Chapter 5

The prognostic and predictive value of Tregs and tumor immune subtypes in postmenopausal, hormone receptor-positive breast cancer patients treated with adjuvant endocrine therapy: A Dutch TEAM Study Analysis

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Breast Cancer Res Treat. 2015 Feb;149(3):587-96

ABSTRACT

Purpose

Evidence exists for an immunomodulatory effect of endocrine therapy in hormone receptor-positive (HR+ve) breast cancer (BC). Therefore, the aim of this study was to define the prognostic and predictive value of tumor immune markers and the tumor immune profile in HR+ve BC, treated with different endocrine treatment regimens.

Methods

2596 Dutch TEAM patients were treated with 5 years of adjuvant hormonal treatment, randomly assigned to different regimens: 5 years exemestane or sequential treatment (2.5 years tamoxifen-2.5 years of exemestane). Immunohistochemistry was performed for HLA class I, HLA-E, HLA-G, and FoxP3. Tumor immune subtypes (IS) (low, intermediate & high immune susceptible) were determined by the effect size of mono-immune markers on relapse rate.

Results

Patients on sequential treatment with high level of tumor-infiltrating FoxP3+ cells had significant (p=0.019, HR: 0.729, 95%CI: 0.560-0.949) better OS. Significant interaction for endocrine treatment and FoxP3+ presence was seen (OS p<0.001). Tumor IS were only of prognostic value for the sequentially endocrine treated patients (RFP: p=0.035, HR intermediate IS: 1.420, 95%CI: 0.878-2.297; HR low IS: 1.657, 95%CI: 1.131-2.428; BCSS: p=0.002, HR intermediate IS: 2.486, 95%CI: 1.375-4.495; HR low IS: 2.422, 95%CI: 1.439-4.076) and OS: p=0.005, HR intermediate IS: 1.509, 95%CI: 0.950-2.395; HR low IS: 1.848, 95%CI: 1.277-2.675).

Conclusion

Tregs and the tumor IS presented in this study harbour prognostic value for sequentially endocrine treated HR+ve postmenopausal BC patients, but not for solely exemestane treated patients. Therefore, these markers could be used as a clinical risk stratification tool to guide adjuvant treatment in this BC population.

INTRODUCTION

Breast cancer (BC) is the most commonly diagnosed female cancer in the developed world and also leading cause of cancer death, responsible for 14% of cancer-related deaths in women of the West [1]. Nowadays, BC treatment consists of a combination of locoregional treatment (i.e. surgery and radiotherapy) and systemic therapy (i.e. chemotherapy and hormonal therapy), to concur present and less evident metastasis. In the USA, an increased tendency of adjuvant treatment allocation using genomic expression assays such as Onco*type* DX (genomic health, redwood city, CA, USA) and Mammaprint (Agendia, Amsterdam, the Netherlands), providing additional information about the risk of relapse and benefit of adjuvant chemotherapy, is seen [2-4]. However, in the Netherlands, decisions regarding the use of adjuvant systemic therapy in primary BC patients are still mainly based on classical prognostic factors, like lymph node status, tumor-size, -grade, hormone receptor (HR) and human epidermal growth factor receptor 2 (HER2) expression [5]. However, currently these do not provide optimal risk-stratification, resulting in over- and undertreatment of certain patients. There is evidence that a host's cellular immune response plays a pivotal role in controlling tumor progression through a number of immunological mechanisms, involving classical human leukocyte antigen (HLA) class I and non-classical HLA-E and HLA-G expression by the tumor, and presence of tumor infiltrating cytotoxic T cells (CTL), Natural Killer (NK) cells and regulatory T cells (Tregs) [6-11], suggesting that complex interactions take place between breast tumor cells and immune cells [12]. Valuable prognostic interactions reported are those between classical HLA class I and Tregs, where loss of HLA class I in combination with presence of Treg in the tumor microenvironment resulted in a worse patient's outcome, and also the interaction between classical HLA class I, HLA-E, and HLA-G tumor expression, where HLA-E and HLA-G expression resulted in worse patient outcome in the co-occurrence of loss of classical HLA class I on the tumor surface [8;9;12]. Together, this emphasizes the importance of research on combinations of markers of immune surveillance together with markers of tumor immune escape.

Our group previously constructed breast tumor immune subtypes (IS) by combining markers of immune surveillance together with markers of tumor immune escape, based on a biological rationale [13]. Data revealed strong associations with patient outcome whereby tumors defined as highly susceptible to immune attack showed favorable clinical outcome compared to patients with tumors harboring a low immune susceptibility profile, independent of known clinicopathological parameters [13]. In the current study we used another approach to define tumor IS. Tumor immune mono-markers in Dutch postmenopausal hormone-sensitive BC patients from the Tamoxifen and Exemestane Adjuvant Multicenter (TEAM) trial were correlated to clinical outcome. Subsequently,

we designed tumor immune subtypes based on statistical effect sizes of the immune monomarkers on relapse rate.

It has already been shown that tumor-infiltrating lymphocytes (TILs) act as an independent predictor of response to chemotherapy treatment [14-16]. Elaborating on this result, evidence also exists for an immunomodulatory effect of tamoxifen; it is thought that tamoxifen induces a shift from cellular (T-helper 1) to humoral (T-helper 2) immunity [17]. Given the fact that T-helper 1 immunity is essential for anti-tumor immune response, a tamoxifen-induced shift away from cellular immunity may represent a significant step in tumor development. This would hamper the cytotoxic effect of tamoxifen and possibly explain the differential effect of aromatase inhibitors versus tamoxifen on clinical outcome [17-19].

The aim of our current study was therefore to investigate the difference in prognostic value of tumor IS in relation with type of hormonal treatment received in HR+ve, postmenopausal BC patients.

PATIENTS AND METHODS

Patients and tumors

Eligibility criteria for the TEAM study have been previously described [20]. In brief, patients were postmenopausal and had HR+ve early BC diagnosed between 2001 and 2006. Patients with bilateral tumors or prior history of cancer were excluded. Patients were randomly assigned in a 1:1 ratio to either exemestane, 25mg daily for five years, or sequential therapy consisting of tamoxifen 20mg daily for 2.5 years followed by exemestane 25mg daily for another 2.5 years [20].

Medical-ethical approval was obtained and the study was conducted in accordance with the Declaration of Helsinki. All TEAM patients gave informed consent prior to enrolment in the study. Surgically resected formalin-fixed paraffin-embedded (FFPE) tumor samples of the Dutch TEAM patients (n=2596) were used. All samples were handled in a coded fashion, according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies).

Data was centrally collected at the Datacenter of the Department of Surgery of the Leiden University Medical Center. For all patients the following data was known: age at diagnosis, histological tumor grade, HR status, tumor size and nodal stage, type(s) of local and systemic treatment, date and type of disease recurrence, death and follow-up data. Reporting of the biomarkers was done according to the REMARK criteria [21].

Immunohistochemistry

Immunohistochemical staining was performed on 4µm FFPE Tissue Micro Array sections consisting of breast cancer tissue of the Dutch TEAM patients (three 0.6 mm^2 tumor tissue punches per patient) [22]. The tissue sections were stained according to the previously described protocol [9]. Sections were incubated at room temperature over night with mouse monoclonal antibodies HCA2 and HC10 (anti-HLA-A and anti-HLAB/C, respectively) [9;23] for the detection of classical HLA class I on the tumor cell surface. Non-classical HLA class I staining was performed using mouse monoclonal antibodies against HLA-E (MEM-E/02 Clone (sc-51621, Santa Cruz biotechnology, Dallas, Texas)) and HLA-G (4H84 Clone (sc-21799, Santa Cruz Biotechnology, Dallas, Texas)) [8]. Mouse monoclonal antibodies against FoxP3 (clone 236A/E7 (ab20034, Abcam, Cambridge, United Kingdom)) were used to identify Tregs [9]. All slides were stained simultaneously to avoid inter-assay variation.

Evaluation of immunostaining

Microscopic quantification of positive tumor cells for HCA2, HC10, HLA-E and HLA-G was performed in a blinded manner by two independent observers (C.C.E., A.S. and A.v.V). The scores of the three tissue cores were averaged. For HCA2 and HC10, the percentage of tumor cells with membranous staining was assessed. Classical HLA class I expression status was determined according to the standard set by the International HLA and Immunogenetics Workshop [24]. According to this standard, HCA2 and HC10 staining were scored in two categories: score 1 (0-5% of tumor cells positively stained) or score 2 (5-100% of tumor cells positively stained). Three groups were defined for classical HLA class I expression: HLA class I loss (both HCA2 and HC10 scored 0-5%); HLA class I downregulation (either HCA2 or HC10 scored 0-5%); and HLA class I expression (both HCA2 and HC10 scored 5-100%) [9]. For non-classical HLA class I markers, both HLA-E and HLA-G were scored based on the percentage of tumor cells with membranous staining and re-categorized in a binary manner. Any specific staining of tumor cells was considered positive and no staining was considered negative for HLA-G. HLA-E expression was divided into quartiles, of which the first quartile was categorized as low HLA-E expression and subsequent quartiles (> first quartile) as high. FoxP3+ nuclear presence per mm^2 in tumor epithelium and surrounding stroma tissue was identified with the use of a Panoramic Midi scanner (3DHistech, Hungary) by means of an automated positive cell count analysis using AxioVision 4.6 (Carl Zeiss Vision, Jena, Germany). FoxP3+ presence was scored by two categories: low (≤49 positive cells) and high (>49 positive cells) Treg infiltration per mm^2, based on the median value.

Statistical analysis

Statistical analyses were performed using statistical package SPSS (version 20.0 for Windows, IBM SPSS statistics). Patients of whom tumor material was lost during staining procedure were excluded from analyses. Cohen's kappa coefficient was used to assess inter-observer agreement in quantification of HCA2, HC10, HLA-E and HLA-G. As BC relapse strongly influences survival rates of BC patients, we designed tumor IS based on the regression coefficient of mono-markers in the Cox-regression using Relapse Free Period (RFP) as clinical endpoint for all tumor samples. The regression coefficient value, indicating either negative or positive clinical effect, served as a penalty or bonus (in case of a negative or positive slope, respectively). All regression coefficients (for HLA-I, HLA-E, HLA-G and FoxP3+) were added up to construct the final score per patient. Ultimately, three groups: low, intermediate and high immune susceptible tumor types were constructed based on tertile (≤33%, >33-≤67% and >67%) cut-off points of the final score.

The χ^2 test was used to evaluate associations between the tumor immune monomarkers, and also between clinicopathological parameters and tumor immune monomarkers and tumor IS. The clinical endpoints were RFP, defined as time from date of randomization in the TEAM-trial until any recurrence (loco-regional recurrence and/or a distant recurrence, whichever came first), Breast Cancer Specific Survival (BCSS), defined as time from date of randomization until death due to BC, and Overall Survival (OS), defined as time from randomization until death by any reason. The Kaplan–Meier method was used for survival plotting and log-rank test for RFP, BCSS and OS curve comparison. Cox proportional hazard analysis was used for univariate analysis and was additionally adjusted for clinically relevant confounders (age, pathological tumor and nodal stage, tumor grade, histology, and treatment). All analyses were stratified for hormonal regimen (exemestane or sequential regimen). Interaction between endocrine treatment and tumor IS was tested in a multivariable model.

RESULTS

Patient and tumor characteristics

The Dutch TEAM cohort consists of 2596 postmenopausal non-metastasized BC patients with a median age of 65 years (range: 38-91y). Median follow-up of patients was 5.9 years. Clinicopathological and treatment characteristics in relation with tumor IS are shown in Table 1. Only for radiotherapy a significant difference (chi-square test, p=0.045) was seen for between tumor IS, showing less radiotherapy treatment for intermediate tumor IS compared to low and high tumor IS. Substantial agreement ($K \geq 0.6$) was observed for quantification of all immunohistochemical stainings.

Table 1: patient and tumor characteristics

	High immune subtype		Intermediate immune subtype		Low immune subtype		*p*-value
	N=501	%	N=318	%	N=817	%	
Age							
<65	259	51.7	164	51.6	425	52.0	0.988
≥65	242	48.3	154	48.4	392	48.0	
Missing	0		0		0		
pT stage							
T1	227	45.4	126	39.6	385	47.2	0.218
T2	244	48.8	169	53.1	387	47.4	
T3-4	29	5.8	23	7.2	44	5.4	
Missing	1		0		1		
pN stage							
N0	143	28.5	107	33.6	277	33.9	0.373
N1	319	63.7	192	60.4	490	60.0	
N2-3	39	7.8	19	6.0	49	6.1	
Missing	0		0		1		
Grade							
I	66	13.9	55	18.5	98	12.6	0.100
II	222	46.8	134	45.1	348	44.8	
III	186	39.3	108	36.4	330	42.6	
Missing	27		21		41		
Histology							
Ductal	391	78.5	249	78.6	664	81.8	0.495
Lobular	65	13.1	40	12.6	79	9.7	
Mixed	18	3.6	14	4.4	37	4.6	
Other	24	4.8	14	4.4	32	3.9	
Missing	3		1		5		
Operation							
Mastectomy	263	52.5	183	57.5	434	53.1	0.318
BCS	238	47.5	135	42.5	383	46.9	
Missing	0		0		0		
Radiotherapy							
Yes	318	63.5	174	54.9	500	61.2	**0.045**
No	183	36.5	143	45.1	317	38.8	
Missing	0		1		0		
Chemotherapy							
Yes	129	25.7	102	32.2	247	30.2	0.097
No	372	74.3	215	67.8	570	69.8	
Missing	0		1		0		
Endocrine therapy							
EXE	257	51.3	154	48.4	410	50.2	0.726
TAM→EXE	244	48.7	164	51.6	407	49.8	
Missing	0		0		0		

Abbreviations: pT: pathological tumor pN: pathological nodal BCS: breast conserving surgery EXE: exemestane TAM: tamoxifen

Classical HLA-I expression and association with prognosis

Microscopic quantification for classical HLA-I was successful in 73% (1891/2596) of tumors (79% (2042/2596) for HCA2 and 80% (2083/2596) for HC-10). Classical HLA-I loss was found in 16% (298/1891), down-regulation in 27% (513/1891) and expression in 57% (1080/1891)(Supplementary Table 1A). In the analyses stratified for endocrine treatment, no significant difference in outcome was seen for HLA-I expression in RFP, BCSS or OS (Supplementary Table 2A).

HLA-E and HLA-G expression and association with prognosis

Successful staining for HLA-E was obtained in 74% of tumors, and in 79% for HLA-G. Low HLA-E was found in 26% (495/1914) and high expression in 74% (1419/1914) of the patients, whereas absence of HLA-G was found in 76% (1558/2042) and expression in 24% (484/2042) of the patients (Supplementary Table 1B). Neither of the two immune markers showed significant association with clinical outcome when stratified for endocrine treatment received (Supplementary Table 2B and 2C).

Presence of FoxP3+ cells and association with prognosis

Automated positive cell count was successful in 93% (2426/2596) of tumors for FoxP3+ cells. Low (≤ median value of 49 cells) number of positive cells was seen in 51% (1241/2426) and high number (>median of 49 positive cells) in 49% (1185/2426) of the patients (Supplementary Table 1A). Patients on sequential hormonal therapy showed a significant (univariate: p=0.026, multivariate: p=0.019, HR: 0.729, 95%CI: 0.560-0.949) preferential outcome for high FoxP3+ presence in OS, but not for RFP or BCSS. No association with clinical outcome was seen for patients in the exemestane only treated arm (univariate OS: p=0.138, HR: 0.821, 95%CI: 0.633-1.065) (Supplementary Table 2D). The multivariable interaction model showed a significant predictive effect for endocrine treatment and FoxP3+ presence (p-value OS: <0.001) in OS.

Tumor immune subtypes and association with prognosis

In view of recent evidence stating that the interaction between tumor cells and cells of the immune system is multifaceted and complex [13], we hypothesized that combined analyses of immune markers may better reflect a patients' outcome by taking into account the interaction between tumor cells and cells of the immune system. First, when the four mono-markers were tested in relation to one another in the chi-square test, results showed a significant association between all four mono-markers (chi-square test, p-values: all <0.001, data not shown). No difference in distribution was observed for the defined risk groups in the two hormonal treatment arms (p=0.726). Based on the tumor IS model described in the material and methods section, which is based on the regression coefficient of the mono-markers in the RFP, high tumor immune susceptibility was char-

acterized by either classical HLA-I expression with HLA-EG presence or absence (HLA-EG absence: both or either HLA-E or HLA-G not expressed; HLA-EG positive: both HLA-E and HLA-G positive) on the tumor surface, known for its activation of Natural Killer (NK) cells [8], or classical HLA-I loss or down-regulation combined with mostly HLA-EG absence. Treg presence was equally distributed in the high IS tumor subtypes. Great variability in Treg presence was also seen in the low and intermediate tumor IS (Supplementary Table 3). The tumor IS showed significant preference for the high immune susceptible tumor types for clinical outcome (RFP: p=0.002, HR intermediate (versus high) tumor IS: 1.539, 95%CI: 1.088-2.178; HR low (versus high) tumor IS: 1.634, 95%CI: 1.235-2.163**;** BCSS: p<0.001, HR intermediate (versus high) tumor IS: 2.119, 95%CI: 1.368-3.283; HR low (versus high) tumor IS: 2.103, 95%CI: 1.456-3.038); OS: p=0.002, HR intermediate (versus high) tumor IS: 1.471, 95%CI: 1.065-2.032; HR low (versus high) tumor IS: 1.602, 95%CI: 1.235-2.077, Figure 1).

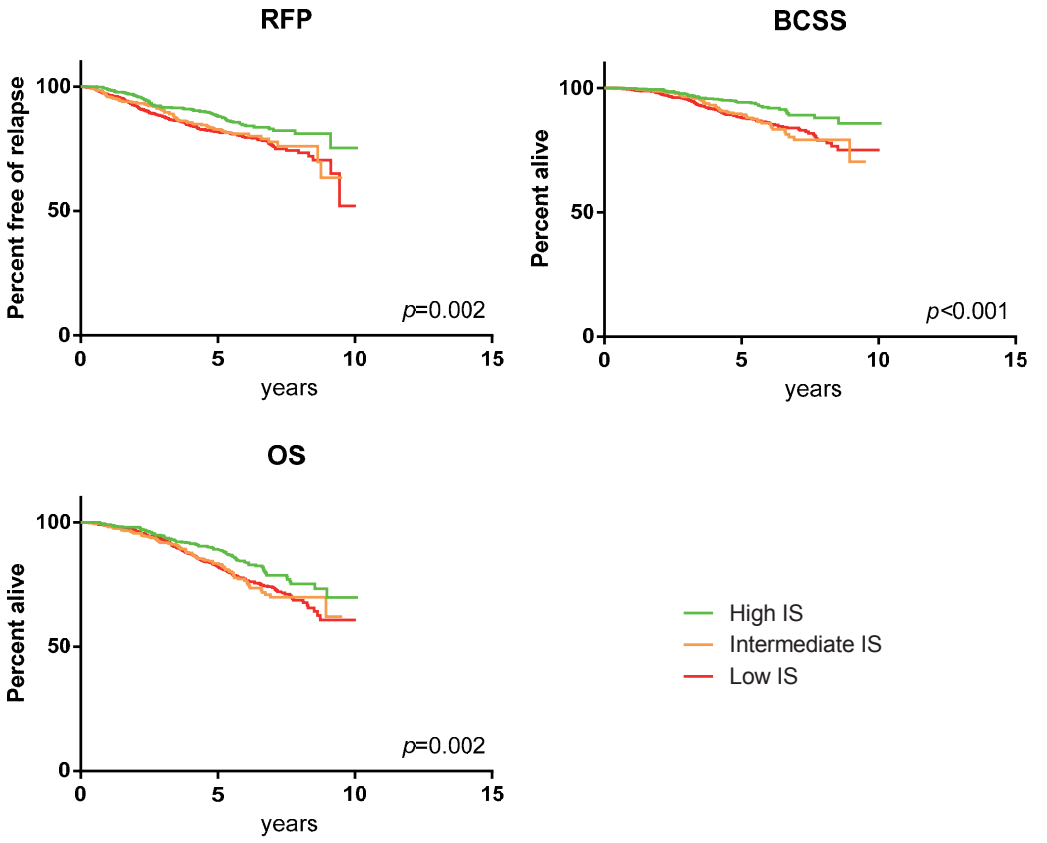

Figure 1: Tumor immune subtypes (high, intermediate and low tumor immune subtypes (IS)) in relation with clinical outcome parameters: Relapse Free Period (RFP); Breast Cancer Specific Survival (BCSS); and Overall Survival (OS), shown with corresponding adjusted (age, pT stage, pN stage, tumor grade, histology, surgery type, chemotherapy, radiotherapy and endocrine therapy) p-values.

Immune subtypes and adjuvant endocrine treatment

Significant differences were seenfor RFP, BCSS, and OS in the sequentially endocrine treated patient group when stratified for adjuvant hormonal treatment. Again, all outcomes are in favor of high tumor immune susceptibility (RFP: sequential treatment: *p*=0.035, HR intermediate IS (versus high): 1.420, 95%CI: 0.878-2.297; HR low IS (versus high): 1.657, 95%CI: 1.131-2.428; BCSS:sequential treatment: *p*=0.002, HR intermediate IS (versus high): 2.486, 95%CI: 1.375-4.495; HR low IS (versus high): 2.422, 95%CI: 1.439-4.076; and OS:sequential treatment: *p*=0.005, HR intermediate IS (versus high): 1.509, 95%CI: 0.950-2.395; HR low IS (versus high): 1.848, 95%CI: 1.277-2.675 ,Table 2 and Figure 2). No prognostic value was seen for the solely exemestane treated patients. A statistical trend was seen for the interaction between endocrine treatment and tumor IS in the multivariable interaction model (*p*-value RFP: 0.15, BCSS: 0. 19 and OS: 0.17).

Table 2:

Out-come	Hormone therapy	Immune subtype	N	Univariate			Multivariate*			Interac-tion *p*
				HR	95%CI	*p*	HR	95%CI	*p*	
RFP	EXE	High	257	1.00		0.113	-	-	-	0.15
		Intermediate	154	1.556	0.958-2.526					
		Low	410	1.464	0.988-2.171					
RFP	TAM→EXE	High	244	1.00		0.086	1.00		**0.035**	
		Intermediate	164	1.343	0.850-2.122		1.420	0.878-2.297		
		Low	407	1.520	1.049-2.203		1.657	1.131-2.428		
BCSS	EXE	High	257	1.00		0.261	-	-	-	0.19
		Intermediate	154	1.482	0.812-2.708					
		Low	410	1.465	0.907-2.367					
BCSS	TAM→EXE	High	244	1.00		**0.002**	1.00		**0.001**	
		Intermediate	164	2.486	1.375-4.495		2.848	1.509-5.375		
		Low	407	2.422	1.439-4.076		2.869	1.651-4.984		
OS	EXE	High	257	1.00		0.204	-	-	-	0.17
		Intermediate	154	1.428	0.925-2.205					
		Low	410	1.311	0.924-1.858					
OS	TAM→EXE	High	244	1.00		**0.024**	1.00		**0.005**	
		Intermediate	164	1.531	0.993-2.362		1.509	0.950-2.395		
		Low	407	1.636	1.144-2.341		1.848	1.277-2.675		

*Adjusted for age, pT stage, pN stage, tumor grade, histology, surgery type, chemotherapy and radiotherapy

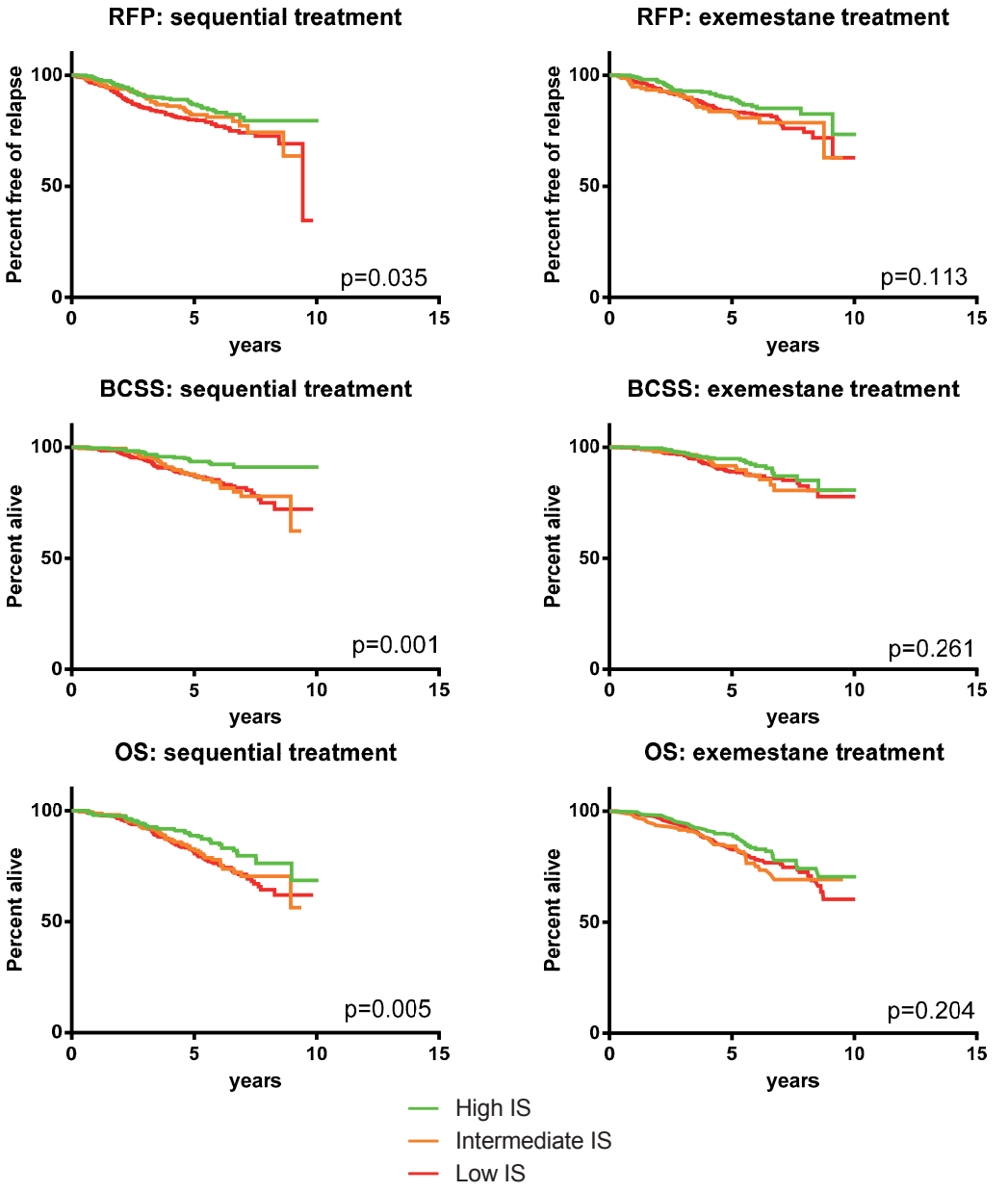

Figure 2: Tumor immune subtypes (high, intermediate and low tumor immune subtypes (IS)) stratified for endocrine therapy in relation with clinical outcome parameters: Relapse Free Period (RFP); Breast Cancer Specific Survival (BCSS); and Overall Survival (OS), shown with corresponding p-values (as seen in Table 3).

DISCUSSION

Evidence is building for an increasingly important role of tumor-immune interaction with regard to clinical outcome of cancer patients [25]. To our knowledge, this is the first study reporting on the effect of endocrine treatment on the prognostic value of Treg cells and tumor IS in a HR+ve BC cohort.

Our data suggest a positive effect of Treg presence on overall survival outcome in the sequentially endocrine treated patient group, which is further supported by a highly significant interaction term for endocrine treatment and Treg presence. This could possibly be explained by recent data indicating that Tregs harbour a dual role in cancer, suppressing anti-tumor immune response (inducible Treg) and suppressing inflammation which is known to promote carcinogenesis (natural Treg) [26;27]. These same studies suggest that the clinical and prognostic significance of Tregs in cancer depends on its environmental factors. Our investigated patient population harbours a number of pro-inflammatory risk factors, namely, a post-menopausal status which is known to be associated with systemic inflammation, and HR+ve breast tumors [28]. Assuming that HR+ve tumors attract higher estrogen levels in and around the tumor due to an increased tendency of estrogen binding, we hypothesize that this estrogen rich environment leads to higher Adenosine Deaminase Gene expression, which in turn is responsible for the degradation of Adenosine (ADO), a potent anti-inflammatory agent [29;30]. This presumed high inflammatory state in our patient population would assume a preference for natural Tregs, explaining the positive effect of high FoxP3+ presence in the tumors and the loss of prognostic significance in solely exemestane treated patients, as aromatase inhibition leads to lower estrogen levels, which will diminish ADO degradation.

For BC patients treated with sequential endocrine therapy, the tumor IS bare a strong independent significant prognostic value for BC specific survival and also, although to a lesser degree, for relapse rate and overall survival, while this association was not seen for patients treated solely with aromatase inhibition for five consecutive years. These data might imply that the immune profile of the breast tumor in sequentially endocrine treated breast cancer patients could predict BC death and overall death in HR+ve breast disease, and thus additional adjuvant therapy, such as chemotherapy and radiotherapy, could be optimally allocated based on this prognostic indicator. Since no prognostic effect was noted for the tumor IS in the solely exemestane treated patient population, the question remains whether there would be any benefit of additional adjuvant treatment for these patients, suggesting that currently we might have obtained the best attainable clinical outcome with five consecutive years of exemestane treatment, even for the low tumor immune susceptible HR+ve patient population. However, the multivariable interaction term for endocrine treatment and breast tumor immune subtypes hinted to a possible statistical trend for clinical outcome. The lack of significance in this test could be explained by the limited power of the statistical interaction test and also due to the low number of clinical events in our cohort.

In this study it was hypothesized that high immune susceptible tumor types, due to a tamoxifen induced shift from Th1 to Th2 immunity, would have the highest likelihood of showing regression of clinical outcome to mean relapse and survival rates of the overall cohort. Based on the data presented in this manuscript, the difference in prognostic

value of tumor immune subtyping between the two endocrine treatment arms cannot be explained by the previously described tamoxifen driven shift from Th1 to Th2 immunity [17]. In that case it would be expected that the difference in prognosis between the high immune susceptible tumor subtype, which is expected to be strongly dependent on cellular Th1 immunity, and the low and intermediate subtypes would be minimized. Reason for this could be that highly immunogenic tumors have the ability to circumvent the inferior immune response caused by the tamoxifen-induced Th1 to Th2 shift, by means of other immune interactions not requiring Th1 activation. A possible explanation for the loss of prognostic value of the tumor IS in the exemestane-treated patient arm of this cohort could also be Treg dependent. Findings supporting exemestane induced loss of Treg are published by Chan *et al.*, showing a significant increase in the CD8+/Treg ratio in ER+ve patients, responding well to aromatase inhibiting therapy, herewith reflecting the dynamic process in which the hosts immune response to tumor antigens changed in consequence of estrogen depletion caused by the aromatase inhibitor [31]. Similarly, Generali *et al.* observed that FoxP3+ cell counts decreased significantly after letrozole treatment [32]. Therefore, one could hypothesize that in this specific HR+ve, postmenopausal BC cohort, exemestane induced loss of highly prognostic Treg cells could lead to equalization of the clinical outcomes of the three tumor IS in the solely exemestane treated adjuvant treatment arm. If this would be true, one could speculate on the great importance of Treg for inhibition of tumor development in a post-menopausal, HR+ve tumor environment. Thereby proposing that under these conditions, HLA-I, HLA-E and HLA-G seem to merely have a supportive role in relation to Treg cells.

This is the first study that assessed the relation between adjuvant endocrine therapy and the prognostic value of tumor immune markers and tumor IS of postmenopausal HR+ve, early BC patients. Of course, the external validity of our results should be investigated in other large studies with tumor material available of HR+ve BC patients treated with different hormonal regimens, such as, for example the ATAC, BIG, or IES study. [18;33;34] The major strength of this study is the use of data from the TEAM-trial, as this provides well-registered data in a large number of patients. This study, however, also has its limitations. First, one could stress the shortcomings of FoxP3 staining, without co-staining of CD25 and CD4, for the detection of Tregs. Herewith, the margin of error for mistakenly scoring FoxP3+ breast tumor cells is increased [35]. However, based on careful review of the histology of the breast cancer tissue and given the fact that the majority of FoxP3+ cells were seen in the stromal region of the tumor tissue, we can state with reasonable certainty that the majority of positive cells were true Treg cells. Second, there were no standard tumor IS categories available from previous literature. Therefore, we categorized patients by tumor IS based on the regression coefficient of the mono-markers in the Cox-regression using RFP. One could criticize that this is an over-fitted model for RFP, but our results also showed significant association with the

other clinical outcome parameters BCSS and OS. Furthermore, our results did not show a difference in the distribution of the tumor IS for the two hormonal treatment arms, nevertheless, results showed a clear significant difference in the prognostic value of the IS based on the hormonal treatment received. Third, patients on sequential hormonal therapy received exemestane after the first 2.5 years of tamoxifen treatment. It would be desirable to compare two endocrine treatment regimens, consisting of solely exemestane and solely tamoxifen given for five consecutive years, eliminating the potential immune modulating effects of endocrine drugs with a different mode of action. Lastly, the immune contribution on clinical outcome described in this manuscript are all based on surgically derived tumor material, assuming that metastasizing cells harbour the same immunogenic characteristics. It should not be ignored that this approach disregards the possible interplay of systemic immune cells which undoubtedly also play a major role in anti-tumor immunity.

In conclusion, when taking into account the difference in associations of the tumor immune markers and tumor IS per endocrine treatment arm, these data partially support the hypothesis of previous manuscripts stating that endocrine treatment harbours an immune modulating effect [17;31]. Nonetheless, this study merely showed a statistical trend for interaction between tumor IS and type of endocrine treatment, and a strong interaction for FoxP3+ cells present in the tumor and endocrine treatment, implying that Based on the data presented in this manuscript, the difference in prognostic value of tumor immune subtyping between the two endocrine treatment arms cannot be explained by the previously described tamoxifen driven shift from Th1 to Th2 immunity [17]. In that case it would be expected that the difference in prognosis between the high immune susceptible tumor subtype, which is expected to be strongly dependent on cellular Th1 immunity, and the low and intermediate subtypes would be minimized. Reason for this could be that highly immunogenic tumors have the ability to circumvent the inferior immune response caused by the tamoxifen-induced Th1 to Th2 shift, by means of other immune interactions not requiring Th1 activation. A possible explanation for the loss of prognostic value of the tumor IS in the exemestane-treated patient arm of this cohort could also be Treg dependent. Findings supporting exemestane induced loss of Treg are published by Chan *et al.*, showing a significant increase in the CD8+/Treg ratio in ER+ve patients, responding well to aromatase inhibiting therapy, herewith reflecting the dynamic process in which the hosts immune response to tumor antigens changed in consequence of estrogen depletion caused by the aromatase inhibitor [31]. Similarly, Generali *et al.* observed that FoxP3+ cell counts decreased significantly after letrozole treatment [32]. Therefore, one could hypothesize that in this specific HR+ve, postmenopausal BC cohort, exemestane induced loss of highly prognostic Treg cells could lead to equalization of the clinical outcomes of the three tumor IS in the solely exemestane treated adjuvant treatment arm. If this would be true, one could speculate on the great

importance of Treg for inhibition of tumor development in a post-menopausal, HR+ve tumor environment. Thereby proposing that, despite the call for strong immune cell interplay recognition in tumor development, under these specific conditions, HLA-I, HLA-E and HLA-G seem to merely have a supportive role in relation to Treg cells.

To the best of our knowledge, this is the first study showing different associations in the prognostic value of tumor infiltrating Tregs and tumor IS with adjuvant endocrine treatment, and thus could be used as a clinical risk stratification tool in sequentially endocrine-treated HR+ve, postmenopausal BC patients. Therewithal, the results of this study add to previous studies on tumor-immune interactions in BC [6;13;17;36;37]. More research is needed to further elucidate this clinically relevant matter.

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SUPPLEMENTARY TABLES

Supplementary Table 1A: Clinicopathologic characteristics of the Dutch TEAM cohort for the expression of classical HLA class I and FoxP3+.

	Total population		HLA class I loss		HLA class I downregulation		HLA class I expression		*p*	FoxP3+ low		FoxP3+ high		*p*
	N	%	N	%	N	%	N	%		N	%	N	%	
	2596	100	298	100	513	100	1080	100		1241	100	1185	100	
Age in years														
< 40	1	0.0	0	0.0	0	0.0	1	0.1	0.534	1	0.1	0	0.0	0.484
40-50	57	2.2	11	3.7	11	2.1	19	1.8		31	2.5	22	1.9	
50-60	824	31.7	94	31.5	160	31.2	332	30.7		387	31.2	385	32.5	
≥60	1714	66.0	193	64.8	342	66.7	728	67.4		822	66.2	778	65.7	
Missing	0		0		0		0			0		0		
pT stage														
T1	1173	45.3	114	38.3	239	46.7	494	45.8	0.152	529	42.6	557	47.1	0.095
T2	1247	48.1	165	55.4	240	46.9	521	48.3		625	50.5	555	46.9	
T3/4	171	6.6	19	6.4	33	6.4	64	5.9		84	6.8	71	6.0	
Missing	5		0		1		1			3		2		
pN stage														
N0	785	30.2	78	26.2	157	30.6	352	32.6	0.431	369	29.7	383	32.3	0.467
N1	1641	63.2	201	67.4	325	63.4	655	60.6		793	63.9	720	60.8	
N2/3	168	6.6	19	6.4	31	6.0	72	6.7		78	6.4	81	6.8	
Missing	2		0		0		1			1		1		
Grade														
1	398	16.4	52	18.3	91	18.7	117	11.6	**0.001**	215	18.5	146	13.1	**<0.001**
2	1148	47.3	137	48.2	218	44.8	480	47.5		580	50.0	488	43.8	
3	883	36.4	95	33.5	178	36.6	414	40.9		365	31.5	479	43.0	
Missing	167		14		26		69			81		72		

Supplementary Table 1A: (continued)

	Total population		HLA class I loss		HLA class I downregulation		HLA class I expression		*p*	FoxP3+ low		FoxP3+ high		*p*
	N	%	N	%	N	%	N	%		N	%	N	%	
	2596	100	298	100	513	100	1080	100		1241	100	1185	100	
Histology														
Ductal	1936	75.2	229	78.2	388	75.9	861	80.2	0.172	892	72.8	929	78.8	**<0.001**
Lobular	409	15.9	44	15.0	72	14.1	122	11.4		227	18.5	146	12.4	
Mixed	123	4.8	7	2.4	29	5.7	46	4.3		52	4.2	60	5.1	
Other	106	4.1	13	4.4	22	4.3	45	4.2		55	4.5	44	3.7	
Missing	22		5		2		6			15		6		
ER status														
Positive	2543	98.0	295	99.0	503	98.1	1054	97.6	0.315	1223	98.5	1153	97.4	**0.041**
Negative	52	2.0	3	1.0	10	1.9	26	2.4		18	1.5	31	2.6	
Missing	1		0		0		0			0		1		
PGR status														
Positive	1884	76.9	204	72.6	379	78.3	785	76.9	0.188	907	77.2	859	77.0	0.900
Negative	567	23.1	77	27.4	105	21.7	236	23.1		268	22.8	257	23.0	
Missing	145		17		29		59			66		69		
HER-2 status														
Overexpression	5	6.3	0	0	2	9.5	3	7.3	0.701	1	2.9	4	9.1	0.272
No overexpression	75	93.7	7	100	19	90.5	38	92.7		33	97.1	40	90.9	
Missing	2516		291		492		1039			1207		1141		
Hormone Therapy														
TAM-EXE	1298	50.0	145	48.7	244	47.6	552	51.1	0.382	621	50.0	593	50.0	0.968
EXE	1298	50.0	153	51.3	269	52.4	528	48.9		620	50.0	592	50.0	
Missing	0		0		0		0			0		0		

Supplementary Table 1A: (continued)

	Total population		HLA class I loss		HLA class I downregulation		HLA class I expression		*p*	FoxP3+ low		FoxP3+ high		*p*
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**		**N**	**%**	**N**	**%**	
	2596	**100**	**298**	**100**	**513**	**100**	**1080**	**100**		**1241**	**100**	**1185**	**100**	
Surgery type														
Mastectomy	1426	55.0	175	58.7	293	57.1	557	51.6	**0.027**	722	58.2	604	51.0	**<0.001**
Local Excision	1169	45.0	123	41.3	220	42.9	523	48.4		519	41.8	580	49.0	
Missing	1	0	0		0		0			0		1		
Axillary dissection														
Yes	1996	76.9	236	79.2	395	77.0	814	75.6	0.363	965	77.8	886	74.8	0.083
No	600	23.1	62	20.8	118	23.0	266	24.6		276	22.2	299	25.2	
Missing	0		0		0		0			0		0		
Chemotherapy														
Yes	771	29.7	97	32.6	154	30.0	300	27.8	0.246	364	29.4	357	30.1	0.678
No	1824	70.3	201	67.4	359	70.0	779	72.2		876	70.6	828	69.9	
Missing	1		0		0		1			1		0		
Radiotherapy														
Yes	1590	61.3	170	57.0	290	56.6	681	63.1	**0.021**	719	58.1	765	64.6	**0.001**
No	1002	38.7	128	43.0	222	43.4	398	36.9		519	41.9	420	35.4	
Missing	4		0		1		1			3		0		

Abbreviations: pT: pathological tumor pN: pathological nodal ER: estrogen receptor PGR: progesterone receptor HER2: human epidermal growth factor receptor 2 TAM: tamoxifen EXE: exemestane

Supplementary Table 1B: Clinicopathologic characteristics of the Dutch TEAM cohort for the expression of HLA-E and HLA-G.

Total	Total population		HLA-E low		HLA-E high		*p*	HLA-G absence		HLA-G expression		*p*
	N	%	N	%	N	%		N	%	N	%	
	2596	100	495	100	1419	100		1558	100	484	100	
Age in years												
< 40	1	0.0	0	0.0	1	0.1	0.087	1	0.1	0	0.0	0.643
40-50	57	2.2	16	3.2	24	1.7		35	2.2	9	1.9	
50-60	824	31.7	142	28.7	462	32.6		480	30.8	162	33.5	
≥60	1714	66.0	337	68.1	932	65.7		1042	66.9	313	64.7	
Missing	0		0		0			0		0		
pT stage												
T1	1173	45.3	212	43.0	646	45.6	0.176	708	45.5	205	42.4	0.487
T2	1247	48.1	243	49.3	693	48.9		750	48.2	247	51.1	
T3/4	171	6.6	38	7.7	78	5.5		97	6.2	31	6.4	
Missing	5		2		2			3		1		
pN stage												
N0	785	30.2	131	26.5	484	34.1	**0.010**	475	30.5	171	35.4	0.160
N1	1641	63.2	324	65.5	849	59.8		986	63.3	281	58.2	
N2/3	168	6.6	40	8.1	85	6.0		96	6.2	31	6.4	
Missing	2		0		0			1		1		
Grade												
1	398	16.4	95	20.5	160	11.9	**<0.001**	236	16.1	51	11.2	**<0.001**
2	1148	47.3	236	50.9	598	44.5		704	48.0	189	41.4	
3	883	36.4	133	28.7	585	43.6		526	35.9	217	47.5	
Missing	167		31		76			92		27		
Histology												
Ductal	1936	75.2	345	70.3	1156	82.0	**<0.001**	1194	77.3	394	81.7	**0.002**
Lobular	409	15.9	99	20.2	144	10.2		217	14.0	41	8.5	
mixed	123	4.8	25	5.1	57	4.0		76	4.9	18	3.7	
Other	106	4.1	22	4.5	53	3.8		58	3.8	29	6.0	
Missing	22		4		9			13		2		
ER status												
Positive	2543	98.0	488	98.6	1385	97.7	0.222	1527	98.0	472	97.7	0.697
Negative	52	2.0	7	1.4	33	2.3		31	2.0	11	2.3	
Missing	1		0		1			0		1		
PGR status												
Positive	1884	76.9	376	80.0	1011	75.8	0.066	1135	77.5	348	75.7	0.404
Negative	567	23.1	94	20.0	322	24.2		329	22.5	112	24.3	
Missing	145		25		86			94		24		

Supplementary Table 1B: (continued)

Total	Total population		HLA-E low		HLA-E high		*p*	HLA-G absence		HLA-G expression		*p*
	N	%	N	%	N	%		N	%	N	%	
	2596	100	495	100	1419	100		1558	100	484	100	
HER-2 status												
Overexpression	5	6.3	0	0.0	5	9.1	0.226	5	8.8	0	0.0	0.220
No overexpression	75	93.7	15	100	50	90.9		52	91.2	16	100.0	
Missing	2516		480		1364			1501		468		
Hormone Therapy												
TAM-EXE	1298	50.0	241	48.7	719	50.7	0.447	762	48.9	238	49.2	0.919
EXE	1298	50.0	254	51.3	700	49.3		796	51.1	264	50.8	
Missing	0		0		0			0		0		
Surgery type												
Mastectomy	1426	55.0	272	54.9	753	53.1	0.478	845	54.2	257	53.2	0.692
Local Excision	1169	45.0	223	45.1	665	46.9		713	45.8	226	46.8	
Missing	1	0	0		1			0		1		
Axillary dissection												
yes	1996	76.9	380	76.8	1060	74.7	0.359	1190	76.3	369	76.2	0.949
no	600	23.1	115	23.2	359	25.3		368	23.6	115	23.8	
Missing	0		0		0			0		0		
Chemotherapy												
yes	771	29.7	129	26.1	441	31.1	**0.035**	454	29.1	148	30.6	0.527
no	1824	70.3	366	73.9	977	68.9		1104	70.9	335	69.4	
Missing	1		0		1			0		1		
Radiotherapy												
yes	1590	61.3	302	61.0	871	61.5	0.857	950	61.0	292	60.5	0.838
no	1002	38.7	193	39.0	546	38.5		608	39.0	191	39.5	
Missing	4		0		2			0		1		

Abbreviations: pT: pathological tumor pN: pathological nodal ER: estrogen receptor PGR: progesterone receptor HER2: human epidermal growth factor receptor 2 TAM: tamoxifen EXE: exemestane

Supplementary Table 2A: Cox univariate and multivariate analysis for RFP, BCSS and OS stratified for endocrine therapy for HLA-I expression of the tumor.

				Univariate			Multivariate*		
Outcome	**Hormone therapy**	**HLA-I**	**N**	**HR**	**95%CI**	***p***	**HR**	**95%CI**	***p***
RFP	EXE	Loss	153	1.00		0.795	-	-	-
		Downregulation	269	1.180	0.715-1.947				
		Expression	528	1.153	0.731-1.817				
RFP	TAM→EXE	Loss	145	1.00		0.265	-	-	-
		Downregulation	244	1.512	0.919-2.485				
		Expression	552	1.353	0.857-2.137				
BCSS	EXE	Loss	153	1.00		0.988	-	-	-
		Downregulation	269	0.962	0.527-1.758				
		Expression	528	0.993	0.851-1.696				
BCSS	TAM→EXE	Loss	145	1.00		0.323	-	-	-
		Downregulation	244	1.528	0.838-2.784				
		Expression	552	1.215	0.696-2.120				
OS	EXE	Loss	153	1.00		0.600	-	-	-
		Downregulation	269	1.090	0.705-1.685				
		Expression	528	0.925	0.620-1.379				
OS	TAM→EXE	Loss	145	1.00		0.094	1.00		0.183
		Downregulation	244	1.474	0.940-2.312		1.390	0.880-2.195	
		Expression	552	1.084	0.712-1.649		1.055	0.689-1.615	

Abbreviations: RFP: relapse free period BCSS: breast cancer specific survival OS: overall survival TAM: tamoxifen EXE: exemestane

*Adjusted for age, pT stage, pN stage, tumor grade, histology, surgery type, chemotherapy and radiotherapy

Supplementary Table 2B: Cox univariate and multivariate analysis for RFP, BCSS and OS stratified for endocrine therapy for HLA-E expression of the tumor.

				Univariate			Multivariate*		
Outcome	**Hormone therapy**	**HLA-E**	**N**	**HR**	**95%CI**	***p***	**HR**	**95%CI**	***p***
RFP	EXE	Low	254	1.00		0.793	-	-	-
		High	700	1.049	0.734-1.500				
RFP	TAM→EXE	Low	241	1.00		0.103	-	-	-
		High	719	1.345	0.942-1.919				
BCSS	EXE	Low	254	1.00		0.505	-	-	-
		High	700	1.171	0.736-1.862				
BCSS	TAM→EXE	Low	241	1.00		0.101	-	-	-
		High	719	1.459	0.929-2.291				
OS	EXE	Low	254	1.00		0.621	-	-	-
		High	700	0.921	0.666-1.274				
OS	TAM→EXE	Low	241	1.00		0.869	-	-	-
		High	719	1.027	0.750-1.406				

Abbreviations: RFP: relapse free period BCSS: breast cancer specific survival OS: overall survival TAM: tamoxifen EXE: exemestane

*Adjusted for age, pT stage, pN stage, tumor grade, histology, surgery type, chemotherapy and radiotherapy

Supplementary Table 2C: Cox univariate and multivariate analysis for RFP, BCSS and OS stratified for endocrine therapy for HLA-G expression of the tumor.

				Univariate			Multivariate*		
Outcome	**Hormone therapy**	**HLA-G**	**N**	**HR**	**95%CI**	***p***	**HR**	**95%CI**	***p***
RFP	EXE	Absence	796	1.00		0.821	-	-	-
		Presence	246	0.960	0.676-1.364				
RFP	TAM→EXE	Absence	762	1.00		0.441	-	-	-
		Presence	238	0.876	0.625-1.227				
BCSS	EXE	Absence	796	1.00		0.958	-	-	-
		Presence	246	0.988	0.641-1.526				
BCSS	TAM→EXE	Absence	762	1.00		0.108	-	-	-
		Presence	238	0.698	0.451-1.082				
OS	EXE	Absence	796	1.00		0.165	-	-	-
		Presence	246	0.786	0.560-1.104				
OS	TAM→EXE	Absence	762	1.00		0.255	-	-	-
		Presence	238	0.830	0.601-1.144				

Abbreviations: RFP: relapse free period BCSS: breast cancer specific survival OS: overall survival TAM: tamoxifen EXE: exemestane

*Adjusted for age, pT stage, pN stage, tumor grade, histology, surgery type, chemotherapy and radiotherapy

Supplementary Table 2D: Cox univariate and multivariate analysis for RFP, BCSS and OS stratified for endocrine therapy for FoxP3+ expression.

Outcome	Hormone therapy	FoxP3+	N	Univariate HR	95%CI	*p*	Multivariate* HR	95%CI	*p*	Interaction *p*
RFP	EXE	Low	621	1.00		0.230	-	-	-	
		High	592	0.843	0.637-1.114					
RFP	TAM→EXE	Low	620	1.00		0.874	-	-	-	-
		High	593	0.979	0.755-1.270					
BCSS	EXE	Low	621	1.00		0.745	-	-	-	
		High	592	0.944	0.664-1.340					
BCSS	TAM→EXE	Low	620	1.00		0.197	-	-	-	-
		High	593	0.808	0.585-1.117					
OS	EXE	Low	621	1.00		0.138	-	-	-	
		High	592	0.821	0.633-1.065					
OS	TAM→EXE	Low	620	1.00		0.026	1.00		0.019	**<0.001**
		High	593	0.752	0.586-0.966		0.729	0.560-0.949		

Abbreviations: RFP: relapse free period BCSS: breast cancer specific survival OS: overall survival TAM: tamoxifen EXE: exemestane

*Adjusted for age, pT stage, pN stage, tumor grade, histology, surgery type, chemotherapy and radiotherapy

Supplementary Table 3: Composition of tumor immune subtypes based on the regression coefficient of the mono-markers in the relapse free period.

Tumor immune subtype category	HLA-I	HLA-G	HLA-E	FoxP3+	N	Regression coefficient range
High	Loss	Negative/Positive	Negative	Low/High	152	(-0.485) – (-0.108) (1st tertile)
	Loss	Positive	Positive	Low/High	6	
	Down-regulation	Positive	Negative	Low/High	14	
	Expression	Negative/Positive	Negative	Low/High	116	
	Expression	Positive	Positive	High	213	
Intermediate	Loss	Negative	Positive	Low/High	100	(-0.086 – (0.011) (2nd tertile)
	Down-regulation	Negative	Negative	Low/High	127	
	Expression	Positive	Positive	Low	91	
Low	Down-regulation	Negative/Positive	Positive	Low/High	288	(0.065) – (0.287) (3rd tertile)
	Expression	Negative	Positive	Low/High	529