	69	58 (18 - 85)		68	8 (2 - 27)	
Unknown	15	90 (78 - 98)		16	0 (0 - 48)	
Metastatic site			0.943			0.458
Visceral	75	58 (28 - 87)		76	10 (2 - 27)	
Non-visceral	46	60 (22 - 88)		45	6 (1 - 25)	
Unknown	7	81 (35 - 97)		6	4 (0 - 39)	

Abbreviation: AR, androgen receptor; ER, oestrogen receptor; IQR, interquartile range; N, number; PR, progesterone receptor; TAMs, M2 tumour-associated macrophages; * age at diagnosis of breast cancer.

Correlation between the studied markers

Tumour AR expression was negatively correlated with M2 TAMs, CD3+ and CD8+ cell infiltration of the tumour. The strongest negative correlation was observed between AR and M2 TAMs (*Pearson's* $r = -0.361$, $P < 0.001$). Correlation between the studied markers is shown in Table 3.

Table 3 Correlation and correlation coefficient between the studied markers in the primary tumours of all patients with HER2-positive breast cancer.

All	AR		TAMs		CD3		CD8		TILs	
	Corr	Corr coeff	Corr	Corr coeff	Corr	Corr coeff	Corr	Corr coeff	Corr	Corr coeff
All tumours										
TAMs	<0.001[&]	-0.361								
CD3	0.030[&]	-0.199	<0.001	0.367						
CD8	0.021[&]	-0.212	<0.001	0.327	<0.001	0.945				
TILs	0.105[&]	-0.160	0.019	0.235	<0.001	0.498	<0.001	0.493		
PD-L1	0.269[&],*	-0.098*	0.004*	0.255*	<0.001*	0.370*	<0.001*	0.386*	0.001*	0.332*
ER positive tumours										
TAMs	0.011[&]	-0.311								
CD3	0.035[&]	-0.266	<0.001	0.433						
CD8	0.039[&]	-0.261	<0.001	0.460	<0.001	0.976				
TILs	0.018[&]	-0.330	0.259	0.163	0.001	0.470	0.001	0.459		
PD-L1	0.979[&],*	-0.003*	0.001*	0.423*	0.002*	0.400*	<0.001*	0.454*	0.044*	0.285*
ER negative tumours										
TAMs	0.028[&]	-0.312								
CD3	0.546[&]	-0.088	0.031	0.311						
CD8	0.251[&]	-0.169	0.241	0.174	<0.001	0.894				
TILs	0.766[&]	-0.045	0.108	0.243	0.001	0.493	0.001	0.503		
PD-L1	0.252[&],*	-0.159*	0.846*	-0.027*	0.005*	0.400*	0.009*	0.371*	0.003*	0.426*

Abbreviation: AR, androgen receptor; corr, correlation; corr coeff, correlation coefficient; PD-L1, programmed cell death ligand 1; TAMs, M2 tumour-associated macrophages; TILs, tumour-infiltrating lymphocytes; [&], negative correlation; *, AR used 10%, other markers used their median numbers as a cut-off point (TAMs: 10 cells / mm^2; CD3: 85 cells / mm^2; CD8: 35 cells / mm^2; TILs: 10%) to determine positivity or high infiltration, chi-square test was performed; correlation is expressed as the *P*-values of the correlation analysis.

In patients with ER-positive tumours, a similar negative correlation between AR expression and immune cell infiltration was observed. In patients with ER-negative tumours, however, AR expression was negatively correlated with only M2 TAM infiltration. Based on the above-mentioned correlation between tumour AR expression and immune cell infiltration, we created heatmaps to dissect the AR expression and immune cell composition in individual tumours (Figure 2 and Supplementary Figure 2). We identified a group of tumours with low AR expression and high immune cell infiltration in around 20% of the patients; around 15% of the tumours with high AR expression and high immune cell infiltration; about half of the tumours with high AR expression and low immune cell infiltration (Figure 2A). This pattern of patient classification was observed similarly for both ER-positive and ER-negative tumours (Supplementary Figure 2). For the immune cell composition, we identified a group of tumours with high or low infiltration of CD8+ T cells and high infiltration of M2 TAMs and/or PD-L1+ cells in around 30% of the patients. Less than 10% of the tumours had high CD8+ T cell infiltration and low infiltration of M2 TAMs and PD-L1+ cells. In 55% of the tumours there was low infiltration of all three immune related markers (M2 TAMs, CD8+

T cells and PD-L1+ cells) (Figure 2B). Subgroup analysis demonstrated that ER-negative tumours have a slightly higher percentage of patients with tumours (around 40%) with high infiltration of M2 TAMs and/or PD-L1+ cells compared to patients with ER-positive tumours (around 30%). In contrary, the ER-positive subgroup has more patients with tumours with low infiltration of all three immune related markers (around 60%) compared with the ER-negative subgroup (around 50%) (Supplementary Figure 3).

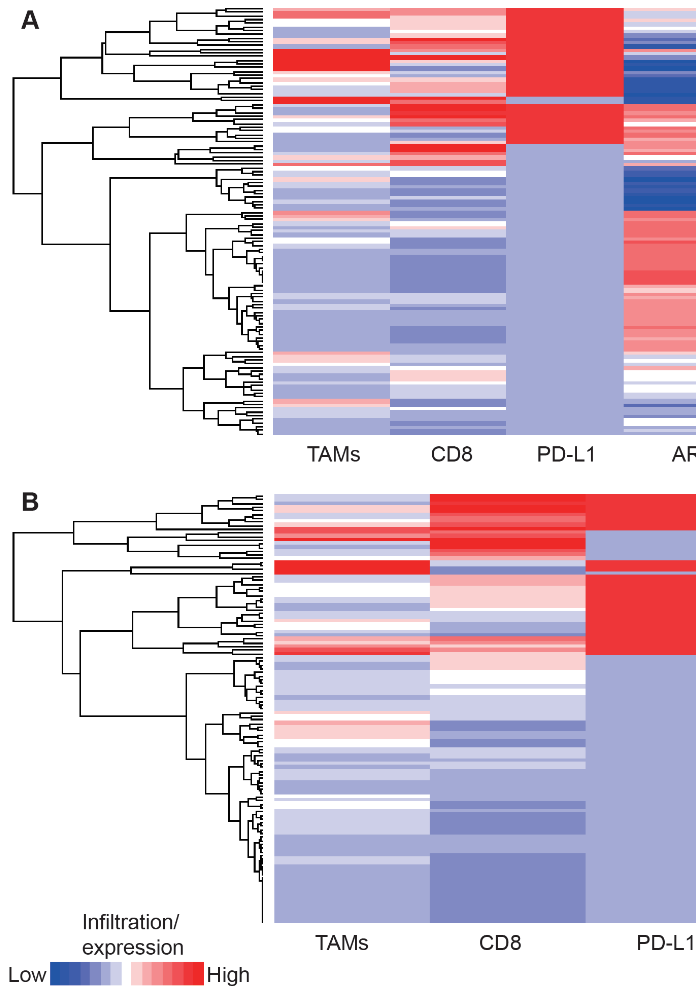

Fig. 2. Heatmap of AR expression and immune cell composition in individual tumours. The heatmaps were created based on AR expression and M2 TAMs, CD8+, and PD-L1+ immune cell infiltration in the primary tumours. The change from high infiltration/expression (red) to low infiltration/expression (blue) is reflected by the colour key. On the x-axis the markers are indicated, and on the y-axis the 116 tumours (upper heatmap; 117 tumours for the lower heatmap) are depicted, for whom data of all the markers are available. Each line represents the AR expression and/or immune cell composition of a tumour. Abbreviation: AR, androgen receptor; PD-L1, programmed cell death protein 1 ligand 1; TAMs, M2 tumour-associated macrophages.

Correlation of identified subgroups with overall survival

Based on the heatmap results for the tumour AR expression and immune cell infiltration, we classified the patients into four subgroups. The median follow-up was 63.8 months. OS is longer in the subgroup of patients with high AR expression and high immune cell infiltration ($n = 27$) in the primary tumour compared to patients having tumours with low AR expression and high immune cell infiltration ($n = 32$) (89.7 months *vs* 66.4 months, $P = 0.01$, HR 2.26, 95% CI (1.19 - 4.30). There are no differences in OS between the other subgroups (Supplementary Figure 4).

DISCUSSION

This is the first study to show that AR expression correlated inversely with M2 TAMs, CD3+ and CD8+ T cell infiltration in primary tumours of patients treated with trastuzumab for HER2-positive metastatic breast cancer.

Interesting this novel finding in breast tumours was also observed tumours of patients with castration-resistant prostate cancer. Tissues of bone metastasis were obtained from 65 patients during surgery for metastatic spinal cord compression. In total 83% of these patients received prior chemotherapy. Tumour AR protein expression was inversely correlated with CD3+ and CD8+ T cell infiltration and CD68 and CD163 mRNA expression.[23] In breast cancer the only available study taken into account AR and immune infiltration was performed in 107 non-metastatic triple negative breast cancer patients. Cluster analysis of RNA gene-expression profile of the primary tumour revealed that AR expression was related to a low immune response defined by the Teschendorff's gene-expression signature.[24] This information combined with our finding in HER2-positive breast tumours, suggests that tumour AR expression coincides with an immune deserted environment. This could possibly indicate that targeting AR is a rational option in AR high expressing HER2-positive tumours, while incorporating immunotherapy may be of potential interest for AR low expressing tumours, which are enriched with immune cells.

As only a small proportion of patients with HER2-positive breast cancer respond to the combination of immune checkpoint inhibitor and trastuzumab[18], better understanding of the immune cell composition in tumour microenvironment may help predict treatment response and optimize treatment strategy. We found in the exploratory analysis a longer OS in patients with high AR expression and high immune cell infiltration in the primary tumour compared to patients having tumours with low AR expression and high immune cell infiltration. Although this difference is found in a group containing small number of patients receiving systemic treatment as part of standard care, it could indicate that the proposed tumour classification is relevant to clinical outcome. The OS difference we found may indicate a role for the AR signalling in anti-HER2 treatment response. In a preclinical study exploring the role of AR signalling in HER2-positive, ER-negative breast cancer cells, high AR expression was associated with increased sensitivity to anti-HER2 treatment most likely based upon impaired androgen stimulated tumour cell growth, which was mediated by HER2/HER3 signalling activation.[25]

We found in this study tumours with relatively high presence of PD-L1+ and CD8+ T cells, and tumours that show high M2 TAM infiltration. In the first group, the PD-1 / PD-L1 axis could be a possible target for therapy, while in the second group macrophage-targeted therapy might support to reverse the immunosuppressive environment. In clinical trials the combination of macrophage-targeted therapy with trastuzumab or anti- PD-1 / PD-L1 axis therapy is currently studied (NCT03013218 and NCT01042379).

In breast cancer, AR functions differently depending on the ER status of the tumour cells. In ER-positive/AR-positive cell lines, ligand-bound AR causes cell apoptosis by binding to oestrogen-related element, whereas in ER-negative/AR-positive cell lines AR causes cell proliferation by binding to androgen-related element in the nucleus.[26] In our study, we found that in ER-positive tumours, AR expression was negatively correlated with all studied immune cells except for PD-L1+ immune cells. In ER-negative tumours, AR expression was not correlated with any of the immune cell fractions except for M2 TAMs. Therefore, the correlation between tumour AR expression and immune cell infiltration may also be affected by the ER signalling. This is consistent with prior findings indicating that the prognostic value of infiltrating immune cells depends on ER status and/or HER2 status.[27, 28] In a cohort of 12,439 patients with breast cancer, presence of CD8+ T cells in the tumour was associated with a 28% reduction of breast cancer-specific mortality in patients with ER-negative tumours, irrespective of HER2 status, and a 27% reduction in patients with ER-positive/HER2-positive tumours. For the entire group of patients with ER-positive tumours no such association was found.[29]

We used anti-PD-L1 SP142 in our study which it is one of the four Food and Drug Administration approved PD-L1 immunohistochemistry assays for predicting PD-1 / PD-L1 inhibitor response.[30] It is however well known that different PD-L1 antibodies provide different results.[31]

This is a small retrospective study of primary tumour samples from patients with synchronous- or asynchronous breast cancer metastases. Our findings must be interpreted as hypothesis generating. A larger number of primary HER2-positive breast cancers, including tumours that have not metastasised, is needed to test the strength of our findings.

Conclusion

AR expression inversely correlates with immune cell infiltration in HER2-positive breast cancer. New treatment strategies can potentially be explored in groups with different AR expression and immune cell infiltration.

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SUPPLEMENTARY FILES

Supplementary Table 1. Clinicopathological characteristics in relation to CD3+ and CD8+ T cell infiltration, tumour infiltrating lymphocytes and PD-L1 expression in the primary tumour.

Characteristics	CD3			CD8			TILs			PD-L1		
	N	Median number of cells/mm^2 (IQR)	*P*-value	N	Median number of cells/mm^2 (IQR)	*P*-value	N	Median number of cells/mm^2 (IQR)	*P*-value	Positive (%)	Negative (%)	*P*-value
Total	139	85 (0 - 311)	-	138	35 (0 - 149)	-	106	10 (5 - 16)	-	36	93	-
Age*			0.889			0.640			0.167			0.235
≤ 50	66	107 (5 - 228)		66	35 (0 - 90)		52	10 (5 - 15)		14 (38.9)	47 (50.5)	
> 50	73	78 (0 - 387)		72	30 (0 - 169)		54	13 (5 - 20)		22 (61.1)	46 (49.5)	
Tumour type			0.003			0.005			0.169			0.514
Ductal	124	107 (0 - 366)		123	42 (0 - 156)		101	10 (5 - 18)		34 (94.4)	82 (88.2)	
Others	15	0 (0 - 120)		15	0 (0 - 7)		5	5 (3 - 13)		2 (5.6)	11 (11.8)	
Clinical T stage			<0.001			<0.001			0.359			0.987
T1	44	177(57 - 464)		44	71 (19 - 215)		43	10 (5 - 20)		13 (40.6)	31 (40.8)	
T2-4	75	21 (0 - 209)		74	2 (0 - 90)		46	10 (5 - 15)		19 (59.4)	45 (59.2)	
Unknown	20	129 (5 - 355)		20	73 (0 - 158)		17	10 (5 - 23)		4	17	
Tumour ER expression			0.321			0.719			0.952			0.557
Positive	72	85 (0 - 270)		72	37 (0 - 151)		51	10 (5 - 15)		17 (51.5)	50 (57.5)	
Negative	57	124 (0 - 428)		56	51 (0 - 149)		49	10 (5 - 20)		16 (48.5)	37 (42.5)	
Unknown	10	0(0 - 119)		10	0 (0 - 80)		6	13 (5 - 16)		3	6	
Tumour PR expression			0.553			0.311			0.245			0.694
Positive	53	120 (0 - 285)		53	57 (0 - 149)		41	10 (5 - 15)		16 (48.5)	36 (44.4)	
Negative	70	66 (0 - 347)		69	18 (0 - 135)		54	8 (5 - 16)		17 (51.5)	45 (55.6)	
Unknown	16	34 (0 - 347)		16	16 (0 - 164)		11	15 (5 - 20)		3	12	
Tumour histological grade			0.653			0.642			0.751			0.273
I-II	42	131(43 - 445)		41	64 (7 - 236)		40	10 (5 - 20)		11 (31.4)	33 (42.3)	
III	68	161(33 - 366)		68	73 (12 - 154)		63	10 (5 - 15)		24 (38.6)	45 (57.7)	
Unknown	29	0 (0 - 0)		29	0 (0 - 0)		3	5		1	15	
Metastatic site			0.270			0.509			0.435			0.185
Visceral	85	57 (0 - 309)		84	28 (0 - 149)		59	10 (5 - 20)		18 (52.9)	58 (65.9)	
Non-visceral	47	131 (0 - 382)		47	57 (0 - 156)		41	10 (5 - 18)		16 (47.1)	30 (34.1)	
Unknown	7	42 (0 - 173)	7	7	18 (0 - 88)		6	13 (4 - 16)		2	5	

Abbreviation: ER, oestrogen receptor; IQR, interquartile range; N, number; PR, progesterone receptor; * age at diagnosis of breast cancer.

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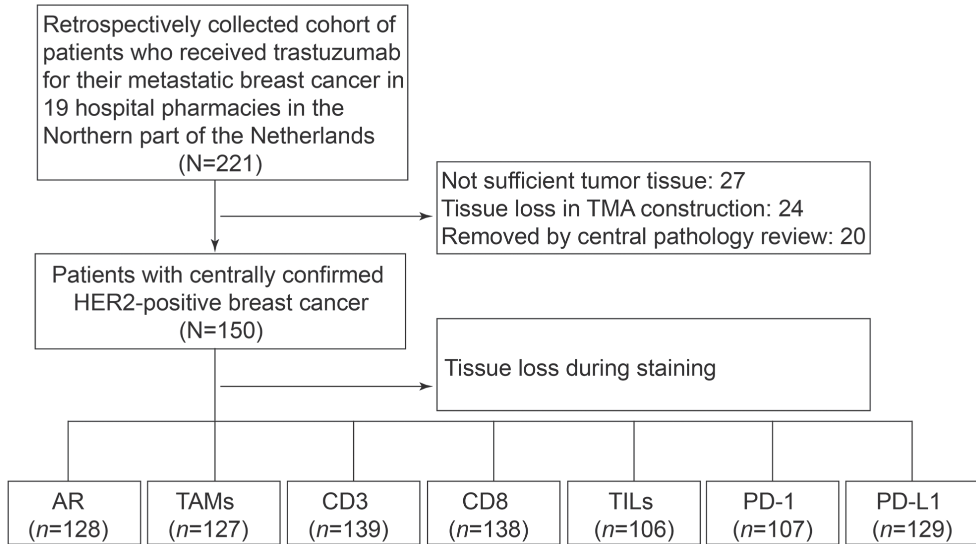
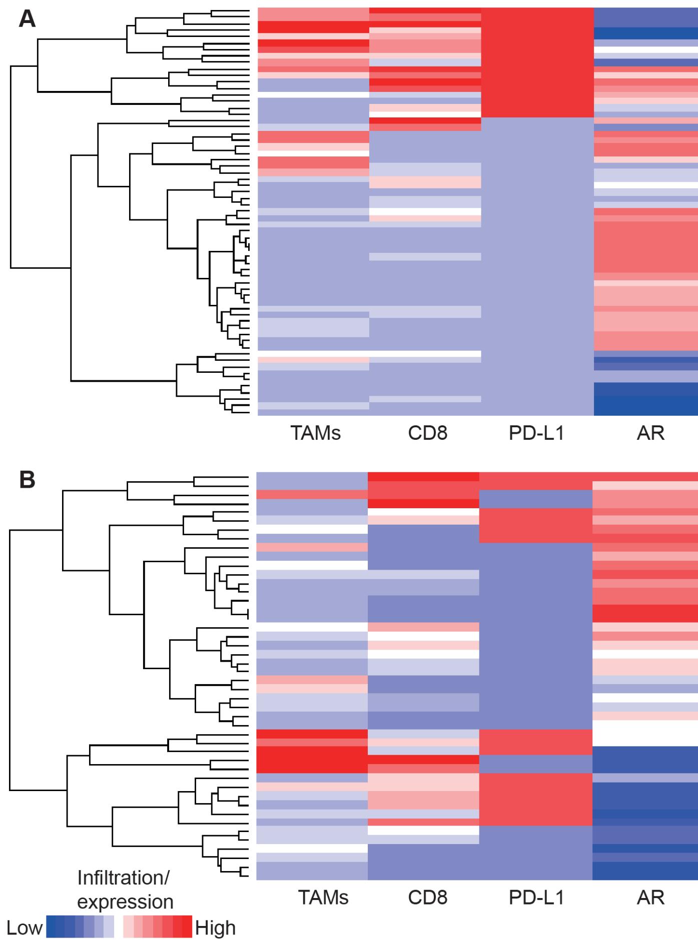
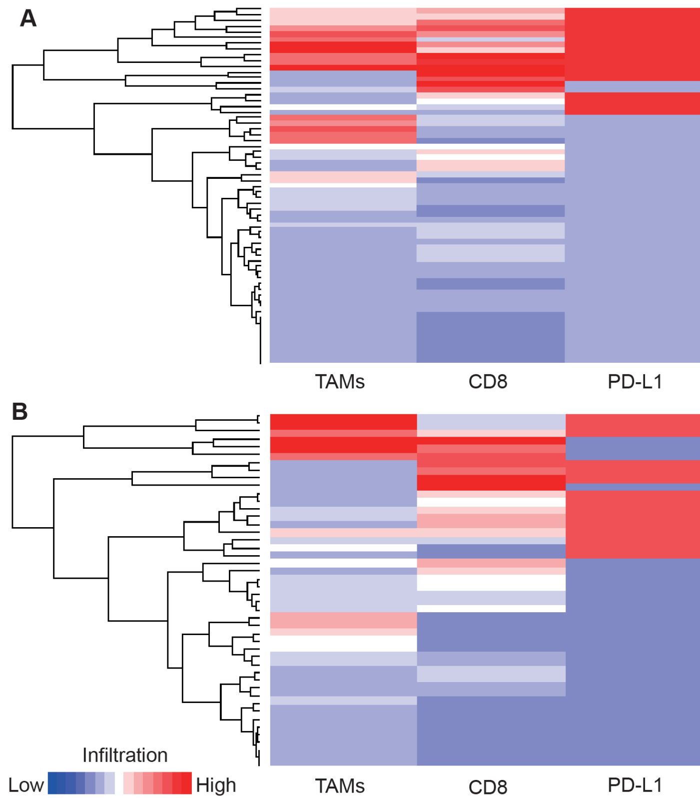


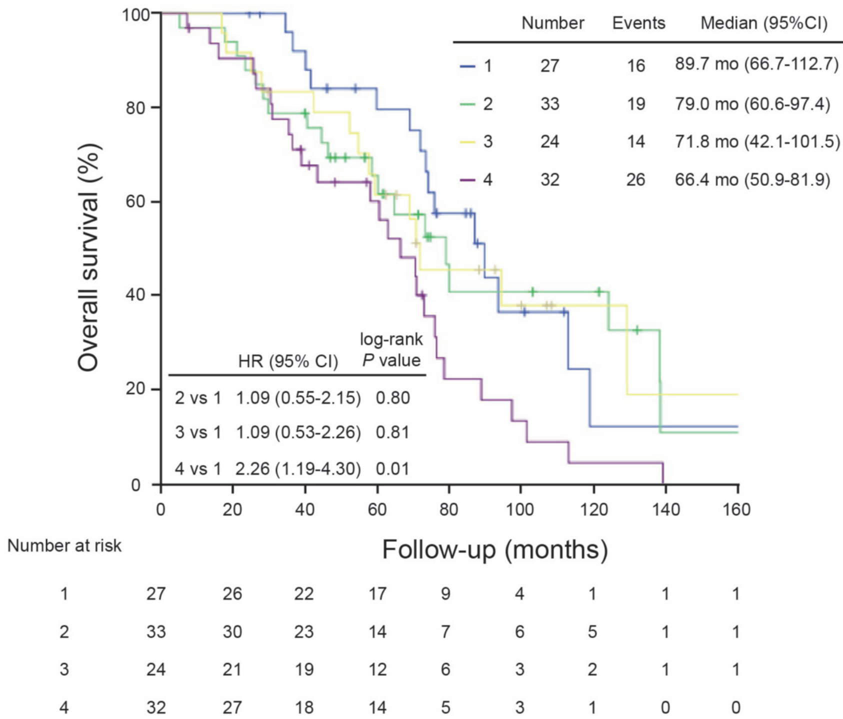
Supplementary Fig. 1. Flow diagram of the studied patients and number of primary HER2+ tumours available for analysis for each marker. Abbreviation: AR, androgen receptor; HER2, human epidermal growth factor receptor 2; N, number of patients; n, number of tumours available for analysis per marker; PD-1, programmed cell death protein 1; PD-L1, programmed cell death 1 ligand 1; TAMs, M2 tumour-associated macrophages; TILs, tumour-infiltrating lymphocytes; TMA, tissue micro-array.



Supplementary Fig. 2. Heatmap of AR expression and immune cell composition in individual tumours with different ER status (A: ER-positive tumours; B: ER-negative tumours). The heatmaps were created based on AR expression and M2 TAMs, CD8+, and PD-L1+ immune cell infiltration in the primary tumours. The change from high infiltration/expression (red) to low infiltration/expression (blue) is reflected by the colour key. On the x-axis the markers are indicated, and on the y-axis of the upper heatmap the 63 ER-positive tumours (46 ER-negative tumours for the lower heatmap) are depicted, for whom data of all the markers are available. Each line represents the AR expression and/or immune cell composition of a tumour. Abbreviation: AR, androgen receptor; ER, estrogen receptor; PD-L1, programmed cell death protein 1 ligand 1; TAMs, M2 tumour-associated macrophages.



Supplementary Fig. 3. Heatmap of immune cell composition in individual tumours with different ER status (A: ER-positive tumours; B: ER-negative tumours). The heatmaps were created based on M2 TAMs, CD8+, and PD-L1+ immune cell infiltration in the primary tumours. The change from high infiltration (red) to low infiltration (blue) is reflected by the colour key. On the x-axis the markers are indicated, and on the y-axis of the upper heatmap the 63 ER-positive tumours (46 ER-negative tumours for the lower heatmap) are depicted, for whom data of all the markers are available. Each line represents the immune cell composition of a tumour. Abbreviation: ER, oestrogen receptor; PD-L1, programmed cell death protein 1 ligand 1; TAMs, M2 tumour-associated macrophages.



Supplementary Fig. 4. Kaplan-Meier analysis for overall survival of subgroups of patients classified based upon tumour androgen receptor (AR) expression and immune cell infiltration. A high AR expression was defined when AR expression was higher than 60% (mean AR expression 59%). A high immune cell infiltration was defined of at least positivity for M2-TAM (number of infiltrating cells more than the mean of 120 cells per tumour), CD8 (number of infiltrating cells more than the mean of 20 cells per tumour) or PD-L1. 1, high AR expression and high immune cell infiltration; 2, high AR expression and low immune cell infiltration; 3, low AR expression and low immune cell infiltration; 4, low AR expression and high immune cell infiltration; HR, hazard ratio.