Chapter 4

Immunological subtypes in breast cancer are prognostic for invasive ductal but not for invasive lobular breast carcinoma

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ABSTRACT

Background

Classical patient and tumor characteristics are the benchmark of personalized breast cancer (BC) management. Recent evidence demonstrated that immune and molecular profiling of BC may also play an important role. Despite evidence of differences between invasive ductal (IDC) and lobular (ILC) BC, they are infrequently accounted for when making treatment decisions for individual patients. The purpose of this study was to investigate the relevance of the tumor immune response in the major histological subtypes of BC. We also assessed the relationship between immune responses and molecular subtypes and their prognostic potential.

Methods

Immunostains were done for HLA-I, HLA-E, HLA-G, Treg, NK-cells and CTL for the composition of the immune profiles and Ki67, EGFR, CK5/6, ER, PR and HER2 for molecular profiles in 714 breast cancer patients who underwent primary surgery.

Results

No significant association was found between IDC (90.6%) and ILC (9.4%) and tumor immune subtypes (p=0.4) and molecular subtypes (p=0.4). However, for relapse free period (RFP) tumor immune subtyping was prognostic (p=0.002) in IDC, but not ILC. Contrary to ILC, IDC patients frequently expressed higher cleaved Caspase-3 and Ki67, which was prognostic. Intermediate immune susceptible IDC expressing high cleaved Caspase-3 or Ki67, showed worse RFP than low expression hereof (Caspase-3:p=0.004; Ki67:p=0.002), this was not seen for ILC or in high or low immune susceptible tumor types for neither IDC nor ILC.

Conclusion

Tumor immune characteristics and host immune responses are prognostic in IDC, but not ILC. To add, tumor immune profiles were only prognostic in Luminal A tumors.

INTRODUCTION

Nowadays, breast cancer is the most commonly diagnosed cancer type and the leading cause of cancer related death in the female population in the West [1]. Invasive ductal carcinoma (IDC) is by far the most common type of breast cancer. The second largest group comprises invasive lobular carcinoma (ILC), and reports indicate that 10 to 15 per cent of breast tumors are ILC [2]. Investigations into the differences between IDC and ILC have consistently shown that lobular carcinomas have a particular single-file growth pattern, tend to be larger, more often ER- and progesterone receptor (PR)-positive, and less aggressive than their ductal counterparts [2;3]. Nevertheless, these two types of breast cancer are treated similarly with regard to systemic adjuvant therapy, which is based on tumor size, histological grade, hormone receptor status and human epidermal growth factor receptor 2 (HER2) status.

Gene expression studies have identified several distinct breast cancer subtypes with marked differences in patient prognosis [4-6].This molecular classification proposed four different classes of breast tumors: Luminal A and B, basal-like and tumors overexpressing HER2. Luminal B tumors differ from Luminal A by a lower quantative content of hormone receptors. Basal-like tumors are triple negative tumors and HER2 overexpressing tumors cluster near these basal-like tumors [4-6]. Studies have shown that basal-like and HER2 overexpressing tumors have a more aggressive character, resulting in an unfavourable patient outcome compared to the Luminal tumor types.

With respect to over- and undertreatment, no optimal risk stratification exists for the allocation of the (individual) breast cancer patient to the most appropriate therapeutic regimen. It is likely that the different gene expression profiles explain the observed survival differences seen in the breast cancer population, even after controlling for tumor stage [7]. However, there is also strong evidence that the breast cancer host's adaptive immune system plays a crucial role in the control of tumor growth and progression [8]. On the other hand, breast tumor cells are able to adapt in order to escape the immune system and thus acquire characteristics to evade immunological recognition [9]. Several studies have attempted to elucidate this highly immunogenic disease by pointing out the great variety of immune reactions found in breast cancer.

Cytotoxic T-lymphocytes (CTL) are capable of recognizing tumor-associated antigens presented by classical human leukocytes antigen (HLA) class I on the tumor surface [10]. In order to avoid immune recognition from CTL, cancer cells may lose expression of this classical HLA class I [10]. However, this makes them more prone to natural killer (NK) cell recognition [11]. Non classical HLA class I molecules (HLA-E and HLA-G) play a crucial role in immune surveillance by NK-cells. Expression of these molecules on the cell surface

causes an inhibitory effect on NK-cell attack [11-13]. Another tumor escape mechanism of immunosurveillance is attraction and induction of immunosuppressive regulatory T cells (Treg) in the tumor microenvironment [14]. Together, these studies suggested that complex interactions take place between breast tumor cells and cells of the immune system. We recently reported on these complex interactions in a study on immune subtypes of breast cancer, representing adaptive immune escape variants based on tumor-associated antigens (classical HLA class I and non-classical HLA-E and HLA-G) and tumor-infiltrating lymphocytes (CTL, Treg and NK cells) [15]. In this study a clear association was observed between patient outcome and three tumor immune subtypes (low-, intermediate and high immune subtypes)(Appendix 1).

Our research group also demonstrated that the level of tumor cell proliferative and apoptotic signalling are important predictors in determining tumor development and thus predicting clinical outcome [16], and could thus very well add to the value of immunosubtypes in breast cancer. Assuming that healthy tissue signifies a fine proliferative-apoptotic balance, we propose that tumor growth may be more accurately determined by the outcome of the balance between tumor cell proliferation (Ki67) on one side and apoptosis (cleaved caspase-3) on the other.

With the increasing ability of earlier diagnosis and subsequently the low relapse rate in early breast cancer patients, in combination with the increasingly demanding nature of the contemporary patient population, the bar is raised for clinicians regarding optimal treatment [17]. Therefore, individualized estimation of the true therapeutic benefit is of crucial importance, in order to avoid over- and under-treatment.

Although the two major histological subtypes are frequently treated as similar entities, there are obvious differences in tumor-biological and prognostic characteristics.

The purpose of this study was to investigate the relevance of the host immune response, the apoptotic-proliferative interaction and molecular tumor types in the two major histological subtypes of breast cancer and in different molecular tumor types.

MATERIAL AND METHODS

Patients and tumors

Non-metastatic breast cancer patients who underwent primary surgical treatment at the Leiden University Medical Center (LUMC) between 1985 and 1996, with or without adjuvant systemic treatment were included in the present cohort. Only patients with ILC or IDC were included in the study. Patients with bilateral tumors or an earlier history of cancer (other than basal cell carcinoma or cervical carcinoma *in situ*) were excluded. Data on age, histological type, tumor grade, TNM stage, ER, PR and HER2 were assembled. In

addition, we collected information concerning local and systemic therapy and follow-up until loco-regional and/or distant recurrence and/or death. All tumors were graded according to current pathological standards by a single breast cancer pathologist (VS). Approval for the study was obtained with the LUMC Medical Ethics Committee. All samples were non-identifiable and coded according to the national ethical guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies).

Immunohistochemistry

Formalin fixed paraffin-embedded tumor blocks of the primary tumor were collected at the pathology department. H&E stained sections with clear histopathological tumor representation were used for assembling of tumor tissue microarray (TMA) paraffin blocks. From each donor breast tumor tissue block, three 0.6 mm^2 tissue cores were punched from tumor areas and transferred into a recipient paraffin block using a custom-made precision instrument. Sections of 4 µm were cut from FFPE tumor TMA material. Tissue sections were deparaffinised and rehydrated. Immunohistochemical staining was performed according to previously described standard protocols [18]. As previously described, sections were incubated overnight with anti-Ki67 (mouse anti human, M7240 Clone MIB-1: Dako, NL) , anti-cleaved Caspase-3 (Rabbit anti human, Anti-Asp175 #9661: Cell Signaling, USA) [16], anti-CD8 (mouse anti human, ab 17147, clone 144B: AbCam, UK), anti-PEN5 (mouse anti human, IM2354, clone 5H10.21.5: Beckman Coulter, NL), mouse monoclonal anti HCA2 and HC10 directed against Classical HLA class I (anti HLA-A and anti HLA-B/C, respectively) and non-classical HLA class I molecules using mouse monoclonal antibodies against HLA-E (ab2216, clone MEM-E/02: AbCam, UK) and HLA-G, ultimately Treg infiltration was determined using anti-FoxP3 antibody (ab 20034, clone 236A/E7: AbCam, UK) with the predetermined optimal dilutions [15;18;19]. For the molecular profiles additional staining was performed for EGFR (NCL-EGFR, Novocastra, UK) and CK5/6 (Clone D5/16 B4, Dako, NL). Immunohistochemical staining and quantification of ER, PGR and HER2 were performed previously. For each staining, all slides were stained simultaneously to avoid inter-assay variation. Negative controls were tissue sections that underwent the whole immunohistochemical staining with omission of the primary antibody.

Evaluation of the immunostaining

Expression of all markers were previously categorized in loss *versus* expression for classical HLA class I; no expression *versus* expression for HLA-E and HLA-G; infiltration absent *versus* infiltration present for Treg cells; presence *versus* absence for PEN5 [15;18;19].The absolute number of infiltrating CD8-positive cells was microscopically assessed per mm^2 and classified into two groups based on two thirds of patients with the lowest number

of CD8 infiltration/mm^2 *versus* the one third of patients with the highest number of CD8 infiltration/mm^2 [15]. For cleaved Caspase-3 staining the mean expression grade of positively stained cells in the TMA was defined as absent, low, intermediate and high scores. Cut-offs for low versus high expression of Ki67, EGFR and CK5/6 were based on the median expression level [16].

Tumor immune subtypes

Tumor immune subtypes, representing tumor adaptive immune escape variants were constructed with data from all known immunological variables of this patient cohort: classical HLA-I (HCA2 and HC10) and non-classical HLA-E and HLA-G expression and Treg (FoxP3), CTL (CD8) and NK cell (PEN5) infiltration in the tumor material [15]. As described by de Kruijf *et al.* , initially seven tumor immune subtypes were defined in ascending order from high immune susceptibility to low immune susceptibility. However, to facilitate clinical applicability the seven immune subtypes were brought back to a more simplified tumor immune subtype variable: high immune susceptibility, intermediate immune susceptibility and low immune susceptibility [15]. Only latter subdivision was used in this experimental design.

Molecular Subtypes

The IHC molecular profiles were previously developed by Carey *et al.* and validated for inter-assay agreement using a gene expression assay [7]). The IHC profile comprised of the markers ER, PGR, HER2, Ki67, EGFR and CK5/6. The Luminal A profile was defined as: ER+ and/or PGR+, HER2- and Ki67-; Luminal B: ER+ and/or PGR+ and HER2+ and/or Ki67+; ERBB2: ER-, PGR- and HER2+; Basal-like: ER-, PGR-, HER2- and EGFR+ and/or CK5/6+ and lastly the unclassified type: ER-, PGR-, HER2-, EGFR- and CK5/6-.

Statistical Analysis

Missing data were imputed (multiple imputation) using a model with IDC/ILC, grade, stage, age, follow-up and recurrence status, tumour immune subtypes, Ki67, Caspase3, molecular subtypes, ER, PR and HER2. With respect to multiple imputation, we generated 25 iterations and combined the estimates and standard errors using Rubin's Rules (micombine in STATA). Prior to running the model, checks were performed to test whether the data was missing at random. Multiple imputation by chained equations was used which assumes a multivariate distribution exists without specifying its form. In STATA the ICE module was used to perform the multiple imputation. Univariable and multivariable binary logistic regression analyses were used to identify differences between IDC and ILC. All variables with a $p \leq 0.1$ in univariable analyses were entered in the multivariable model. Relapse Free Period (RFP) was calculated using multivariable Cox proportional hazard models with any recurrence (locoregional recurrence and/or

distant recurrence, whichever came first) as event, with results stratified for IDC and ILC. Additional analyses were performed and stratified by age, tumor grade, tumor stage and nodal status. With respect to molecular subtypes, regression analyses were performed to assess proportional differences between molecular subtypes and immunological subtypes, Caspase3 and Ki67. In addition, Cox proportional hazard models were used to assess RFP in relation to immunological subtype, and stratified by molecular subtype. STATA/SE 12.0 version was used for all analyses.

RESULTS

Patient and tumor characteristics

Tumor material was available for 87% (704/822) of the patients. Median follow-up was 10 years (range= 0.02-22years), and median age in this cohort was 58 years (range 23-96 years). Clinicopathological and treatment characteristics for the original and imputated cohorts are shown in table 1.

IDC and ILC: differences in associations with clinicopathological parameters

No statistically significant difference was seen between IDC and ILC with regard to the association with tumor immune subtypes (p=0.4) and molecular subtypes (p=0.4). For the classical prognostic variables tumor grade (p<0.001) and pathological tumor stage (p=0.0002), a significant difference was seen between lobular and ductal breast tumor histology. ILC had significantly more grade II tumors and a higher pathological tumor stage. Both remained independent prognostic factors in the multivariate correction (grade: p<0.001, hazard ratio (HR): 12.6, 95% confidence interval (CI): 3.5-44.8); pathological tumor stage: p<0.0001, (HR pT2: 2.3 (95%CI: 1.1-4.8), HR pT3: 9.1 (95%CI: 3.1-26.4), HR pT4: 10.3 (95%CI: 3.0-35.5)). Also, compared to IDC, ILC showed a significantly lower expression pattern for both cleaved Caspase-3 (p=0.0004, HR low: 0.2 (95%CI: 0.1-0.6), HR intermediate: 0.4 (95%CI: 0.1-0.9), HR high: 0.1 (95%CI: 0.01-0.4)) and Ki67 (p=0.03, HR: 0.4, 95%CI: 0.2-0.9) following multivariable analyses.

Interaction with breast cancer histology

An interaction test for RFP and histological subtype was performed to test for differences in effect between IDC and ILC. Results showed a significant effect modification for RFP for immune subtype (p<0.001). In addition, similar results were observed with regard to active Caspase-3 (p<0.001) and molecular subtype (p=0.0005). With regard to Ki67, no effect modification was observed (p=0.09).These findings indicate a possible influence of breast cancer histology on the prognostic value of immune subtype, active Caspase-3 and molecular subtype.

Table 1: Baseline characteristics and distributions in the original and imputated datasets

		Original dataset		Multiple imputations
		N	%	%
Age	<45	137	19.2	19.2
	45-54	175	24.5	24.5
	55-64	157	22.0	22.0
	65+	245	34.3	34.3
Year	1985-1988	251	35.1	35.1
	1989-1992	232	32.5	32.5
	1993-1996	231	32.4	32.4
ER	Negative	288	40.4	42.7
	Positive	393	55.0	57.3
	Missing	33	4.6	
PR	Negative	316	44.3	48.0
	Positive	351	49.1	52.0
	Missing	47	6.6	
HER-2	No overexpression	520	72.8	83.2
	Overexpression	59	8.3	16.8
	Missing	135	18.9	
Grade	Grade I	116	16.3	16.8
	Grade II	342	47.9	48.7
	Grade III	244	34.2	34.5
	Missing	12	1.7	
pT stage	pT1	289	40.5	41.4
	pT2	328	45.9	47.2
	pT3	44	6.2	6.5
	pT4	33	4.6	4.9
	Unknown	20	2.8	
pN stage	Negative	381	53.4	54.9
	Positive	313	43.8	45.1
	Unknown	20	2.8	
Histological subtype	IDC	638	89.4	90.6
	ILC	66	9.2	9.4
	Missing	10	1.4	
Surgery	Mastectomy	416	58.3	58.3
	BCS	298	41.7	41.7

Abbreviations: pT stage: pathological Tumor stage, pN stage: pathological Nodal stage, IDC: invasive ductal carcinoma, ILC: invasive lobular carcinoma; BCS: breast conserving surgery

Relapse-free period in relation to IDC and ILC

Immunological profile was found to be prognostic for RFP in patients with IDC but not ILC, revealing a hazard ratio (HR) of 3.16 for low immune susceptibility compared to high immune susceptible tumor types for IDC only (p=0.002) (table 2). With regard to ILC a statistically significant association was only found in relation to immune subtype when stratified by tumor grade (grade I&II: p<0.001 and grade III: p=0.01 (data not shown).

For both high expression of apoptotic Caspase-3 (p=0.02, HR1.6, 95%CI: 1.1-2.4) and high expression of proliferative Ki67 (p=0.03, HR1.33, 95%CI: 1.02-1.74) a significantly worse association was found with RFP in IDC, but not for ILC (table 2). When stratified by pathological tumor stage, a significant association with the RFP was only found for stage I and II tumors (high Ki67 HR: 1.37, 955CI 1.01-1.84, p=0.04 and high caspase-3 HR: 1.85, 95%CI: 1.21-2.81, p= 0.0004) in IDC. With regard to IDC stage III and IV and ILC, no significant association was observed for Ki67 and caspase-3 (data not shown).

Table 2: Association between breast cancer histological subtype and active caspase-3, Ki67 and immunological subtypes in relation to relapse-free period

	Ductal breast cancer		Lobular breast cancer	
	HR* (95%CI)	**p-value**	**HR* (95%CI)**	**p-value**
All patients				
Immune subtypes				
High	1 (ref)	**0.002**	1 (ref)	0.3
Intermediate	1.95 (1.09-3.48)		2.10 (0.51-8.73)	
Low	3.16 (1.59-6.25)		3.24 (0.73-14.38)	
Active caspase-3				
Negative	1 (ref)	**0.02**	1 (ref)	0.2
Low	1.04 (0.72-1.52)		1.4 (0.4-4.7)	
Intermediate	1.55 (1.07-2.25)		3.8 (0.8-17.3)	
High	1.58 (1.06-2.36)		4.2 (0.7-24.2)	
Ki67				
Low	1 (ref)	**0.03**	1 (ref)	0.7
High	1.33 (1.02-1.74)		0.83 (0.29-2.37)	

* Adjusted for age, pT and pN

(Statistical interaction tests for histological subtype (IDC-ILC) and immune subtypes: p<0.001; histological subtype (IDC-ILC) and active caspase-3: p<0.001; histological subtype (IDC-ILC) and Ki67: p=0.09; histological subtype (IDC-ILC) and molecular subtypes: p=0.0005)

When Caspase-3 and Ki67 were combined, a statistically significant association was observed in relation to RFP for IDC (p=0.003), but not for ILC (p=0.07) (data not shown).

The highest HR was seen for high caspase-3 expression combined with a high proliferative Ki67 rate (HR2.0, 95%CI: 1.2-3.3, p=0.003). When stratified by immune subtype, intermediate tumor immune phenotypes were significantly associated with Caspase-3 (p=0.004) and Ki67 (p=0.002) expression regarding RFP. With increasing expression rate, both factors showed higher hazard ratios (Caspase-3 high: HR: 2.0, 95%CI: 0.9-4.2 and Ki67 high: HR: 2.2, 95%CI: 1.3-3.6). This was not observed in high or low tumor immune subtypes (data not shown).

Table 3: Associations between molecular subtypes and immune subtypes(**A**), active caspase-3(**B**) and Ki67(**C**)

A. Molecular subtypes (p=0.6)	**Immune High**	**Immune Intermediate**	**Immune Low**
Unclassified	14.0	74.5	11.4
Luminal A	17.0	60.9	22.1
Luminal B	19.7	61.9	18.4
HER2	16.0	58.4	25.6
Basal	19.5	58.1	22.5

B. Molecular subtypes (p<0.001)	**Caspase-3 Negative**	**Caspase-3 Low**	**Caspase-3 Intermediate**	**Caspase-3 High**
Unclassified	36.0	34.2	20.4	9.4
Luminal A	37.8	35.5	19.0	8.7
Luminal B	25.1	34.2	21.9	18.9
HER2	34.2	19.6	19.9	26.4
Basal	18.9	25.2	24.7	31.2

C. Molecular subtypes (p<0.001)	**Ki67 low**	**Ki67 high**
Unclassified	63.8	36.2
Luminal A	92.6	7.4
Luminal B	8.9	91.1
HER2	49.4	50.6
Basal	34.4	65.6

Molecular subtypes: immune profiles and prognosis

There were no proportional differences between molecular subtypes and tumor immune subtypes (p=0.6) (Table 3A). Luminal A tumors frequently did not express Caspase-3, while high expression was more prominent in Basal-like tumors (p<0.001) (Table 3B). Basal-like tumors also expressed higher levels of Ki67 (p<0.001) (Table 3C). As expected, luminal A tumors expressed low levels of Ki67, while luminal B tumors expressed high

Table 4: Associations of molecular and immunological subtypes with Relapse Free Period

		All Histological BC types	
Molecular subtypes	**Immune subtypes**	**HR* (95%CI)**	**p-value**
Luminal A			
	High	1 (ref)	**0.006**
	Intermediate	1.8 (0.8-4.4)	
	Low	3.9 (1.5-10.1)	
Luminal B			
	High	1 (ref)	0.4
	Intermediate	1.8 (0.8-4.2)	
	Low	2.0 (0.7-5.9)	
Basal-like			
	High	1 (ref)	0.1
	Intermediate	2.3 (0.7-7.7)	
	Low	3.8 (1.2-12.5)	

* Adjusted for age, pT and pN; HER2 excluded due to too few numbers

Ki67 levels (Table 3C). Needless to say, immune profiles were strong prognostic indicators in Luminal A tumors, but not in Luminal B, HER2, or Basal-like tumors (Table 4). Luminal A tumors with low immune susceptibility showed a worse RFP than patients with high immune susceptible tumors (high vs. low: HR 3.9, 95%CI:1.5-10.1, p=0.006).

DISCUSSION

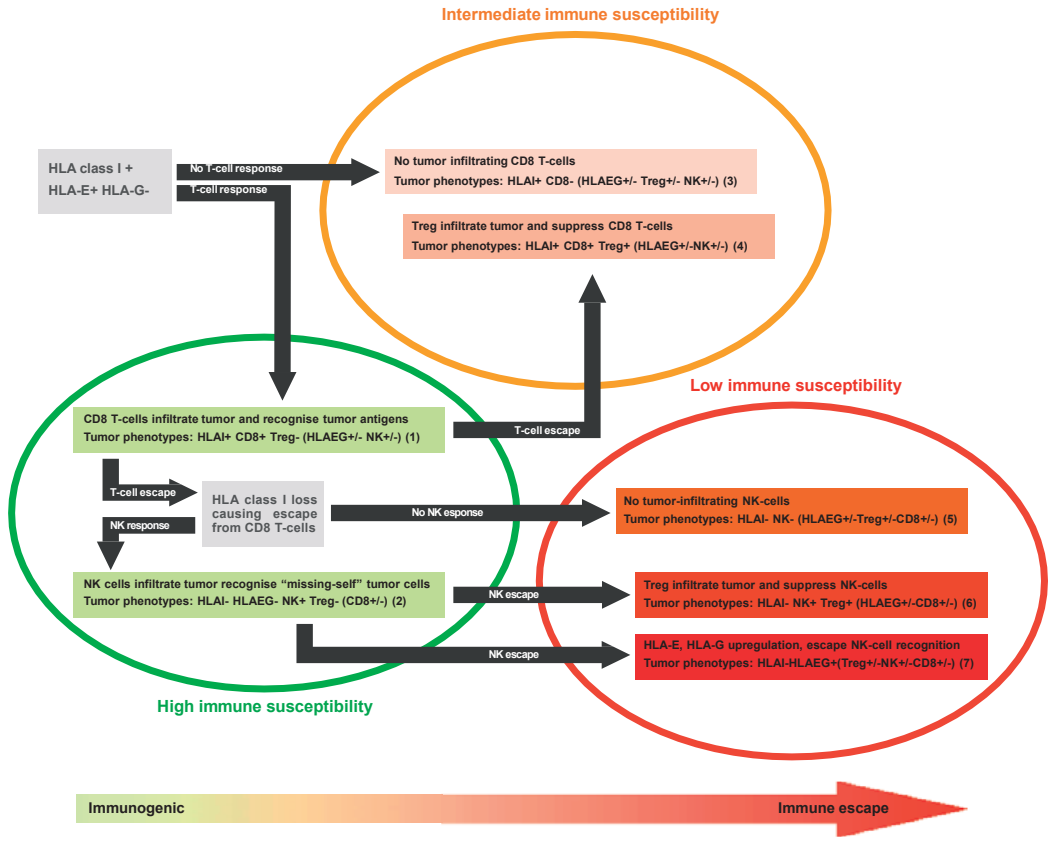
Previously, our study group reported that breast cancer patients with particular immunological profiles were more susceptible to unfavourable outcomes, demonstrating that patients with low immune susceptible tumors had a poorer prognosis when compared with intermediate or high susceptible tumors [15]. Multiple research groups demonstrated that molecular profiling of breast cancer also has important prognostic value [4;5]. The current study focused on distinguishing the major histological subtypes to assess whether tumor immune and molecular profiles were of prognostic value. Our results show that tumor immune profiles are prognostic indicators in different histological subtypes of breast tumors.

IDC and ILC, by far, constitute the largest group of breast tumors, comprising up to 95% of all breast cancers. Although current treatment regimens do not distinguish between these histological subtypes, IDC and ILC are considered to be distinctive entities, and differentiating between these two subtypes may play a role in prognosis and the optimisation of breast cancer treatment in addition to tumor size, histological grade, lymph node, ER/PR and HER2 status. The role of the immune response in cancer prognosis

has been speculated on previously. Several studies have demonstrated a correlation between single immune markers and patient outcome. CD8+ lymphocytes, one of the most studied immune markers worldwide revealed that in various types of cancer the presence hereof results in advantageous outcomes [20]. De Kruijf *et al.* demonstrated that the presence of classical HLA class I and high amounts of Treg infiltration affect prognosis in chemotherapy-treated breast cancer patients only [18]. In all probability, chemotherapy may selectively eliminate Treg, thus enabling CTLs to kill tumor cells that have retained HLA-I expression [18]. The same group demonstrated that presence of non-classical HLA subtypes E and G were associated with a worse relapse-free period [19]. This highly prognostic relation in breast cancer was also seen when the immune markers were combined into immunological subtypes [15]. However, in none of the previous studies the distinction was made between IDC and ILC. Differences in histological subtype may evoke diverse responses on breast cancer cells, thereby rendering one subtype more susceptible to the host immune response than another. In IDC patients, our analyses showed that low immune susceptibility as well as high Caspase-3 and Ki67 expression, were associated with a worse RFP, while this could not be demonstrated for ILC. These results also suggest that neither the apoptotic or proliferative marker, nor immune profiling applies to ILC, again suggesting that these tumors differ biologically from IDC.

With regard to molecular subtype, no correlation was observed between tumor immune subtype and molecular subtype. Based on previous studies, we know that tumors overexpressing HER2 and basal-like tumors generally present with more aggressive clinical characteristics than Luminal A and Luminal B tumors [4;5]. Our results confirm that tumor aggressiveness, as established by molecular subtypes of breast cancer, is not dependent on a tumor's immunological profile. In addition, immunological profiling was found to be prognostic only for Luminal A tumors. Luminal A tumors make up the largest group of IDC. Therefore it is not surprising that these results show a similar prognostic association within the immune profiles. Jung *et al.* proposed that ILC is frequently strongly ER-positive, HER2-negative and presents with low Ki67 expression, making it more likely to be characterized as a Luminal A molecular subtype [21]. This finding may lead to the assumption that outcomes for molecular and histological subtypes are similar, but this was not confirmed in our analyses. This implies that a simple extrapolation cannot be made and that histological subtypes are presumably far more complex.

In this report we investigated the relationship of the clinical outcome of breast cancer patients with immunological and histological profiles. Our results show that tumor immune biology differs greatly between IDC and ILC patients, confirming that ILC and IDC are completely different entities. Further studies are needed to validate these differences between IDC and ILC.



Appendix 1: Tumor immune subtypes: showing a schematic overview of different stages of immune surveillance and tumor immune escape classified into 7 immune subtypes, graded from (1) to (7) in ascending order from highly immunogenic and therefore high immune susceptibility (green) to high immune escape and low immune susceptibility (red), concerning combinations of CTL infiltration, NK-cell infiltration, Treg infiltration, classical HLA class I tumor expression, and HLA-EG tumor expression (de Kruijf *et al.*, BCRT 2013).

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