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Review

Breast cancer genomics and immuno-oncological markers to guide immune therapies

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ABSTRACT

There is an increasing awareness of the importance of tumor – immune cell interactions to the evolution and therapy responses of breast cancer (BC). Not surprisingly, numerous studies are currently assessing the clinical value of immune modulation for BC patients. However, till now durable clinical responses are only rarely observed. It is important to realize that BC is a heterogeneous disease comprising several histological and molecular subtypes, which cannot be expected to be equally immunogenic and therefore not equally sensitive to single immune therapies. Here we review the characteristics of infiltrating leukocytes in healthy and malignant breast tissue, the prognostic and predictive values of immune cell subsets across different BC subtypes and the various existing immune evasive mechanisms. Furthermore, we describe the presence of certain groups of antigens as putative targets for treatment, evaluate the outcomes of current clinical immunotherapy trials, and finally, we propose a strategy to better implement immuno-oncological markers to guide future immune therapies in BC.

1. Introduction

Cancer immunotherapy is a rapidly emerging field, which has proven successful in the treatment of various tumor types, such as lymphoma, melanoma, renal cell carcinoma, and non-small cell lung cancer [1]. Initially, breast cancer (BC) has been considered a poorly immunogenic tumor type and has therefore not been extensively investigated for its susceptibility to immune therapies. During the past years, however, it became evident that certain cases of BC are strongly infiltrated by immune cells and that the presence of these immune cells has significant prognostic and predictive value. Although many studies are currently examining immune therapies for BC, still only a minority of patients appear to respond, and little is known about the underlying mechanisms of treatment efficacy. Thus, there is an unmet need to get better understanding of the interaction of breast cancer and the immune system in order to identify potential immuno-oncological prognostic and predictive markers as well as novel leads for effective mono or combination immune therapies.

Genomics has improved our understanding of BC biology and revealed 4 intrinsic molecular subtypes: luminal A (resembling the histological phenotype: ER+, PR+, HER2-, Ki67-), luminal B (ER+, PR+, HER+/−, Ki67+), HER2 (ER-, PR-, HER2+), and basal-like subtype (ER-, PR-, HER2-). The classification of BC into subtypes bears clinical relevance. For instance, in the treatment of the hormone receptor (HR) positive subtypes (those that are positive for ER and/or PR) endocrine therapy, including aromatase inhibitors or selective estrogen receptor mediators such as Tamoxifen, play an important role. HER2 over-expressing tumors are generally treated with HER2-targeting drugs such

Abbreviations: APC, antigen presenting cell; APOBEC, apolipoprotein B mRNA editing enzyme catalytic polypeptide-like; BRCA1/2, breast cancer 1/2; BCSS, breast cancer specific survival; B2M, β-2-microglobulin; CAF, cancer associated fibroblast; CCL, chemokine ligand; CD, cluster of differentiation; CMV, cytomegalo virus; CTLA4, cytotoxic T lymphocyte associated protein 4; CR, Complete response; CXCL, CXC-motif chemokine ligand; DC, dendritic cell; DCIS, ductal carcinoma in situ; DFS, disease free survival; EBV, epstein-barr virus; ECM, extracellular matrix; ELF5, E75 like ETS transcription factor 5; EMT, epithelial − mesenchymal transition; GBP1, interferone induced guanylate binding protein 1; GRZM, granzyme; H & E, hematoxylin and eosin; HER2, human epidermal growth factor receptor 2; HERV-K, human endogenous retrovirus K; HLA, human leucocyte antigen; HPV, human papiloma virus; HR, hormone receptor or hazard ratio; hTERT, telomerase reverse transcriptase; IDC, invasive ductal carcinoma; IDO1, indoleamine-pyrrole 2,3-dioxygenase; IFN, interferon; IGK, immunoglobin kappa locus; IGLL5, immunoglobin lambda like polypeptide 5; JAK1/2, Janus kinase 1/2; LAG3, lymphocyte activation gene 3; MDSC, myeloid derived suppressor cell; MEK, map kinase kinase; MFS, metastasis free survival; MHC, major histocompatibility complex; MMTV, mouse mammary tumor virus; MUC1, mucin 1; MV, measles virus; NK, natural killer cell; NO, nitric oxide; OCLN, occludin; OR, objective response or odds ratio; OS, overall survival; PC, plasma cell; PD1, programmed cell death protein 1; PDL1, programmed death ligand; PI3K, phosphoinositol 3-kinase; PR, progesteron receptor; PTEN, phosphatase and tensin homolog; RFS, relapse-free survival; ROS, reactive oxygen species; SD, stable disease; STAT1, signal transducer and activator of transcription 1; TAA, tumor associated antigen; TAP, transport associated protein; TIL, tumor infiltrating leukocytes; TLS, tertiary lymphoid structures; TGFB, transforming growth factor beta; TNBC, triple negative breast cancer; TNFa, tumor necrosis factor alpha; Treg, Regulatory T cell; Tγδ, gamma delta T cell

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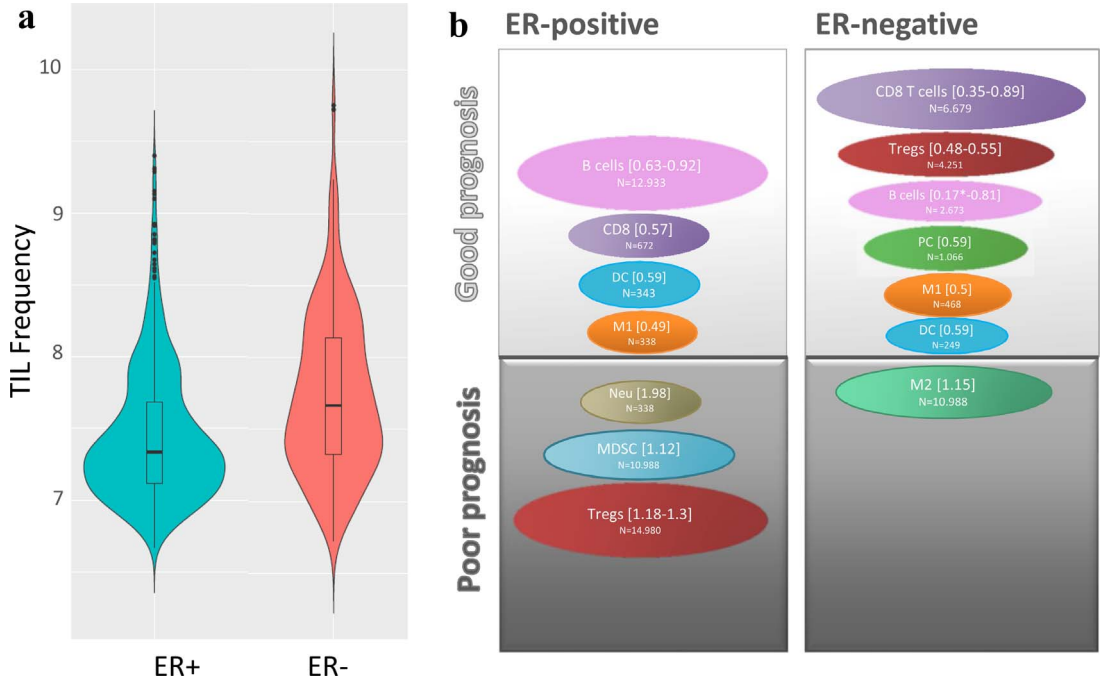

Fig. 1. TIL frequencies and prognosis in ER+ and ER- BC: Violin plots based on average RNA expression of TIL gene signature [> 100 leukocyte related genes, manuscript in preperation] on a log scale, per patient based on ER-status. (Data from NCBI's Gene Expression Omnibus, accessions GSE2034, GSE5327, GSE2990, GSE7390 and GSE11121.) (A). Leukocyte subsets which are significantly correlated ($p < 0.05$) with overall survival, or metastasis free survival (*), in ER+ and ER- tumors. Hazard ratios of multivariant regression analyses are shown between brackets [HR]. Circle sizes are indicative of cohort-size (N), based on numbers of patients evaluated in one or more studies [15, 20–23, 26–46] . Studies include gene expression based analysis, immunohistochemistry and/or flow cytometry (B).

as trastuzumab and pertuzumab, whereas triple negative BC (TNBC, largely resembling the basal-like BC subtype) is mostly treated with standard cytotoxic therapies.

Notably, and the focus of the current review, these molecular subtypes also differ with respect to quantity and composition of tumor infiltrating leukocytes (TILs). In BC, an enormous number of studies have been performed in order to evaluate the prognostic and predictive values of TILs, and their specific subsets. Although mononuclear cells can easily be identified by H & E-stainings upon routine diagnostics, this technique does not allow accurate assessment of different immune subsets. Immune stainings have enabled the phenotypic distinction of various cell types, but are often limited to those markers for which well-characterized antibodies are available. Recent advances in immunogenomics have paved the way towards enhanced understanding of specific immune subsets and their interactions with tumor cells based on gene expression data [2–5]. In addition, emerging DNA sequencing data has made it possible to explore mutational landscapes of BC and investigate their relationship with TILs and immune pathways. Here, we discuss TIL profiles, prognosis and prediction based on TIL subsets, antigenicity, immune evasive mechanisms, and current immunotherapy trials. Finally, we propose a strategy to select and implement immune-oncological markers to improve therapy choices for BC patients.

2. Normal breast versus (pre)malignant breast tissues: quantity and quality of TILs

2.1. Normal breast tissue

Immune cells in the healthy mammary gland form an active and dynamic barrier against microbes in the mucosal layer [6]. In addition, immune cells take part in mammary gland remodeling and are considered to play a role in cancer immune surveillance [7]. In normal breast tissue, one generally finds low numbers of leukocytes, including T cells (typically expressing the markers CD3, CD4 or CD3, CD8), B cells (CD20), macrophages (CD68) and dendritic cells (CD11c) [6]. These immune cells are not found in interlobular stroma but are restricted to the lobules, where T cells directly associate with the epithelial layer [8]. While frequencies of macrophages and CD4 T cells are rather constant, frequencies of CD8 T cells depend on hormonal changes and peak within the luteal phase of the menstrual cycle, coinciding with epithelial cell turnover [9].

2.2. Pre-malignant breast tissue

BC formation is a multistep process, including premalignant stages of hyperplasia and ductal carcinoma in situ (DCIS) and the malignant stage of invasive ductal carcinoma (IDC) [10]. The transition from normal breast tissue to malignancy is typically accompanied by an increased infiltration of leukocytes, including myeloid cells, B cells and cytotoxic CD8 T cells [8]. First, in premalignant DCIS, an increased lymphocytic infiltration is observed [11], which is significantly higher in HER2+ and TN DCIS compared to HR+ DCIS [12]. In DCIS, numbers of neutrophils are significantly increased compared to normal tissue, however in this tumor stage activated T cells represent the dominant lymphocyte population [13], followed by B cells and the immune suppressive regulatory T cells (Tregs: CD4, CD25, FOXP3) [14]. While in normal and premalignant BC the CD4/CD8 T cell-ratio is approximately 2, in IDC this ratio is shifted towards 0.3 [15,16].

2.3. Malignant breast tissue

A common feature in IDC is a high overall quantity of TILs. Interestingly, high lymphocytic numbers relate to better prognosis and predict a favorable response to neo-adjuvant chemotherapy [17–19] (see also Sections 3 and 4). In fact, in highly inflamed tumors, TIL frequency was found to be a superior prognostic marker in comparison to HR status and lymph node involvement in patients with primary operable BC [15]. Notably, characteristics of TILs vary across molecular subtypes of BC [20,21]. The frequency of TILs is usually high in the more aggressive types of BC, including the ER- subtypes (HER2 and

basal) as well as the highly proliferating luminal B subtype, but are low in the less aggressive luminal A subtype [22,23] (Fig. 1A). Even though, the evaluation of overall TIL frequencies, based on H & E stainings, in feasible and clinically relevant [24,25], it is noteworthy, that TILs represent a heterogeneous collection of immune cells, and not all types or subsets of immune cells are associated with a favorable clinical outcome (Fig. 1B and explained in more detail in Section 3) Techniques that go beyond H & E, such as PCR, flow cytometry and in situ stainings, may be required to define the composition of TILs more accurately.

3. Prognosis of breast cancer based on TILs

Numerous studies have investigated the prognostic values of TILs and specific subsets by means of H & E- and immune stainings, flow cytometry or analyses of gene expression. We evaluated 33 of such studies and schematically categorized different TIL subsets based on hazard ratios (HR) for ER- and ER + BC (Fig. 1B).

3.1. Prognostic TILs in ER- breast cancer

ER- tumors typically show higher numbers of TILs when compared to ER + tumors. Especially numbers of T- and B cells, macrophages and myeloid derived suppressor cells (MDSC) are higher in ER- compared to ER + BC [21].

3.1.1. Favorable outcome

Adaptive immune cells, including cells of T- and B cell lineages, are typically found in sites of prior, or ongoing immune reactions. High numbers of such lymphocytes are associated with a better prognosis in lymph node negative, primary BC patients including those with stages I–III [15,26–28]. Moreover, numerous studies show that high frequencies of CD8 effector T cells and T helper type-1 gene signatures (Th1: IFNG, STAT1, GRZM, CXCL9) are correlated with favorable clinical outcome, particularly in ER- tumors [22,23,25,30]. In contrast, high numbers of Tregs in tumor tissue and blood are correlated with favorable outcome in ER- tumors, which may reflect the initiation of negative feedback since numbers of Tregs strongly correlate with those of CD8 T cells and are correlated with poor prognosis in the absence of CD8 T cells [30–32]. B cell and plasma cell (PC: CD138) gene signatures are especially significant prognostic factors in ER- BC, but also in highly proliferating luminal B BC [22]. Macrophages are enriched in basal-like BC and associate with survival according to immune stainings [15,18,28]. In agreement, myeloid and macrophage/dendritic cell signatures (oa. MHCII, CD11c, CD11b) were found to have overall prognostic value in BC according to large gene-expression cohorts [22,33]. Notably, higher blood lymphocyte to monocyte ratio (LMR) correlates with overall survival (OS) in 1570 BC patients (HR: 1.63, 95% CI: 1.07-2.49), in particular in TNBC patients (HR: 3.05, 95% CI: 1.08-8.61) [34].

3.1.2. Unfavorable outcome

Frequencies of immature immune cells, such as MDSC (CD33) which can originate from monocytic or granulocytic lineages, are enriched in highly proliferating ER- tumors [21], and intra-tumoral numbers of these cells are correlated with poor survival in ER- tumors [35]. Elevated numbers of MDSCs are also found in peripheral blood of BC patients when compared to healthy controls [36]. Strikingly, also in the blood compartment frequencies of MDSCs are associated with later stage tumors, metastatic tumor burden, and are correlated with reduced survival [37,38]. Also, numbers of intra-tumoral neutrophils (N, CD16) are associated with poor BC-specific survival [15], and meta-analysis revealed significant unfavorable prognosis in case of a high neutrophil to lymphocyte ratio (NLR, HR(OS): 2.03, 95% CI: 1.41-2.93) [39]. High frequencies of undifferentiated macrophages and alternatively activated, M2 macrophages (CD163) are inversely correlated with survival [35].

3.2. Prognostic TILs in ER + BC

In comparison with ER- BC, less studies found significant correlations between immune cell subsets and clinical outcome in ER + BC. Overall, mostly innate immune cells cluster to the ER +, luminal A tumors and correlate with good prognosis [21].

3.2.1. Favorable outcome

NK cells are shown to have anti-tumor activity in ER + BC [40,41], yet their numbers are decreased in later tumor stages [42]. Signatures of B cells including plasma cells, plasmablasts and immunoglobulin not only correlate with favorable outcome in ER-, but also ER + tumors [20,32,40].

3.2.2. Unfavorable outcome

Gamma delta T cells (Tγδ, TCRγ/δ) are more frequent in BC compared to other immunogenic tumors, such as melanoma, suggesting a unique role of these T cells in BC [45]. Moreover, numbers of a subset of Tγδ cells, the so-called regulatory Tγδ, correlate with advanced cancer stages, lymph node involvement and numbers of FOXP3 + cells in ER + BC, whereas numbers of this subset inversely correlate with those of CD8 T cells in these tumors [46]. It is important to note that while Tregs are correlated with good prognosis in ER- tumors, these cells are strongly associated with adverse clinical outcome in ER + tumors [30,32]. Even though numbers of MDSC are generally lower in ER + tumors, their presence is correlated with poor OS [35].

4. Prediction of breast cancer therapies based on TILs

Many studies show that standard neo-adjuvant therapies can recruit TILs and modify the tumor microenvironment. *Vice versa*, TILs, when present prior to therapy, were found to be predictive for clinical response to neo-adjuvant therapies.

4.1. Prediction of neo-adjuvant therapies based on TILs

Besides surgical resection and radiotherapy (RT), primary operable BC patients are frequently treated with neo-adjuvant chemotherapy (NAC) and/or targeted therapies. It is of interest to note that numerous studies suggest that the immune system is required to boost the efficacy of NAC. Sequential treatment with anthracycline- or taxane-based drugs is a common form of NAC used to treat BC, with pathological complete responses (pCR) ranging from 10 to 30%. NAC based on anthracyclines and taxanes can directly induce immunogenic tumor cell death, resulting in increased antigen presentation. Moreover, NAC was found to induce inflammatory pathways in tumor associated fibroblasts, such as interferon, Wnt and TGFβ signaling pathways [47], which can enhance recruitment of TILs. Consequently, immune gene signatures have been revealed to predict the response to NAC across various studies, regardless of molecular subtypes or treatment regime [22,48,49]. Also, high TIL frequencies (> 60%), as assessed by H & E stainings were predictive for response to NAC [50]. In fact, a 10% increase in TIL frequencies resulted in 16% increase in pCR rates in TNBC (OR: 1.16), 13% in HER2 (OR: 1.13), and 33% in ER + /HER2- BC (OR: 1.31). In the latter subtype no survival benefit was noted, which may be attributed to differences in TIL composition (as explained in more detail in Sections 3.1 and 3.2). The predictive value of TILs in the setting of NAC is mainly attributed to high numbers of CD8 T cells (odds ratio (OR) for pCR: 1.59–3.36, [51,52]) but also the presence of follicular T helper cells (Tfh: CD200, CXCL13), were found to have predictive value in ER- (OR (pCR): 1.34–1.85) as well as ER + (OR(pCR): 2.52) BC patients, across different studies, using both immune stainings and genomic approaches [35,41,51]. *Vice versa*, chemotherapy can change the immune cell composition in tumor tissue and blood. For example, within 2 weeks after NAC, B-, T- and NK cells were found significantly depleted from peripheral blood compared to pretreatment levels, with numbers of B

and CD4 T cells remaining low up to 9 months post chemotherapy [53], whereas numbers of MDSCs were increased [37]. Numbers of intra-tumoral CD68 macrophages were found significantly decreased to NAC, while those of intra-tumoral CD8 T cells were increased compared to pre-NAC frequencies [18,37]. Strikingly, high intra-tumoral numbers of CD3, CD4 and CD20 as well as high CD4/CD8 ratios prior to therapy predict clinical benefit following NAC independently of subtype or clinical parameters (OR(pCR): 17.84, [18]). In ER- tumors, pre-therapy T- and B cell signatures were found to predict long-term (> 6 year) outcome to anthracycline-based chemotherapy (OR(pCR):6.33, [54]).

Similar to NAC, RT can also induce immunogenic cell death, antigen release and local inflammation, and consequently evoke an innate and adaptive immune response directed against the tumor [55]. Interestingly, in an ER-, HER2+ patient, who showed a clinical complete response following neo-adjuvant (paclitaxel) and RT, the production of Th1-type cytokines by tumor-specific T cells was enhanced compared to pre-treatment [56]. Immune responses may also predict clinical responses to endocrine therapy [57,58]. In example, OS of post-menopausal women treated with aromatase inhibitors, which block the conversion of androgens into estrogens, is correlated with high numbers of TILs, in particular high numbers of Tregs [59]. In contrast, treatment with tamoxifen (an ER antagonist) shifts immune response from Th1-towards Th2-type T cell responses in an estrogen-independent manner, and may promote immune escape [60]. Treatment with trastuzumab, a humanized antibody directed towards HER2, is at least in part dependent on the immune system as this treatment induces influx of T cells, macrophages and NK cells into tumor tissue and promotes cell-mediated cytotoxicity [61]. *Vice versa*, pre-existing lymphocytic infiltrate can predict response to trastuzumab treatment [62,63], although clinical studies provide contradictive data. While in certain trials higher TIL frequencies [64], or high expression of TIL gene signatures [65] at diagnosis were significantly associated with good response when trastuzumab was combined with CT, in another large clinical trial the presence of TILs was not associated with survival following the same treatment combination [66]. Interestingly, in the same study, expression of genes related to immune function, did correlate with survival when trastuzumab was combined with CT [67], suggesting that particular TIL subsets, rather than bulk TIL predict response. These conflicting results between different studies, may be explained by differences in treatment regime and HR status of patients [68]. In fact, the latter correlates with both, TIL frequency and composition, as well as CT responses, potentially favoring that patients treated with trastuzumab should be stratified according to HR status. Further research is required to better define the predictive value of particular TIL subsets in this patient group.

Overall, the above findings suggest that standard care of treatments can modulate the tumor microenvironment and may sensitize tumors towards immune therapies. In fact, combination of RT and NAC with immune therapies has already shown promising results in mouse models of BC, and is currently investigated in a number of clinical trials (Table 1), [69–71].

4.2. Prediction of immune therapies based on TILs

Thus far, immunotherapeutic approaches to treat BC include: peptide vaccines; autologous transfer of T cells, NK cells or DCs; and application of checkpoint inhibitors. An overview of these treatments is given in Table 1. Vaccinations in BC have been focusing mainly on targeting the over-expressed HER2/neu antigen, for which successful treatment has been achieved in DCIS, usually resulting in lesion shrinkage along with activation of HER2-specific CD8 T cells [72–74]. In later stage tumors, however, at best stable disease (SD) has been achieved using similar approaches. Adoptive transfer of autologous HER2-specific T cells resulted in the killing of BC cells that were metastasized to bone marrow, but these T cells were unable to penetrate and resolve liver metastases [75,76]. In contrast, adoptive transfer of allogeneic T cells or NK cells to metastatic BC patients (all subtypes) did not result in T cell persistence and frequently led to graft versus host disease [77], [78]. Promising clinical responses have been observed for checkpoint inhibition in the advanced metastatic BC setting. For example, blockade of CTLA-4 (Tremelimumab) has led to SD for > 12 weeks in 42% of heavily pre-treated ER+ patients [79]. Even better responses, including a few complete responses and several partial responses, were observed upon treatment with a PD-1 blocking antibody (Pembrolizumab) in TNBC patients with PD-L1-positive tumors in 2 trials (objective response rate (ORR): 18.5%, [80]; ORR: 23%, [81]). Combinations of CTLA-4 (Tremelimumab) and PD-L1 (Durvalumab) blockade even reached OR in 43% of stage IV, TNBC patients, however, no OR was observed in any of the 11 HR + patients, [82] which may be due to low numbers of CD8 T cells in these tumors (Fig. 3). In contrast, blockade of PD-L1 (antibody not specified) in a small group of 4 stage IV BC patients (unknown HR status) did not result in any clinical response [83]. Notably, in that study, PD-L1 expression had not been assessed prior to PD-L1 treatment, which may have contributed to these contradicting results. Another large trial with a PD-L1 blocking antibody (Avelumab), in 168 BC patients, which were not selected for BC subtype nor PD-L1 expression, resulted in a low ORR of 4.8%, including 1 CR and 7 PR [84]. When evaluating BC subtypes in that study, TNBC patients had an ORR of 8.6% while HR+ patients had an ORR of 2.8%. Even though > 10% PD-L1 expression on immune cells in TNBC tumors correlated with response, interestingly, there was no overall association of PD-L1 expression and OR [85]. Due to the dynamic nature of PD-L1 expression (explained in Section 5), we propose to take caution when considering to use this molecule to stratify BC patients for treatment with checkpoint inhibitors. The presence of TILs, in particular CD8 T cells, and (co-) expression of checkpoint molecules on these cells may provide more discriminatory markers for therapy response when compared to tumor cell PD-L1 expression. In fact, high stromal TIL numbers were significantly correlated with a better response to PD-1 blockade (Pembrolizumab) when administered as monotherapy in a first-line setting for metastatic TNBC (ORR: 39.1% above median stromal TIL; ORR: 8.7% below median stromal TIL), while PD-L1 expression did not add to the response prediction in that cohort [86]. Promising results have also been observed when combining checkpoint blockade with standard chemotherapies in the neo-adjuvant, as well as the advanced disease setting of TNBC: Upon combination of neo-adjuvant paclitaxel and PD-1 blockade (Pembrolizumab), an impressive ORR of 71% was observed in stage II/III TNBC patients, and an ORR of 28% was seen in HR+ patients, which were both significantly increased when compared to paclitaxel monotherapy (ORR: 19% and 14% in both patient groups, respectively) [87]. In addition, combination of nab-paclitaxel and PD-L1 blockade (Atezolizumab) in metastatic TNBC reached comparable results (ORR: 70%) independent of PDL-1 status [88]. Notably, ORR where higher in early lines of therapy in patients with a lower disease burden, reaching 11% CR and 78% PR in patients with one lesion, in contrast to 0% CR and 43% PR in patients with 3 lesions [88]. When treating mainly HR+ metastatic BC with a combination of LAG3Ig fusion protein (IMP321) with paclitaxel, an ORR of 50% was achieved which was 25% higher compared to a historical paclitaxel treatment results [89]. These data strongly encourage the rational of combination therapies, particularly in BC where initial TIL numbers are low (HR+) and sensitization of the tumor micro environment may be required for effective immune therapies (Fig. 4).

At the moment, an increasing number of clinical studies are focusing on immune therapies for BC of various subtypes. A main category of immune treatments is represented by (combinations of) antibodies blocking the checkpoints PD-1, PD-L1, CTLA-4, and LAG-3. In addition to the checkpoint blockade studies mentioned above, another 91 trials are currently being scheduled (blockade of PDL-1: 13x; CTLA-4: 10x; PD-1: 62x; LAG3: 6x, according to clinicaltrials.gov). In addition to checkpoint blockers, vaccine studies are performed directed against over-expressed antigens other than HER2, such as hTERT, surviving and

Table 1
Overview of immune therapy clinical trials in BC.

DC vaccination	Target	Stage/type	Patients	Clinical outcome	References
Her2/neu peptides (MHCI and II)	HER2	0/HER2	11	PR: 64%	[72]
Her2/neu peptides (MHCI and II)	HER2	0/HER2	27	PR: 88%, CR: 40%(ER-); 5.9%(ER+)	[73,74]
autologous APC + Her2/neu cDNA	HER2	IV/HER2	18	PR: 5%, SD: 16%	[151]
autologous DC		II,IIIA/ER-, PR-	31	PD: 100%	[152]
wt p53 peptide (MHC II)	P53	IV	26	SD: 30%	[153]
Vaccination (not DC)					
Mam-A cDNA	Mam-A	IV	7	NA	[154]
E75 Her2 peptide (HLA-A2/A3)	HER2	0/HER2	182	DFS: 94.3%	[155]
MDA-MB-231 (CD80+, HLA-A2, HER2) cell line	HER2	IV	30	SD: 30%	[156]
AE37 Her2/neu peptide (MHCII)	HER2	0	15	NA	[157]
Checkpoint inhibitors					
anti PD-L1 (not specified)	PDL1	IV	4	PD: 100%	[83]
tremelimumab	CTLA4	IV/ER+	26	SD: 42%	[79]
pembrolizumab	PD1	IV/TNBC	27	CR: 2.7%, PR: 15%, SD:26%	[80]
pembrolizumab	PD1	IV/TNBC	52	CR: 4% PR: 19%, SD: 17%	[81]
avelumab	PDL1	II/IV	168	CR:0.6% PR: 4.8% SD:24%	[84]
pembrolizumab + paclitaxel	PD1	II, III/Her2-	69	CR: 71.4% (TNBC), CR: 28% (HR+)	[87]
durvalumab + tremelimumab	PDL1, CTLA4	IV/ER+, TNBC	18	PR: 43% (TNBC), 0% ER+	[82]
atezolizumab + nab-paclitaxel	PDL1	IV TNBC	32	CR: 4,2% PR:66.7% SD: 20.8%	[88]
Adoptive Transfer of immune cells					
autologous T cells	HER2	IV/HER2	1	NA	[75]
allogenic T cell mix		IV	9	PR: 56%	[77]
autologous T cells + bispecific antibody	HER2, CD3	IV/HER2+/−	23	SD: 27%	[76]
allogenic NK cells		IV	6	NA	[78]
Other therapies					
oxidized mannam MUC1	MUC1	II/MUC1+	31	NA	[158]
zoledronate (ydT cell agonist) + IL2		IV	10	PR: 10%, SD: 20%	[159]
IMP321 (LAG3Ig fusion protein) + paclitaxel	MHCII	IV	30	PR: 50% SD:40%	[89]

NA, not assessed; PR, partial response; CR, complete response; SD, stable disease; PD, progressive disease; DFS, disease free survival; DTH, delayed type hypersensitivity; Tγδ, gamma delta T cell.

p53. And finally, adoptive transfer studies with T cells have started, either those with T cells engineered with a Chimeric Antigen Receptor (directed against: HER2 (3x), EpCAM, ROR1, MUC1 and CD133) or a T cell Receptor (directed against: survivin or Cancer Germline Antigens: Mesothelin, NY-ESO1:3x, MAGE-A4, PRAME and SSX1), (according to clinicaltrials.gov).

5. Immunogenicity of breast cancer knows several flavors

Immunogenicity of tumor tissue determines the initiation of an anti-tumor adaptive immune response, and depends on various factors, including the quantity and quality of TAA and their presentation to infiltrating immune cells. TAAs are typically categorized in different groups of antigens, including shared antigens which are generally over-expressed in tumors, but not restricted to malignant tissues (and also expressed by normal tissues). Some shared antigens, such as oncoviral antigens and Cancer Germline Antigens (CGAs), are predominantly expressed in tumors and, in case of CGAs, also in immune privileged tissues of the germline. Besides shared antigens, TAAs also include non-shared antigens, such as tumor-specific neo-antigens, which derive from mutated proteins, and are absent in normal tissues.

Most of these groups of TAAs have been exploited for their use as immunotherapeutic targets in many different tumors. In BC most experience has been gained with the targeting of over-expressed antigens. Even though over-expressed antigens are not tumor-specific, cancer vaccines directed towards such antigens, including HER2, MUC1, and hTERT, could induce partial regression and induce immune responses against these antigens in a number of BC patients without major side effects (reviewed in [90,91]). Virus specific DNA can drive tumor formation and lead to expression of oncoviral antigens. Virus specific DNA (EBV, HPV and MMTV) is significantly more frequently detected in BC compared to normal breast tissues [92]. For instance, expression of human retrovirus type K (HERV-K) is enriched in BC, including BC cell lines, and antibody titers are significantly increased in women with DCIS and IDC when compared to healthy controls [93]. Also, Measle Virus (MV) was detected in 64% of BC including DCIS, and its expression correlated with younger age and lower grade tumors [94]. Notably, human cytomegalovirus (CMV) is expressed in 100% of primary BC specimens and 95% of lymph node metastases [95], while it is generally not expressed in normal tissues [96]. Although in general the presence and reported immunogenicity of viral antigens is evident, the therapeutic potential of this class of TAAs in BC is not clear, nor have these antigens yet been targeted in BC patients. CGAs have not yet been targeted frequently either, while the majority of BC express at least a single CGA [97]. Although CGAs are expressed throughout all tumor stages, including DCIS and all histotypes [98], expression levels and number of expressed CGAs are significantly increased in high grade and ER- BC (highest in basal-like BC) (Fig. 2A). Interestingly, especially TNBC patients and BRCA carriers often co-express multiple CGAs [99,100]. Besides their high and tumor-specific expression of at least some CGAs, these antigens represent therapeutically relevant target antigens since they have been reported to elicit humoral immune response and were shown in some instances to contribute to BC development. In example, patients with CGA+ BC have demonstrated enhanced antibody titers against these antigens, and CGAs, have been reported to be associated with increased EMT, genomic instability, angiogenesis and tissue invasion in BC [101–103]. Not surprisingly, expression of these CGAs is often linked to adverse outcome. With respect to neo-antigens, expression of these antigens is governed by the mutational load of tumors. Compared to other cancer types, BC has an average mutational load of 1 somatic mutation per Mb, which ranks these tumors among the lower half of a large series of different human cancer types [104]. A mutational load of 10 somatic mutations per Mb (=150 non-synonymous mutations in all expressed genes) is considered sufficient to elicit a T cell response in melanoma [105]. This suggests that the overall chance of T cells recognizing neo-antigens in BC is

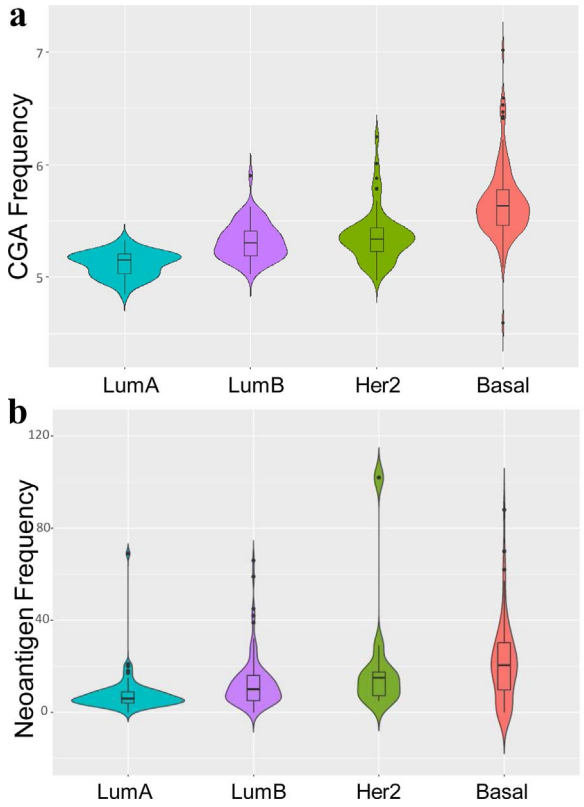

Fig. 2. Antigen expression across BC subtypes. Violin plots show average CGA gene expression on a log scale, per patient, based on molecular subtypes. Differences in CGA frequency per molecular subtype are significant ($p < < 0.0001$, Kruskal Wallis test). CGA genes list was derived from CT Database and include CGA genes that were available on Affymetric U133a chip, data from GSE2034, GSE5327 (A). Violin plots show the total number of predicted neo-antigens [109] per patient, based on molecular subtypes. Differences in neoantigen frequency per subtype are significant ($p < 0.0001$, Kruskal Wallis test) (B).

rather low. Within BC, however, the median mutational load increases upon higher tumor grades, and the mutational load is significantly increased in ER- subtypes (highest in Basal-like BC), compared to ER+ subtypes, regardless of BC histotypes [106], (Fig. 2B). These findings suggest that more aggressive, ER- BC may be susceptible for the immunological targeting of neo-antigens. Besides the number of mutations, some mutational signatures were found to be more immunogenic than others. The most prevalent mutational signatures in BC are age-, APOBEC- and BRCA1/2-related signatures [104]. APOBEC3 B (A3B) expression is enhanced in ER-, HER2+ subtypes, and correlates with lymph node metastasis [107] and poor prognosis [108]. Interestingly, we have shown previously that APOBEC signatures may create positively charged, neo-antigens, which are associated with increased T cell infiltration in ER + BC [109]. A3B deletion, on the other hand, leading to hyper-mutation, correlates with IFNy/STAT1 expression and immune cell signatures [107]. The exact mechanism and role of A3 B and APOBEC mutagenisis in BC immunogenicity requires further research.

6. Immune evasion of breast cancer counteracts effective therapy

High expression levels of tumor associated antigen (TAA) in late stage and HER2+, ER- BC or TNBC, and high frequencies of TILs in these subtypes do not correlate with each other [110], suggesting that either not all TAAs are equally immunogenic and/or that these tumors have undergone immune editing. The latter generally refers to the shaping of tumor antigenicity under the selective pressure of effector immune cells, which may precede, although not in a causative manner, the establishment of immune evasive mechanisms [111–113]. Such immune evasive mechanisms may include down-regulation of antigen presentation, lack of immune effector cells, enrichment of immune suppressor cells, and up-regulation of checkpoint molecules [2,3].

6.1. Antigen presentation

Critical for the recognition of tumor cells by T cells is that peptides derived from TAAs are presented on human leukocyte antigen (HLA) molecules expressed on the surface of tumor cells or antigen-presenting cells. In fact, expression of genes related to the HLA-A antigen presentation pathway correlates with expression of genes related to T cells, and was found to be most significantly associated with survival within TNBC patients [114]. Especially higher grade BC often (30–40% of tumors) down-regulate classical HLA-A, HLA-B, HLA-C molecules, which are required for the activation of CD8 T cells, and up-regulate non-classical HLA-E, HLA-F, HLA-G molecules, which may promote immune escape [115–117]. Besides HLA-A, expression of transport-associated proteins (TAP1/TAP2), which are required for proper antigen loading, is also down-regulated in high-grade BC [118]. TAP1/TAP2 down-regulation, however, is independent from HLA-A, B, C down-regulation [119], pointing to lack/absence of redundancy of various components of the HLA antigen presentation pathway with respect to immune escape. Besides downregulation of gene expression, mutations in antigen presentation and IFN response genes may provide yet another mechanism of immune escape. Mutations in β2-microglobulin (B2 M), a component of MHC class I, and JAK1/2, kinases downstream of IFN receptors, can lead to resistance to checkpoint blockade [120,121] and potentially other immune therapies. While JAK1/2 mutations affect only a minority of primary BC, and only truncated mutations (1.3% of BC) are associated with poor prognosis [120], BC metastases were found to have acquired additional JAK/STAT driver mutations [122].

6.2. Immune effector and suppressor cells

The frequency of clonally expanded, activated T cell is decreased in IDC compared to DCIS [13], suggesting that in IDC the level of antigenicity or T cell recruitment is less, and/or that the level of T cell suppression is high. In general, exclusion from tumor tissue or compromised activity of intra-tumoral CD8 T cells may in some cases be the direct consequence of aberrant expression of chemokines, adhesion molecules and/or extracellular matrix components (ECM), which to our knowledge has not been investigated yet in BC. Furthermore, there is increasing evidence that oncogenic pathway alterations can contribute to T cell exclusion or comprised T cell activity [112]. Interestingly, loss of PTEN and a highly active PI3 K pathway, which was found to correlate with a lack of T cells in melanoma [123], frequently occur in basal-like BC (35%) [124]. More over, PI3 K pathway alterations are the most common driver mutations in BC, affecting 49% of luminal A tumors and 7% of basal-like BC [124], and may therefore contribute to heterogeneiety with respect to TILs in these BC subtypes. In addition, in TNBC, a lack of T cells has been reported to be associated with RAS/MAPK pathway activation [125]. Exclusion or compromised activity of CD8 T cells, in other cases, may also be the indirect consequence of enhanced presence of M2 macrophages, MDSC, Tregs and/or cancer associated fibroblasts (CAFs) [126]. CAFs can promote angiogenesis and/or endothelial to mesemchymal transition (EMT), and release suppressive cytokines, such as IL1, IL6 and TGFβ, which can drive the formation of immune suppressor cells [127,128]. In BC, immune suppressor cells, including MDSC and M2, can promote tumor growth and metastasis and suppress T- and NK cell function by releasing suppressive mediators, such as IL10, IDO1, reactive oxygen species (ROS) and nitric oxide (NO) [129,130]. Enhanced recruitment of MDSC is considered to be related to increased expression of ELF5 and CCL2 in ER- BC, and enhanced IFN-signaling was found to induce immune suppressive activities of MDSC [131]. Tregs are recruited by CCL5 and

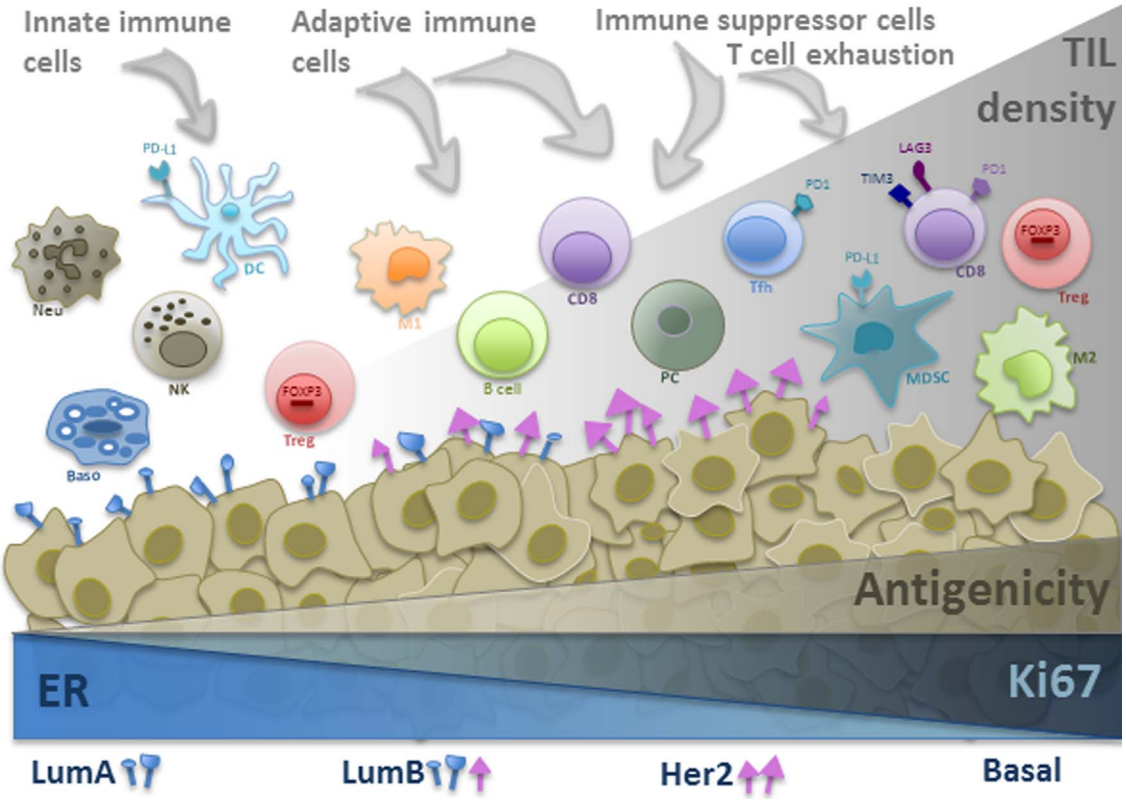

Fig. 3. Schematic illustration of immunity and evasive mechanisms in BC. BC subsets are categorized according to hormone receptor ER and PR (blue) or HER2 (pink) expression of tumor cells (brown). Antigenicity (ao. CGAs and neo-antigens) increases from luminal to basal type BC. Overall TIL quantity (gray background) increases from lumA to basal type BC, and its increase is related to tumor cell proliferation (Ki67). With respect to TIL quality, lumA tumors have relatively more innate immune cells, whereas the highly proliferating lumB, her2 and basal BCs are enriched for adaptive immune cells and immune suppressor cells. In particular, basal BC is enriched for exhausted CD8 T cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CCL22, which are produced by CD8 T cells and DC [132]. Next to inhibition of CD8 T cells, Tregs can directly promote BC metastasis in a paracrine manner [132].

6.3. Checkpoint molecules

As a consequence of an ongoing adaptive immune response, CD8 T cells, but also their target cells, up-regulate the expression of a number of immune checkpoint molecules, which slow down and ultimately inhibit active tumor killing by T cells. PD-L1, for instance, is expressed in a quarter of all BCs and high expression levels correlate with poor OS across all subtypes [133]. PD-L1 expression is particularly high in inflammatory BC (IBC, defined by symptoms resembling an inflammation, mostly ER-), and correlates with T- and B- cell signatures, most significantly those covering cytotoxic T cells, interferon and TNFα pathways [134]. Early BC stages, such as DCIS do not yet express PD-L1, however, in triple negative DCIS, APCs do already show strong PD-L1 expression [14]. Besides acquired expression of PD-L1 by the inducers IFNα/β or IFNγ, which are well-recognized products of activated immune cells or resident stromal cells, also mutations in PTEN and PI3 K which occur in 30% and 40% of BC, respectively, were found to provide inherent expression of PD-L1 [135]. Moreover, EMT was found to induce PI3 K and MEK-dependent up-regulation of PD-L1 in BC [136]. PD-L1 expression in BC is accompanied by expression of other immune suppressive checkpoints, like IDO1 and TGFb, as well as the expression of T cell co-inhibitory receptors, such as PD-1, CTLA-4, TIM-3 and LAG-3 [134,136]. PD-1 expression is commonly up-regulated after T cell activation and PD-1 positive T cells can be detected in blood of early stage BC patients, while peripheral changes in the expression of other checkpoint molecules such as CTLA-4 are not observed [137]. Within tumors, T cells positive for PD-1 are generally restricted to tertiary lymphoid structures (TLS), which are present in tumor stroma and composed of B- and T cells. TLS are often representative of a strong and ongoing immune response, and are present in 60% of BC, including all molecular subtypes [138]. In TNBC, the expression of PD-1 and LAG-3 tends to be associated with good prognosis. PD-1 and LAG-3 positive TILs were found in 30% and 18% of BC, respectively, and 15% of tumors were double positive for these markers, most likely indicating the presence of exhausted T cells [139]. Checkpoint molecules are not only up-regulated on CD8 T cells as PD-1 and TIM-3 were also found to be up-regulated on CD4+ Tfh cells in BC, which was associated with both a reduced CXCL13 production and a reduced capability of stimulating B cells [140]. Interestingly, in metastatic lesions, only 5% and 3% were found positive for the PD-1 and PD-L1, respectively [141], suggesting that immune evasive mechanisms described for primary tumors may not always be present in metastases, arguing that other immune escape mechanism may be more dominant in advanced diseases.

7. Future therapies should combine tumor sensitization and T cell treatments

Here we propose a strategy that implements immune-oncological markers to better select immune therapies in BC subtypes, and rationalize whether or not there is a requirement for sensitization for immune therapies based on our current understanding of BC's immune evasion and immunogenicity. In Fig. 4, we have distinguished ER+ and ER- BC, and described steps in selecting (combination) immune therapies:

Across BC subtypes, ER+ tumors, in particular luminal A BC, are the least immunogenic since they have the lowest numbers of TILs and the lowest expression levels of CGAs and neo-antigens (Fig. 3). Because of the low abundance of antigen, immune therap ies targeting TAAs in

ER + BC require extensive screening for pre-defined antigens, which is costly and time consuming. Therefore, immune therapies using checkpoint inhibition, which do not directly target TAAs, but rather TILs, may show more potential in ER + BC, since the presence of TILs can easily be assessed by H & E or immune stainings of routine biopsies. Thus far checkpoint blockade as monotherapy in ER + tumors has resulted in SD at best (see Section 4.2). In a subset of ER + BC patients with deficiency in DNA mismatch repair (MMR) genes [142], mutational load may represent an independent parameter for therapy selection. In general, however, we argue that the presence of TILs rather than mutational load serves as a more robust marker for patient stratification in BC, making it at this point in time not opportune to measure MSI for BC. Even though TILs in ER + BC are generally scarce and composed of innate rather than adaptive immune cells, it is important to note that significant heterogeneity exists with respect to quantity and quality of TILs (own observations; manuscript in preparation). The presence of effector CD8 T cells, and the expression of immune checkpoint molecules on these T cells are indicative of an antigen-initiated immune response, which is anticipated to robustly predict success of checkpoint blockade in patients with these tumors. Therefore the presence of these markers, in particular CD8 T cells (which reflects an ongoing immune response) should be assessed in the first step when designing therapies (Fig. 4 step1). In case CD8 T cells are absent one could opt for combinitation therapies, since NAC was found to increase TIL levels [143] and to enhance the CD8/Treg ratio (see Section 4.1), and therefore may further enhance treatment efficacy in ER + BC. In fact, such combinations have shown to increased pCR rates by 13–25%, when compared to NAC monotherapy (Table 1). The immunogenicity of tumors may also be increased by epigenetic drug treatment, including DNA-methyltransferase and/or histone deacetylase inhibitors, which were found to promote expressions of CGAs, MHC-I as well as co-stimulatory molecules in particular in tumor cells [144], [145]. A few clinical studies are currently examining the combination of epigenetic drugs and checkpoint inhibitors in ER + BC [146]. Even though results have not yet been published, combining cytotoxic therapies and/or epigenetic drugs with checkpoint inhibitors should be considered interesting strategies to treat ER + BC.

In contrast to ER + tumors, ER- tumors (HER2, TNBC) are intrinsically more immunogenic. Among all BC subtypes, TNBC bear the highest numbers of T cells, which are accompanied by the highest frequencies of neo-antigens and CGAs, and intra-tumoral CD8 T cells are often present with an exhausted T cell phenotype (Fig. 3). Thus, TNBCs may represent a subtype of BC most sensitive to immune therapeutic interventions. However, antigenicity does not always predict response to checkpoint inhibition [147]. Even though clinical trials have resulted in higher response rates to checkpoint blockade in TNBC tumors when compared to ER + tumors (Table 1), the majority of metastatic patients, however, does not show any clinical benefit to checkpoint blockade as monotherapy. This lack of response may be due to heterogeneity with respect to expression of checkpoint molecules or numbers of TILs. Indeed, high numbers of TILs and CD8 T cells were predictive for response to checkpoint inhibitors as first-line and second-line (following irradiation and chemotherapy) treatment for metastasized TNBC [86,148]. Therefore, also in ER- tumors, the presence of CD8 T cells should be assessed first. Most likely T cells are present. In case checkpoint molecules are present, one could again opt for therapy with checkpoint inhibitors. Multiple checkpoint molecules should be evaluated, since ER- tumors often co-express these molecules, which may prevent an effective monotherapy-approach. Indeed, the combination of durvalumab and tremelimumab resultated in an about 2-fold increased ORR of 43% in TNBC patients [82] when compared to monotherapy approaches. In case these checkpoint moelcules are not expressed, but immune suppressor cells are present (assessed in step 3), inhibitors of these suppressor cells provide a therapeutic option [149,150]. In some cases CD8 T cells are absent. Underlying reasons for CD8 T cell exclusion in a subset of TNBC patients, despite expression of TAAs, could be lack of or a compromised antigen presentation by tumor cells and/or activation of oncogenic pathways. When CD8 T cells are absent, we therefore suggest to assess MHC class I expression (which reflects capability of antigen presentation). In case MHC class I is expressed, then in the next steps assessments of TAAs and corresponding T cells are informative toward the option of adoptive therapy of T cells. In case MHC class I is not expressed, one could opt for therapy with PI3 K and MEK-inhibitors that are found to up-regulate expression of MHC

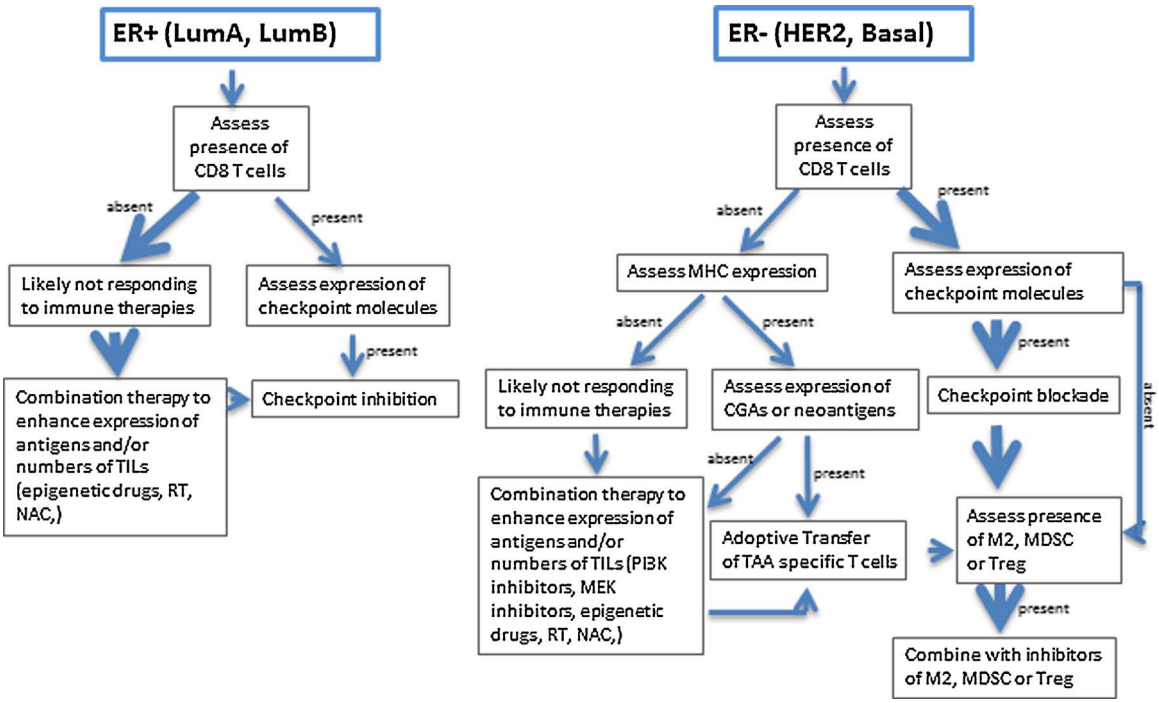

Fig. 4. Strategy to optimally implement immuno-oncological markers to guide selection of therapies for ER + and ER- BC patients. Thick arrows indicate the most likely path. Strategies are explained in more detail in Section 7.

class I and PD-L1 in TNBC [150]. In more general terms, epigenetic drugs, RT and/or NAC also represent therapeutic options to sensitize tumors for T cell treatments, such as adoptive T cell therapy.

In conclusion, BC subtypes are heterogenous with respect to quantity and quality of TILs, occurrence of immune evasive mechanisms, and antigenicity. Therefore all these factors should be assessed and taken into account when designing and selecting optimal (combination-) immune therapies for a selected group of BC patients.

Conflict of interest

The authors declare that there are no conflicts of interest.

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