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REVIEW

Refining patient selection for breast cancer immunotherapy: beyond PD-L1

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Therapies that modulate immune response to cancer, such as immune checkpoint inhibitors, began an intense development a few years ago; however, in breast cancer (BC), the results have been relatively disappointing so far. Finding biomarkers for better selection of BC patients for various immunotherapies remains a significant unmet medical need. At present, only tumour tissue programmed death-ligand 1 (PD-L1) and mismatch repair deficiency status are approved as theranostic biomarkers for programmed cell death-1 (PD-1)/PD-L1 inhibitors in BC. However, due to the complexity of tumour microenvironment (TME) and cancer response to immunomodulators, none of them is a perfect selector. Therefore, an intense quest is ongoing for complementary tumour- or host-related predictive biomarkers in breast immuno-oncology. Among the upcoming biomarkers, quantity, immunophenotype and spatial distribution of tumour-infiltrating lymphocytes and other TME cells as well as immune gene signatures emerge as most promising and are being increasingly tested in clinical trials. Biomarkers or strategies allowing dynamic assessment of BC response to immunotherapy, such as circulating/exosomal PD-L1, quantity of white/immune blood cell subpopulations and molecular imaging are particularly suitable for immunotreatment monitoring. Finally, host-related factors, such as microbiome and lifestyle, should also be taken into account when planning integration of immunomodulating therapies into BC management. As none of the biomarkers taken separately is accurate enough, the solution could come from composite biomarkers, which would combine clinical, molecular and immunological features of the disease, possibly powered by artificial intelligence.

Key words: breast cancer, immunotherapy, immune checkpoint inhibitors, biomarkers, PD-L1

INTRODUCTION

Therapeutic approaches which modulate the immune response to cancer have contributed to an unprecedented improvement of patient survival, transforming many life-threatening tumours into durably controlled diseases. The spectrum of cancer immunotherapies encompasses molecular and cellular treatments designed either to stimulate anticancer actions of immune cells or to remove their blockade developed during cancer immunoediting.[1] The latter is represented by a number of drugs which interact with the regulators of the checkpoints of physiological immune response, members of the immune synapse.[2] These drugs, known as immune checkpoint inhibitors (ICIs), have shown spectacular results in some metastatic cancers [melanoma, non-small-cell lung cancer (NSCLC) or renal cancer] and have been integrated into their standard of care.[3-5] Furthermore, certain ICIs are being introduced/clinically tested in preoperative/neoadjuvant cancer treatment.[6,7] However, the clinical benefit rate of ICIs, as either monotherapy or combined with chemotherapy, is still variable (15%-60%), their adverse effects can be severe and the treatment cost is high.[8,9]

To improve patient selection for cancer immunotherapy, biomarkers should be employed in order to identify patients most likely to experience prolonged survival. The biomarkers currently used have insufficient positive and negative predictive ability, so there is an urgent need to develop better tools for guiding clinical decisions in immuno-oncology.

Breast cancer (BC) has become an indication for ICIs later than melanoma, lung, or clear-cell renal cancer. The crucial element to make a rationale for immunotherapy development in BC was the discovery of various amounts of tumour-infiltrating lymphocytes (TILs) in malignant breast tumours, suggesting the existence of natural immune response to a number of breast neoplasms. The seminal work of Denkert et al.,[10] as well as many published later, have shown that BC patient survival and response to

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neoadjuvant cytotoxic therapy (NACT) are better if their tumours contain more TILs.[11,12] The existence of active or suppressed immune response to BC cells was further confirmed by gene expression (GE) analysis; signatures containing immune genes were found highly expressed in triple-negative BCs (TNBCs) and HER2-enriched BCs, and significantly associated with better patient outcomes.[13-19] In addition, several studies have shown that BCs express various levels of immune checkpoint regulators among which the programmed cell death-1 (PD-1)—programmed death-ligand 1 (PD-L1) pair is most extensively studied.[20-25] PD-1 was found expressed by TILs, whereas PD-L1 was observed on either immune or cancer cells, either due to the suppression of adaptive immunity or due to oncogenic action.[26,27] Finally, recent studies revealed that a subgroup of BCs, mostly of metaplastic high-grade histology, has very high PD-L1 levels which are due, in rare cases, to an amplification of the PD-L1 gene (*CD274*).[28-30] As the number of approved PD-1/PD-L1 inhibitors has been rapidly increasing in non-BC indications, an extensive testing of this type of immunotherapy is ongoing in BC as well. Atezolizumab, an anti-PD-L1 antibody (TECENTRIQ; Roche, Basel, Switzerland), has been approved, in combination with nab-paclitaxel, as first-line therapy in unresectable, locally advanced or metastatic TNBC, on the basis of the phase III IMpassion130 trial results. In this study, patient metastasis-free survival was significantly prolonged in the atezolizumab arm, compared with placebo.[31] Recently, an anti-PD-1 antibody, pembrolizumab (Keytruda; Merck & Co. Inc., Kenilworth, NJ), was approved in combination with chemotherapy for treatment of patients with locally recurrent unresectable or metastatic TNBC, on the basis of the phase III KEYNOTE-355 trial, in which pembrolizumab combined with nab-paclitaxel or gemcitabine/carboplatin significantly reduced the risk of metastatic progression and death.[32] In each of these two trials, clinical benefit was observed in the patient population selected by a tumour tissue biomarker, protein expression of PD-L1, evaluated by approved companion tests.

The fraction (percentage) of immune cells alone or of both immune and tumour cells expressing PD-L1 protein is still the only biomarker introduced in the clinic for use of anti-PD-1/PD-L1 agents in BC. Microsatellite instability (MSI)/mismatch repair deficiency (dMMR) served as a theranostic biomarker for approval of tissue-agnostic use of pembrolizumab in unresectable or metastatic solid tumours.[33] This parameter was given 1C level by the European Society of Medical Oncology (ESMO) Scale for Clinical Actionability Scale of Molecular Targets (ESCAT),[34] meaning that it should be considered as a theranostic biomarker for pembrolizumab in BC as well. However, the frequency of dMMR/MSI-high BCs is low, in the range of 0.4% to 3%.[35,36] Therefore finding additional biomarkers for BC immunotherapy is still a significant unmet medical need. Research and development in this field are intense, encompassing analyses of tumour tissue and the circulation compartment, and exploiting a plethora of methods, from single gene/messenger RNA (mRNA) or protein expression assessment to complex high-throughput measurements and mathematical equivalents of molecular expressions (scores). To wisely incorporate immunotherapy in the clinics of BC, we must consider the particularities of the immune response reflectors/markers: presence of various cell subpopulations, temporal and spatial heterogeneity, expression modulation by tumour- and host-related factors.

To prepare this article, we performed a PubMed search of articles published (online publication) from 1 January 2010 to 28 February 2021. The presentations/abstracts of the ASCO and the ESMO 2020 Congress, as well as of the San Antonio Breast Cancer Symposium 2020, were also analysed. We will first elaborate on currently approved biomarkers for immuno-oncology and then present biomarkers in development, in breast and in other cancers potentially relevant for BC management. Table 1 presents a list of those biomarkers and the tests/assays used to measure them, together with brief summaries about clinical significance and the current status of development of each biomarker/biomarker group. As a conclusion, we will give a critical opinion on the current status of immuno-oncology biomarkers and summarize about perspectives in this exciting field.

Clinically approved biomarkers for BC immunotherapy

PD-L1. PD-L1 (also known as B7-H1 or CD274) is the predominant ligand of the co-inhibitory receptor programmed cell death-1 (PD-1). PD-1 binds two ligands, PD-L1 or PD-L2, inducing downregulation of the effector T-cell activity, tolerance to antigens and reduction of cytokine production.[37] PD-L1 is expressed by macrophages, some activated T and B cells, dendritic cells and some epithelial cells, particularly under inflammatory conditions.[38] PD-L1 is expressed in many solid and haematologic tumours as an adaptive mechanism to suppress the immune surveillance of malignant cell growth.[39]

The fraction (percentage) of tumour and/or tumour-infiltrating immune cells expressing the PD-L1 protein has been evaluated as a predictive biomarker in numerous trials testing the efficacy of ICIs; higher percentages were associated with good response in some of these studies.[40-42] However, in both metastatic and neoadjuvant settings, clinical benefit to ICIs has been observed also in PD-L1-negative tumours. Notably, this was the case in two major metastatic setting trials which led to the first approvals of anti-PD-L1/PD-1 inhibitors in BC.[31,32] Furthermore, in the IMpassion031 trial, the use of atezolizumab in combination with NACT in patients with early TNBC improved pathological complete response (pCR) rates compared with placebo regardless of PD-L1 expression.[43] Similar results were observed in the randomized neoadjuvant phase III KEYNOTE-522 trial in early TNBC, with an increased pCR rate achieved in patients treated by chemotherapy and pembrolizumab, independently of PD-L1 status.[44] The discrepancy in PD-L1 predictive value in early and metastatic setting could be partly explained by the induction of PD-L1 expression by NACT; in that case even a PD-L1-negative tumour at the baseline can respond to PD-1/PD-L1

Table 1. Current status of the biomarkers for cancer immunotherapy

Biomarker	Type	Methodology	Clinical significance	Stage of clinical validation	References
PD-L1	Cellular, tumour- and TME-related marker	IHC: percentage of tumour cells and/or immune cells expressing PD-L1	Higher percentage of tumour or immune cells expressing PD-L1 predicts response to ICIs	Clinically approved biomarker	31,32,43-45
MSI/dMMR	Cellular and genomic, tumour-related marker	IHC: expression of MMR proteins on tumour cells, sequencing of tumour sample	The presence of MSI/dMMR predicts response to ICIs	Clinically approved biomarker	59
Quantity of TILs	Cellular, TME-related marker	Assessing quantity of TILs in the tumour microenvironment on a H&E-stained tumour tissue	High quantity of TILs seems to predict response to ICIs	In development, with demonstrated predictive value	42,44,66,67
Tumour mutational burden	Genomic, tumour-related marker	Sequencing of a tumour sample	High TMB seem to be predictive of response to ICIs	In development, with demonstrated predictive value	74,75
CD274 (PD-L1 gene) amplification	Genomic, tumour-related marker	Sequencing of a tumour sample	The presence of a *CD274* amplification seems to confer a good response to ICIs	In development, under evaluation	83-85
BRCA1/2 mutational and HRD status	Genomic, tumour-related marker	Sequencing of a tumour sample	The presence of *BRCA1/2* mutation or a high HRD score is correlated with a high TMB and immunogenicity and may predict good response to ICIs	In development, under evaluation	41,89-91
POLE mutational status	Genomic, tumour-related marker	Sequencing of a tumour sample	The presence of *POLE* mutation is correlated with a high TMB and may predict good response to ICIs	In development, under evaluation	93-96
Immune gene signatures	Genomic, TME-related marker	Sequencing of a tumour sample	High expression of immune genes seems to be predictive of response to ICIs	In development, under evaluation	98-102
Other genomic anomalies: alterations of MAPK, PI3K—AKT—mTOR, WNT-β-catenin, JAK—STAT, TGF beta pathways	Genomic, tumour-related marker	Sequencing of a tumour sample	Alterations in these pathways seem to impact the immune response and may be predictive of response to ICIs	In development, under evaluation	106-109
TIL subpopulation and TLS	Cellular, TME-related marker	IHC on immune cells	The presence of B cells and TLS seems to facilitate response to ICIs	In development, under evaluation	111
Spatial distribution of TILs	Cellular, genomic, TME-related marker	Multiplex IHC/IF, digital labelling of RNA or protein-binding probes and mass spectrometry-based detections (CyTOF) of TILs	May predict response or resistance to ICIs	In development, under evaluation	128-131
Peripheral blood cell subpopulations	Circulating marker	Quantification of serum NLR, PLR or T cells	High NLR is associated with worse OS and PFS in patients treated with ICIs; serum T-cell quantification may help monitoring response to ICIs	In development, under evaluation	134-137
Exosomal/soluble PD-L1	Circulating marker	Quantification of exosomal/soluble PD-L1	Exosomal and soluble PD-L1 seem to reflect the patient immunosuppressive state; decreased exosomal PD-L1 may predict a good response to ICIs	In development, under evaluation	140,141

Continued

Table 1. Continued

Biomarker	Type	Methodology	Clinical significance	Stage of clinical validation	References
Tumour hypoxia/ acidosis	Circulating marker	Quantification of serum LDH levels	High serum LDH levels may predict lower overall response rates to ICIs	In development, under evaluation	145,146
Molecular imaging	Imaging marker	^{18}F-FDG PET imaging of PD-1, PD-L1 or CD8+ TIL quantity	PD-1, PD-L1 or CD8+ TIL quantity changes and dynamics may predict and help monitoring response to ICIs	In development, under evaluation	147-153
Microbiome	Genomic, host-related marker	Sequencing of local breast cancer and gut microbiomes	Local breast cancer and gut microbiomes may influence response to ICIs	In development, under evaluation	154-161
Lifestyle, sociological and metabolic factors: age, gender, smoking, diet, exercise, obesity, diabetes	Host-related marker	Clinical questionnaires, written and oral	May impact tumour response to ICIs	In development, under evaluation	122,163-166

CyTOF, cytometry by time of flight; F-FDG, ^{18}F-fluorodeoxyglucose; H&E, haematoxylin and eosin; HRD, homologous recombination deficiency; ICIs, immune checkpoint inhibitors; IF, immunofluorescence; IHC, immunohistochemistry; LDH, lactate dehydrogenase; MSI/dMMR, microsatellite instability/mismatch repair deficiency; NLR, neutrophil-to-lymphocyte ratio; OS, overall survival; PD-1, programmed cell death-1; PD-L1, programmed death-ligand 1; PET, positron emission tomography; PFS, progression free survival; PLR, platelet-to-lymphocyte ratio; POLE, polymerase epsilon; TILs, tumour-infiltrating lymphocytes; TLS, tertiary lymphoid structure; TMB, tumour mutational burden; TME, tumour microenvironment.

blockade due to the increasing of PD-L1 levels during treatment. In the metastatic setting, PD-L1 level better predicts the response to anti-PD-1/PD-L1 agents; however, the intermetastatic heterogeneity of PD-L1 expression can count for discrepancies between the target expression and therapeutic result.[45]

The biomarker value of PD-L1 remains controversial due to the variability of the technical aspects of its assessment [performance of several antibodies available for immunohistochemistry (IHC), incompletely standardized scoring methods] and the spatial and temporal heterogeneity of PD-L1 expression. To date, five anti-PD-L1 clones have been developed as assays for individual drugs, and have shown different sensibilities [22C3, 28-8 and 73-10 from Agilent Technologies Inc. (Santa Clara, CA, USA); SP142 and SP263 from Roche Tissue Diagnostics (Tucson, AZ, USA)]. Several parameters are behind result heterogeneity and reduced possibility to interchange the assays or the clones. The clones differ by binding epitope, and the assays by antigen—antibody reaction revelation kit and the platform/ automates, as can be seen in Table 2.

An important challenge in using PD-L1 assessment by IHC-based assays is the interobserver reproducibility.[41] Although the scoring of tumour cells resulted in high concordance between pathologists, the scoring of immune cells seems to be less precise.[46-49] Rugo et al.[50,51] evaluated the analytical concordance of SP142 with SP263 and 22C3 IHC assays, and their ability to predict clinical activity in the IMpassion130 trial. Although 22C3 and SP263 assays had a higher sensitivity (81% and 75% of PD-L1-positive cases, respectively) compared with the SP142 assay (46% of PD-L1 positive cases), SP142 was clinically more relevant in identifying patients who benefited from the treatment. Only the patients with double PD-L1-positive tumours (SP142 PD-L1-positive population within the 22C3 or SP263 PD-L1-positive populations) presented a greater clinical benefit, showing that the prediction of atezolizumab benefit was driven by the positive PD-L1 status defined by SP142 immune cell score $\geq$1%.

PD-L1 testing assays have been validated in clinical trials but their implementation into daily practice has been experiencing difficulties. PD-L1 IHC assay introduction into the clinic depends on regulatory approvals and reimbursement by health insurance which vary between countries.[52] Pathology laboratories cannot offer all assays as they are performed in dedicated automates which are not always all available on-site. PD-L1 protein expression assay could also be performed as a laboratory developed test, for practical and economic reasons, but it has to be analytically validated including external and internal quality insurance.[53,54] For larger implementation of PD-L1 laboratory developed tests into routine pathology and clinical practice, international guidelines are needed such as the recommendations published by the Canadian Association of Pathologists or by the French working group on PD-L1 testing harmonization in lung cancer.[53,55] A useful detailed review about how to improve clinical use of PD-L1 as a biomarker has been recently published by the International Immuno-Oncology Biomarker Working Group.[54]

MSI/dMMR. Microsatellite, also called short tandem repeats or simple sequence repeat, consists of repeated sequences of 1-6 nucleotides, widely distributed within human DNA and mostly located near the coding regions. MSI is generated through errors in DNA replication due to deficiencies in the mismatch repair system (dMMR).[35]

MSI/dMMR is most often assessed by IHC, by polymerase chain reaction-based testing of microsatellite loci, or by

Table 2. IHC assays for PD-L1 assessment

Assay	Partner drug	Platform	Scored cell type	Scoring system
SP142[a]	Atezolizumab	Ventana	IC	PD-L1+ IC tumour area positive if ≥1% IC+, in mTNBC
22C3[b]	Pembrolizumab	Agilent	IC and TC	CPS: PD-L1+ IC + PD-L1+ TC TC positive if ≥10%, in mTNBC TPS: PD-L1+ TC TC
SP263	Durvalumab	Ventana	IC or TC	T%: PD-L1+ TC TC IC%: PD-L1+ IC IC
73-3	Avelumab	Agilent	IC or TC	T%: PD-L1+ TC TC IC%: PD-L1+ IC IC
28-8	Nivolumab	Agilent	TC	T%: PD-L1+ TC TC

CPS, combined positive score; IC, immune cells; IHC, immunohistochemistry; mTNBC, metastatic triple-negative breast cancer; PD-L1, programmed death-ligand 1; TC, tumour cells; TPS, tumour positive score; T%, percentage of positive tumour cells.
[a] Companion test for atezolizumab + nab-paclitaxel in first-line metastatic TNBC treatment.
[b] Companion test for pembrolizumab + chemotherapy in first-line metastatic TNBC treatment.

sequencing methylation assays.[56] The IHC test comprises detection of four MMR proteins, MLH1, PMS2, MSH2 and MSH6, where an absence of any of them in tumour tissue indicates a dMMR. However, discrepancies can occur, especially in case of MSH6 loss that is not always leading to dMMR and a high level of MSI (MSI-H). Therefore a combination of IHC and molecular biology analyses is recommended to accurately evaluate the MSI/dMMR status.[35]

MSI-H/dMMR status is relatively rare in BC. Depending on the method used and the cohort analysed, earlier studies have shown higher frequencies of anomalies in the MMR pathway in BC.[57,58] However, a very recent study, in which MSI status was assessed by FoundationOne CDx (Foundation Medicine Inc., Cambridge, MA, USA), found a very low frequency of MSI-H status in breast tumours (0.1%, 0.2% and 0.4% in ER+/HER2−, HER2− and TN subtype, respectively).[36]

The discovery of strong association between MSI-H status and response to pembrolizumab in many solid tumours is the basis for a strong consideration of testing for MSI/dMMR in BC, with the hope of revealing even a small group of patients who will benefit from one or more ICIs. The results validating the use of MSI/dMMR status as theranostic biomarker for PD-1/PD-L1 blockers in BC are still scarce, however, some durable responses to this immunotherapy type in MSI-H tumours have been reported.[59]

Biomarkers in development for breast immuno-oncology

As cancer interacts with local (within tumour bed) and systemic immunity, biomarkers that potentially indicate efficacy of any immune response-modulating cancer treatment can be found at the tumour site and/or in the circulation. Indeed, an extensive research of these two compartments has been discovering numerous candidate biomarkers for immuno-oncology, which are today in different phases of development. Another source, the human microbiome, mainly the one localized in the gut, has been recently added as an important arena of biomarker discovery for immune and nonimmune cancer treatment. Finally, lifestyle factors, such as exercise, exposure to stress, smoking or nutrition habits markedly influence the immune response to cancer, as well as cancer response to immune modulators.

The biomarkers in development for immuno-oncology could be classified into tumour related and host related, although such a separation is not clean-cut. According to the method of assessment, they can be considered as molecular (gene mutations, gene and protein expression), cellular (amount, phenotype and functional status of immune and other cell subpopulations, including bacterial) and clinical/observational (menopausal status, lifestyle, etc.). In the following text, we will present biomarkers in development for breast immuno-oncology according to their demonstrated clinical value. Various categories of biomarkers in use or in development for cancer immunotherapy are presented on Figure 1.

Biomarkers with demonstrated predictive value

Quantity of TILs. TILs are a part of mononuclear cells which infiltrate tumour tissue. They contain several immunophenotypically and functionally different subpopulations, such as CD8+ cytotoxic T cells, CD4+ helper T cells, B cells and natural killer cells.[60]

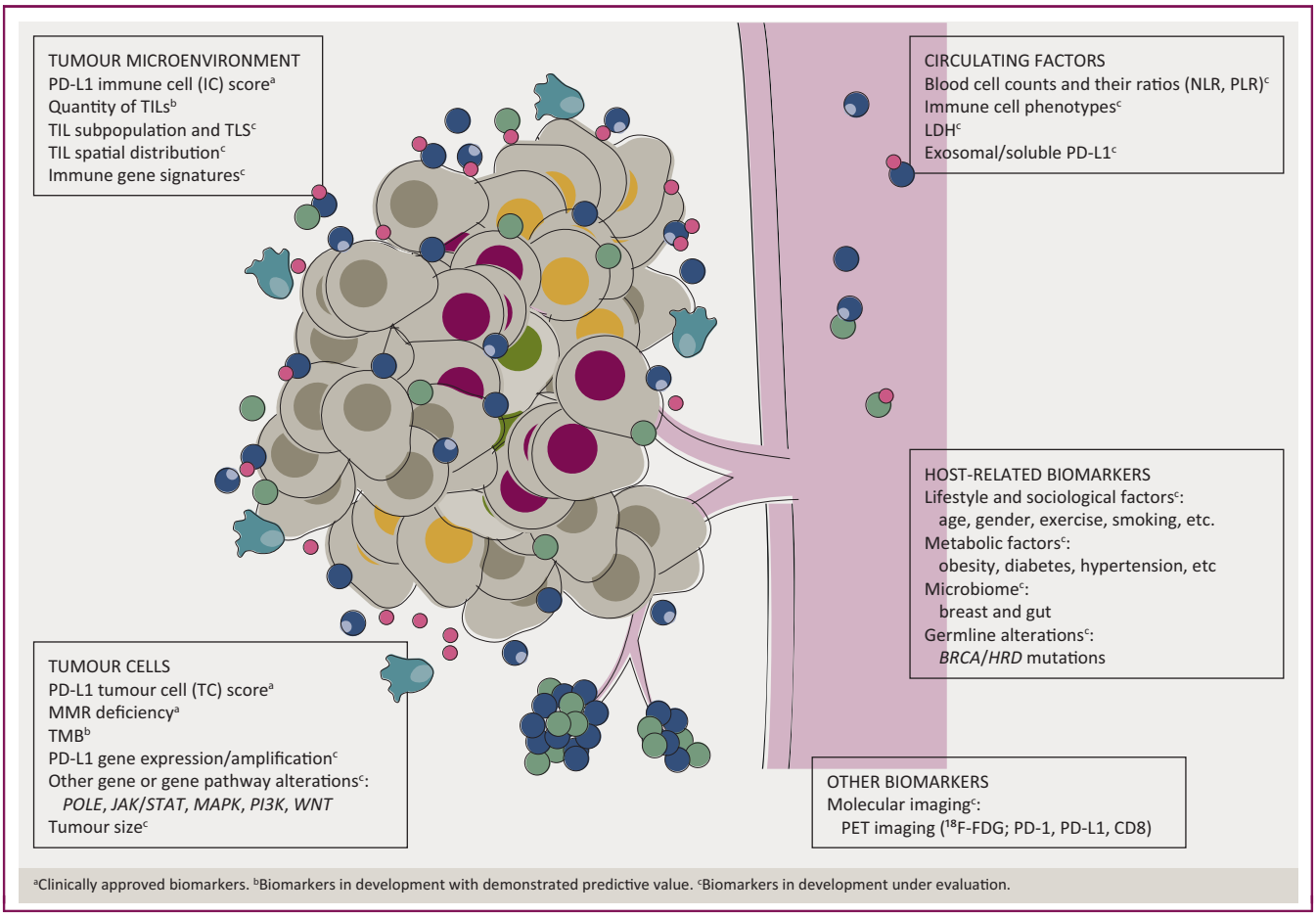

Figure 1. An overview of the main biomarkers for immunotherapy in breast cancer.
^{18}F-FDG, ^{18}F-fluorodeoxyglucose; HRD, homologous recombination deficiency; LDH, lactate dehydrogenase; MMR, mismatch repair; NLR, neutrophil-to-lymphocyte ratio; PD-1, programmed cell death-1; PD-L1, programmed death-ligand 1; PET, positron emission tomography; PLR, platelet-to-lymphocyte ratio; TILs, tumour infiltrating lymphocytes; TLSs, tertiary lymphoid structures; TMB, tumour mutational burden.

TIL quantity/density is assessed on haematoxylin and eosin (H&E)-stained tumour tissue sections. They can be observed within the tumour-associated stroma [stromal TILs (sTILs)] or within the tumour cell nests [intratumoural TILs (iTILs)].[61] A standardized and reproducible method for evaluation of TIL density in BC has been provided by the International Immuno-Oncology Biomarker Working Group.[61] iTILs and sTILs strongly correlate; however, it is recommended to assess only sTILs which are easier to visualize and present in more cases.[61-63] TILs are reported as a percentage of intratumoural stroma occupied by them; this method has been analytically validated, showing a reliable interobserver reproducibility.[54,64] The aforementioned international group also published approaches to avoid pitfalls in TIL scoring and constructed web-based educational tools (for more details, go to www.tilsinbreastcancer.com).[65]

In the randomized phase III KEYNOTE-119 trial, TILs $\geq$5% predicted response to pembrolizumab.[66] Similar results were obtained in neoadjuvant ICI trials in TNBC: in KEYNOTE-173, a phase Ib study investigating pembrolizumab in combination with chemotherapy; in NeoTRIP, a randomized trial assessing the addition of atezolizumab to nab-paclitaxel and in GeparNuevo, a randomized phase II study evaluating addition of durvalumab to chemotherapy.[42,44,67]

TIL assessment is not introduced yet into the routine clinical/pathological practice; however, it is being integrated into biomarker discovery studies associated with new immunotherapy trials as a low-cost parameter which can rapidly give an estimation of tumour immune profile.

Tumour mutational burden. Tumour mutational burden (TMB) is the number of somatic nonsynonymous exon mutations per megabase pair (mut/Mb). Although only a minority of these mutations code for neoantigens, the number of potential neoantigen peptides is bigger if a tumour has more DNA mutations. Therefore TMB is considered as a good, although not perfect, reflector of tumour neoantigen load.[68]

In June 2020, the US Food and Drug Administration (FDA) granted accelerated approval to pembrolizumab for the treatment of adult and paediatric patients with unresectable or metastatic tumours with high TMB (TMB-H, TMB $\geq$10 mut/Mb) that have progressed following prior treatment and who have no satisfactory alternative treatment

options. TMB was determined by an FDA-approved assay (FoundationOne CDx; Foundation Medicine, Inc.).

Compared with other cancers, BC TMB is low; only 5% of BCs have >10 mut/Mb.[69] Using the FoundationOne CDx assay in a cohort of 5475 advanced BCs, Israel et al.[36] found that the rate of TMB-H cases was dependent on molecular subtype, being highest in lobular, inflammatory and HER2+ carcinomas (16%, 14% and 12% of cases, respectively). TMB-H cases were absent in papillary carcinomas and less present in metaplastic and mucinous histotypes (7% in each). Finally, this status was more frequent in metastatic than in primary tumours, as shown earlier by other teams.[70,71] TMB has been found to be higher in ER− than in ER+ subtypes, except for lobular carcinomas, and not consistently correlated with other reflectors of immune response to the tumour.[36,72] One recent study on 3969 primary or metastatic BC samples, assessed by whole exome or gene panel sequencing, confirmed that median TMB in BC is low and varies highly according to the molecular subtype (HR−/HER2− > HER2+ > HR+/HER2−) and sample type (metastatic > primary). High TMB cases represented only 5% of the cohort. The most common mutational processes in hypermutated tumours were APOBEC activity (59.2%) and dMMR (36.4%). Several of the patients with hypermutated tumours achieved an objective and durable response to pembrolizumab-based therapy, indicating that PD-1 inhibitors might be a good option only for those patients.[73]

In the phase II GeparNuevo trial, TMB was predictive of response in both the neoadjuvant durvalumab (anti-PD-L1, Imfinzi; AstraZeneca, Cambridge, UK) and the placebo arm: median TMB was 1.52/Mb (range 0.02-7.65) and significantly higher in patients with pCR than in patients with residual disease (1.87 versus 1.39, respectively, $P = 0.005$). TMB and immune GE profiles had independent value for pCR prediction: the patients with tumours having both high TMB and high expression of immune genes had a pCR rate of 82% [95% confidence interval (CI) 60% to 95%], whereas those with tumours characterized by both low TMB and immune GE had only 28% pCR (95% CI 16% to 43%). These results, obtained in one of the largest series ($n = 149$) of BC patients treated by neoadjuvant ICIs, recommended further development of TMB, in combination with immune parameters, for personalized BC neoadjuvant treatment.[74] One recent large pan-cancer study (TCGA cohort) showed that TMB-H BCs without correlation between CD8 T-cell levels and neoantigen load exhibit a significantly lower overall response rate to ICIs than the TMB-L tumours, strengthening the need to develop TMB and the immune parameters together.[75]

However, development of TMB as a biomarker faces a number of technical and tumour type-related challenges. Several preanalytic variables impact TMB determination and should be standardized between platforms, such as the length of tumour tissue fixation, fixative type, depth of sequencing and length of sequencing reads. In addition, sensitivity of the TMB assay can be reduced by low tumour purity and sampling errors. The impact of panel size on TMB sensibility and specificity seems to be less relevant in BC than in TMB-H cancers such as NSCLC. Conversely, variability of bioinformatic algorithms, which could differ between tests, might hamper comparison of reproducibility. The cumulated experiences of TMB determination in various tumours indicate that the gold standard for assessment of this parameter is still whole exome sequencing (WES). Large panels which use somatic and germline testing can be a good alternative, especially having in mind that they have become increasingly available on the market, so are less expensive.[36,76]

Although TMB has been accepted as a good surrogate for a number of neopeptides, recent studies have pointed out the importance of the quality instead of the quantity of these molecules, as many parameters can influence neoantigens' ability to stimulate effective immune responses.[77] Mutation clonality and subclonality, functional diversity of HLA-I genes and intratumoural heterogeneity can all influence ICI efficacy.[78,79] As shown in a recent study, analyses that combine WES, neoantigen quality, mutation clonality and HLA status could outperform mere TMB assessment.[80]

Taken together, these findings suggest that TMB is an imperfect biomarker like PD-L1 due to many factors related to variability of preanalytical and analytical conditions. TMB is nonredundant with PD-L1 and MSI/dMMR status—each parameter provides clinically valuable information. To introduce TMB in BC treatment by ICIs, additional data and efforts are needed. There is an ongoing initiative to standardize TMB measurement and to define the best cut-off for each cancer type.[81] Combining WES and RNA analysis, or WES and IHC, seem to be a promising approach to better predict patient responses to immunotherapy, in both high- and low-TMB malignancies.[82]

Biomarkers under evaluation

***CD274* (PD-L1 gene) amplification/expression.** Besides PD-L1 protein expression, PD-L1 gene (*CD274*) amplification status has been recently shown to confer a very good response to anti-PD-L1 agents; however, this amplification is much more frequent in non-BC than in BC.[83] In a study comprising 5399 cases from the Memorial Sloan Kettering Cancer Center in New York and from TCGA, TNBCs were confirmed to be enriched for *CD274* amplification (5% of the cases), whereas only 1% of all BCs carried that anomaly. The tumours with amplified *CD274* did not differ in immunity-related features, like mutational load or lymphocytic infiltration.[84] A potential predictive value for PD-L1 gene amplification has been recently shown in an exploratory analysis of the randomized phase II SAFIR02-IMMUNO trial comparing durvalumab with chemotherapy in patients with metastatic BCs.[85] More data are required to confirm whether the assessment of PD-L1 gene amplification status should be incorporated into the standard biomarker panel for patient selection for anti-PD-1/PD-L1 agents.

***BRCA1/2* mutational and homologous recombination deficiency status.** Because of major defects in DNA repair, it was hypothesized that *BRCA*-mutated cancers would have a

higher level of genomic instability, higher TMB and higher immunogenicity, and so have better response to ICIs. Indeed, responders to PD-1 blockade in melanoma, NSCLC or urothelial cancer were significantly enriched in *BRCA* mutations.[86-88]

Until now, in BC, only the IMpassion130 trial has reported on relevance of germline *BRCA1/2* mutations for response to an ICI: the patients with PD-L1+ tumours benefited from atezolizumab and nab-paclitaxel regardless *BRCA1/2* mutational status.[41] *BRCA1/2* deleterious mutations were not linked to PD-L1 immune cell expression, and PD-L1 immune cell status was equally distributed in the 89 *BRCA*1/2-mutated patients. This could be explained, at least partially, by the fact that *BRCA*1/2-mutated BCs are heterogeneous in terms of immunogenicity; more than half of them have low TILs levels[89] and the expression of the immune metagene signatures reflecting intratumour cytotoxicity ranges widely.[90] Kraya et al.[91] have recently shown that homologous recombination deficiency (HRD) scores and hormone receptor subtype are predictive of immunogenicity in *BRCA1/2*-mutated BCs. Several methods and commercialized assays are currently available to assess HRD;[92] however, there are still no data published about their predictive value for a given cancer immunotherapy.

Polymerase epsilon (*POLE*) mutational status. Some microsatellite-stable tumours have a high TMB due to the presence of DNA polymerase epsilon (*POLE*) mutations. The *POLE*-mutated tumours are heavily infiltrated by immune cells, express a high number of neoantigens and are seen as good candidates for benefit from ICIs. In the multicohort phase Ib KEYNOTE-028 trial, which evaluated the safety and efficacy of pembrolizumab in patients with PD-L1-positive advanced solid tumours,[93] the drug demonstrated antitumour activity in endometrial cancer with *POLE*-ultramutated status. In addition, durable clinical responses were observed on both pembrolizumab and nivolumab in this patient category.[94]

The frequency of *POLE*-mutated tumours in BC is lower than 3% and practically all are characterized by high TMB.[95] Reports on the response to ICIs of *POLE*-mutated BC are still missing; however, the high TMB of those tumours presents an opportunity for ICI use.[96] It remains to be seen whether TMB and *POLE* mutational status are redundant in theranostics of ICI and other immunotherapies in BC.

Immune gene signatures. The immune contexture of cancers can be investigated by assessing GE (mRNA quantification), which, in most cases, positively correlates with quantification of protein expression.

One of the most extensively tested immune signatures is tumour inflammation signature (TIS), an 18-gene signature shown to enrich for patients with clinical benefit from pembrolizumab in a pan-cancer manner.[97,98] The expression of TIS is measurable by several GE panels available from Nanostring Technologies (Seattle, WA, USA), developed specifically for immuno-oncology and/or BC research (PanCancer IO360 panel, Breast Cancer 360 panel). TIS itself measures the extent of the adaptive immune response suppression. In the TCGA cohort, TIS scores were found to vary between tumours; however, the tumours with TIS value above the median had significantly better response to pembrolizumab.[98] TIS correlates only minimally with TMB but can be considered as a pan-cancer reflector of the immune-inflamed phenotype. The predictive value of TIS has been confirmed in metastatic NSCLC treated by nivolumab; however, the signature has not been tested enough in BC.[99,100]

Marincola et al.[101] have described a 20-gene signature comprising genes from four functional categories, CXCR3/CCR5 chemokines, Th1 signalling, effector and immune regulatory functions (including CD274, CTLA4, FOXP3, IDO1, PDCD1), which has been shown to be a favourable prognostic marker and a putative predictor of increased responsiveness to immunotherapy in several tumour types.[101,102] Other signatures, or simple expressions of several immune genes, were shown to correlate with good patient prognosis.[10,103-105] It was hypothesized that many of them reflect an immune-favourable cancer phenotype which would be responsive to ICIs. However, it remains to be determined which immune signature is best suitable for this type of BC therapy.

Other genomic anomalies. Several canonical cancer pathways have been recently reported to be implicated in cancer sensitivity or resistance to immunotherapy. The three most involved pathways, both in carcinogenesis and in cancer response to immunomodulation are MAPK, PI3K—AKT—mTOR and WNT-β-catenin pathways and the anomalies of the genes regulating these pathways might be biomarkers of clinical benefit from cancer immunotherapy.

Loi et al.[106] have shown that genetic or transcriptomic alteration resulting in Ras/MAPK activation is associated with lower numbers of TILs in TNBC. Both *in vivo* and *in vitro*, MEK/MAPKK inhibition upregulated tumour cell surface major histocompatibility complex (MHC) expression and PD-L1, and combined MEK and PD-1/PD-L1 inhibition enhanced antitumour immune responses in mouse models of TNBC. These experiments suggested that Ras/MAPK activation and MHC expression may be predictive biomarkers of response to ICIs.

Manguso et al.[107] demonstrated that tumours without key elements of the JAK—STAT pathway fail to upregulate MHC-I molecules and better evade immune surveillance. In BC, the most frequent genomic anomaly of the JAK—STAT pathway is the amplification of *JAK2*, found in ~10% of TNBC residual tumours after NACT.[108] As *JAK2* belongs to the PDJ amplicon, the *JAK2*-amplified BCs are likely to be amplified for PD-L1 gene (*CD274*), which might be a good basis for PD-L1-blocking therapy. It remains, however, to be determined whether a systematic search for anomalies of the JAK—STAT pathway will improve precision of BC treatment by ICIs.

Alterations of a number of other pathways, processes and molecules, such as the transforming growth factor beta pathway, the IDO1 pathway, chromatin remodelling and

cellular metabolic disturbances might be provoked by genomic anomalies assessable by next-generation sequencing. Their biomarker value for cancer immunotherapy is under investigation.[109]

TIL subpopulations. Hammerl et al.[110] underlined the heterogeneity of TIL immunophenotype among BC subtypes. As a result, the subpopulations and the immune phenotypes of TILs are being increasingly identified as good predictors of response to ICIs. For example, the amount of CD8+ iTILs is becoming a better reflector of tumour immunogenicity than total sTILs. In the KEYNOTE-086 study, which tested pembrolizumab in previously treated (cohort A) or untreated (cohort B) metastatic BC, Loi et al.[111] found that each of PD-L1 combined positive score, sTILs, CD8+ iTILs and TMB was associated with good response to pembrolizumab; however, CD8+ iTILs were most significant.

Besides the effector T cells, B cells and tertiary lymphoid structures (TLSs) are coming to light as potential predictive marker of ICIs. The role of tumour-infiltrating B cells has not been well elucidated; the published data are still conflicting, with some studies suggesting that these cells promote tumour progression, whereas others showed a positive association of rich B-cell tumour infiltrates with better clinical outcomes, when B cells were found within TLSs.[112-116] In BC, the presence of TLS appears to correlate with PD-L1 expression and TIL density, notably B-cell density.[117-119] B cells and TLS seem to facilitate response to ICIs as shown in recent publications on melanoma and sarcoma.[114,116,120]

Spatial distribution of TILs. Tumour immune contexture is defined by the density, composition, functional status and spatial organization of the tumour-infiltrating immune cells.[121] Three main spatially defined immune phenotypes of cancer have been described: the immune inflamed, characterized by TILs present within the tumour bed and in close proximity with tumour cells; the immune excluded, characterized by the presence of TILs predominantly in the tumour-adjacent stroma; and the immune deserted, characterized by a paucity of TILs within both tumour stroma and tumour cell nests.[122] These patterns are easily identified on H&E-stained tumour tissue sections and are associated with specific biological mechanisms regulating the tumour microenvironment (TME). While inflamed tumours have been shown to be more responsive to ICIs, both immune-deserted and immune-excluded tumours are considered as noninflamed and rarely respond to those drugs.[122]

A number of novel techniques for analysis of spatial relationships in tumour tissues have been developed over recent several years. Multiplex IHC/immunofluorescence, digital labelling of RNA or protein-binding probes and mass spectrometry-based detections (cytometry by time of flight) can perform very high-content *in situ* tissue analyses.[123-127] In BC, these high-dimensional technologies have already provided a better insight into TME of TNBC.[128,129] Savas et al.[130] showed, by exploiting single-cell and region-based complex immunophenotype analysis, that CD8+ tissue-resident memory cells are crucial contributors to BC immunosurveillance and, likely, the key targets of modulation by immune checkpoint inhibition. Patients with advanced-stage BCs highly infiltrated by CD8+ tissue-resident memory cells have increased response rates to anti-PD-1 antibodies.[131]

Peripheral blood cell subpopulations. Systemic immunity is necessary for effective cancer immunotherapy as elegantly shown by Spitzer at al.,[132] on animal models. The reinvigoration of the immune response against cancer by ICIs is reflected in an increase of the effector T-cell number in tumour tissue, as well as in the bloodstream. However, the changes of peripheral blood immune cell populations are not always concordant with the changes within the tumour bed.[133] The presence of an unfavourable ratio between peripheral blood white cells with anticancer and those with procancer activities, at the beginning, as well as during immunotherapy, reflects a systemic immunosuppressed state which is a difficult terrain for restitution of anticancer immunity within the tumour bed.

Numerous studies have shown that increased neutrophil-to-lymphocyte ratio (NLR) and/or platelet-to-lymphocyte ratio (PLR) in breast and other cancers are predictors of a poor response to cytotoxic therapy and patient survival in general. It is basically explained by a reduction in blood cells with antitumour action (lymphocytes) and/or increase in cells/elements with protumour/prometastatic action (neutrophils, platelets). A recent meta-analysis showed that high NLR is associated with worse overall survival and progression-free survival across all ICIs (ipilimumab, nivolumab and unspecified or pooled pembrolizumab and nivolumab, in melanoma, NSCLC and genitourinary cancer).[134] Li et al.[135] showed that dynamics of NLR during ICI treatment of advanced cancer also indicate clinical outcomes. Namely, the patients with baseline and on-treatment NLR <5 had significantly longer overall survival, whereas those with a significant increase in NLR within the first month of ICI therapy had the shortest overall survival of 5.0 months (95% CI 0.9-9.1). The change in NLR overtime was nonlinear and remained statistically significant after adjusting for age, body mass index, sex, cancer type, performance status and days to repeat NLR measurement.

Several studies have shown that response to ICIs can be monitored by analysing the subpopulation of peripheral blood immune cells, such as CD4+/PD-1+, CD8+/TIM-3+ or CD8+/PD-1+/Ki67+ T cells.[136,137] These findings strongly support further evaluation of peripheral blood T-cell immune phenotype and count dynamics as predictive biomarkers in immunotherapy of solid tumours, including BC.

Exosomal/soluble PD-L1. One of the recent major achievements in understanding cancer response to immune surveillance and to immune therapies was made by Chen et al.[138] who showed that melanoma cells shed their PD-L1 into the bloodstream, packed into exosomes, and expressed on the exosome surface, where it can fight circulating T cells

even before they approach tumour tissue. Exosomal PD-L1 was further found in breast, gastric and prostate cancer, and shown to exert immunosuppressive effects and facilitate tumour growth.[139]

Exosomal PD-L1 is a potentially useful circulating biomarker for cancer immunotherapy, as the level of exosomal PD-L1 mRNA significantly decreased in the plasma of melanoma patients who responded well to anti-PD-1 therapy. Therefore a lack of that decrease might be an indicator of resistance to PD-1 blockade.[140] In head and neck cancer, higher levels of PD-L1 in circulating exosomes were associated with a stronger inhibition of CD8+ effector T cells.[141]

PD-L1 can also be found as in patients' plasma. A recent study by Han et al.,[142] on 208 patients with recurrent/metastatic BC before receiving first-line therapy, showed that high soluble PD-L1 level (≥8.774 ng/ml) and visceral metastasis were independent factors associated with poor prognosis. Both exosomal and soluble PD-L1 seem to properly reflect the level of the patient immunosuppressive state. Therefore measuring exosomal or soluble PD-L1 might represent a suitable, noninvasive way of monitoring the effects of cancer immunotherapy; however, some technical and conceptual issues must be resolved before wider implementation of circulating PD-L1 assessment into clinical trials.[139]

Tumour size and hypoxia/acidosis. Hypoxic/acidic TME, present in most large tumours, has been shown to be immunosuppressive, leading to impairment of T-cell traffic into the tumour and/or T-cell functions.[143,144] Thus the parameters reflecting tumour hypoxia/acidosis might predict a response to immunomodulating agents. In the phase II KEYNOTE-086 trial, testing pembrolizumab as first-line therapy in metastatic TNBC, patients with high serum lactate dehydrogenase levels had lower overall response rates.[145] In the randomized phase II NeoPHOEBE trial testing neoadjuvant trastuzumab with buparlisib and paclitaxel, in HER2+ BC, Loibl et al.[146] showed that an increase in TILs can already be observed 2 weeks after treatment start in cases with a marked reduction of tumour burden. These findings indicate that, in BCs presenting with big tumour mass, parameters reflecting their hypoxia/acidosis status could predict their response to immunotherapies (ICIs or others) and that large tumours should first be effectively shrunk with an appropriate agent, in order to overcome the initial immunosuppression.

Molecular imaging. Molecular imaging has great potential to improve cancer immunotherapy, by visualizing all malignant cell deposits throughout the body and thus providing an insight into intertumour, especially intermetastasis heterogeneity, as well as into the dynamics of cancer response to treatment. Several tracers for PD-1, PD-L1 or CD8 positron emission tomography (PET) imaging have been in preclinical and clinical development.[147-152] PET imaging of CD8+ lymphocytes allows rapid noninvasive monitoring of CD8+ TIL quantity change after immunotherapy start, thus avoiding patient discomfort provoked by multiple biopsies as well as errors in judgement about TIL quantity and distribution provoked by small samples. As CD8+ lymphocytes are major effectors of the immune response to cancer, molecular imaging methods allowing insight into dynamics of tumour infiltration by them are among the top methods for rapid adaptation of immunotherapy protocols to obtain the best clinical response. Besides PET imaging based on tracers for molecules involved in the immune response, metabolic imaging based on fluorodeoxyglucose is being used to identify early nonresponders and pseudoprogressors to cancer immunotherapy.[153] Because BC immunotherapy is an emerging field, the data about use of molecular imaging in personalization of the immunity-based approaches to BC treatment are yet to come; however, based on the experience with other cancers, these will likely be promising.

Microbiome. The microbiome represents a risk for BC and modulates its response to therapy.[154] Benign breast tissue microbiome differs from BC microbiome.[155,156] Several studies have shown that an imbalance in microbial populations, known as microbial dysbiosis, impacts the anticancer immune response.[2,157] Although some bacteria are associated with poor outcomes, they generate proinflammatory stimuli and may create a favourable microenvironment for immunotherapy.[158,159] Manipulating the microbiota has been shown to improve efficacy of PD-1 inhibitors.[160]

Apart from the local BC microbiome, the gut microbiome may also influence response to ICIs in BC.[161] Further investigation is needed before we can use the microbiome as a predictive biomarker as most studies on the subject are still in a preclinical or exploratory phase.

Lifestyle. Lifestyle habits, such as well-balanced diet and exercise, show a positive effect on the immune system and the gut microbiome, whereas alcohol consumption hinders antigen presentation.[122,162] Tobacco use has a dual and opposite impact on immune system, increasing neoantigens while hampering immune response.[163] Obesity, leading to a chronic low-grade inflammatory condition, is an established risk factor of cancer, including BC. Despite reduced numbers of the immune effector cells, obese patients tend to have a higher response to ICIs, but experience more serious side-effects.[164]

Conflicting results have been reported concerning the effect of gender in response to cancer immunotherapy. A major bias is that apart from typically female sex-related cancer such as breast, or gynaecological cancers, the proportion of males in the clinical trials is two-thirds higher. Nevertheless, oestrogen is known to activate several immune cell subpopulations and pathways, including cytotoxic T cells, all leading to a stronger immune environment. On the contrary, progesterone displays anti-inflammatory effects.[165] Furthermore, female tumours seem to be less antigenic. In NSCLC, female patients responded less favourably than males to ICI monotherapy, but when combining ICI with chemotherapy, the reverse was

observed, as the chemotherapy might have enhanced tumour immunogenicity.[166] A world of caution, however: prospective collection of standardized data is mandatory before translating those observations into clinical practice to guide immunotherapy of BC patients.

Taken together, one can speculate that all of the mentioned lifestyle factors might impact response to ICIs positively or negatively, but more studies are required to make sound conclusions in BC.

CONCLUSIONS

Immunotherapy has revolutionized the treatment of melanoma and NSCLC; however, in BC, we are only at the beginning and the results are inferior than expected. On the other side, specificities of BC biology and natural evolution (e.g. a number of indolent hormone-sensitive tumours), as well as the range of available therapies (chemotherapy regimens for tumours with defects in the DDR pathway and several efficacious targeted agents), allow us to consider BC today as a curable disease by the existing standard-of-care options.

To whom is then immunotherapy in BC useful for, and how to detect these individuals? For patients with mTNBC, who have been prognostically the worst for decades, ICIs (atezolizumab, pembrolizumab) have become the game changers. The tumour features behind this success are the fraction of tumour-infiltrating immune cells expressing PD-L1 protein, the presence of CD8+ TILs, the level of genomic instability or the TMB. However, among the 40% mTNBC patients selected for PD-L1 blockade by PD-L1 IHC test, <30% benefit from the treatment. What are we missing?

To answer this question, several important issues of the PD-L1 IHC assays use in BC must be resolved at the first place: standardization, reproducibility, choice of the best assay, which all call for larger comparative studies aimed to identify the best companion test for PD-1/PD-L1 inhibitors. Beyond PD-L1, TIL quantity, immunophenotype and spatial distribution emerge as promising biomarkers. Combined with PD-L1 protein and GE, they might rapidly grow into a powerful predictive tool to select cancer patients for immunotherapeutic strategies.[167] However, focusing on TME might not be enough in BC. Tumour characteristics such as TMB, MSI status, specific gene alterations also emerge as potential theranostic biomarkers, despite their low frequency in this disease.

One biomarker per drug or even one-type-biomarker per drug seems to be a universally nonadapted approach in precision oncology, especially in immuno-oncology. Composite biomarkers, derived by integration of multiomic tissue analysis and host parameter assessment, are already seen as better reflectors of the complexity of cancer immune status and its response to immunomodulators. Furthermore, to properly adapt any anticancer treatment, the close monitoring of the dynamics of tumour and patient body changes is the key. The noninvasive techniques, such as assessment of circulating molecules and cells, as well as whole-body molecular imaging, would be indispensable for early detection of response or resistance to immunotherapy and the clinical decision about further treatment.

We have tried to apply to BC the knowledge cumulated by the work on other tumours' immunology. However, these cancers have a completely different carcinogenesis, where many carcinogens have a high capacity to induce neoantigens (tobacco, ultraviolet rays, viruses etc.). Compared with those malignancies, a great number of BCs are much more tolerated by the immune system. Many questions about breast-specific immune microenvironment remain to be answered, which will likely bring a guiding light to our navigation. The need for deep investigation of BC immunology and response to immunomodulators using appropriated models (humanized patient-derived xenografts, organoids and organs-on-the-chip, etc.) is stronger than ever. The amount of data which will be generated is surely impressive, and the help of powerful computing tools (artificial intelligence) would be necessary for sculpting those findings into each patient and cancer immune identity. These developments will take time, and in the years in front of us, we will likely do best by investing in strengthening of molecular pathology as the prime resource of knowledge indispensable for precision (immuno)oncology.

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