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Review

The breast cancer tumor microenvironment and precision medicine: immunogenicity and conditions favoring response to immunotherapy

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ABSTRACT

Some main recent researches that have dissected tumor microenvironment (TME) by imaging mass cytometry (IMC) in different subtypes of primary breast cancer samples were considered. The many phenotypic variants, clusters of epithelial tumor and immune cells, their structural features as well as the main genetic aberrations, sub-clonal heterogeneity and their systematic classification also have been examined. Mutational evolution has been assessed in primary and metastatic breast cancer samples. Overall, based on these findings the current concept of precision medicine is questioned and challenged by alternative therapeutic strategies. In the last two decades, immunotherapy as a powerful and harmless tool to fight cancer has received huge attention. Thus, the tumor immune microenvironment (TIME) composition, its prognostic role for clinical course as well as a novel definition of immunogenicity in breast cancer are proposed. Investigational clinical trials carried out by us and other findings suggest that G0-G1 state induced in endocrine-dependent metastatic breast cancer is more suitable for successful immune manipulation. Residual micro-metastatic disease seems to be another specific condition that can significantly favor the immune response in breast and other solid tumors.

1. Introduction

Breast cancer is the most common malignancy worldwide, with about 2.3 million new cases and 700,000 deaths estimated in 2020.[1] Thus, its social impact highlights the relevance of any advance in treatment. Recently, tumor microenvironment (TME) in primary and metastatic breast cancer samples has been dissected and structural phenotypic composition and genetic alteration have been studied.[2-4] Besides, in the last two decades, immunotherapy became an increasingly therapeutic strategy for many researchers and it has been successful for different solid tumors, mainly melanoma, renal cell carcinoma, non-small cell lung cancer,[5] and also breast cancer. In human epidermal growth factor receptor 2 (HER2)$^+$ breast cancer subtype, passive immunotherapy with trastuzumab first, a humanized IgG1 mAb against HER2,[6] and successively with pertuzumab, has become a mainstay. Non-specific and specific active immune therapies also have been investigated, the former promoted by cytokines and other cell signaling molecules and the latter using a vaccine platform. However, these immune-therapies got limited results[7,8] and the former almost has been abandoned. Recently, immune-checkpoint inhibitors (ICIs) and adoptive cell immunotherapies (ACT) have gained consent.[9] Immune-checkpoints are inhibitory receptors and PD-1/PD-L1 is the most well studied receptor-ligand pair. Immunotherapy with ICIs has been successfully tested in some clinical trials mainly conducted in triple negative breast cancer (TNBC).[10-13] Most ACT and chimeric antigen receptor (CAR) therapies are under evaluation in a few ongoing clinical trials[9] and all these immune-therapies have been recently revised by us and others.[9,10,14,15] Breast cancer is considered scarcely immunogenic and the estrogen receptor (ER)$^+$ subtype immunologically "cold" due commonly to a low rate of tumor infiltrating lymphocytes (TILs) and tumor mutational burden (TMB) as well as low expression of PD1-/PD-L1 molecules. In this review, the main different cellular phenotypes in the TME of primary breast cancers within the ER$^+$ subtype and between ER$^+$ and the others were examined along with their hierarchical classification, the main genetic aberrations and sub-clonal heterogeneity. Mutational evolution also has been assessed in metastatic breast cancer samples. Overall, the current concept of precision medicine has been questioned and challenged by more specific conditions favoring the response to therapy. The tumor immune microenvironment (TIME), its prognostic role as well as the immunogenicity in breast cancer were also considered. Finally, G0-G1 state and minimal residual disease as conditions favoring the immune response were discussed.

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2. Main cellular phenotypes and structural features of TME by imaging mass cytometry within the ER$^+$ subtype and between ER$^+$ and the others

2.1. Epithelial tumoral cells phenotypes

In a recent study,[2] imaging mass cytometry (IMC) profiling of 144 primary breast cancer samples was carried out. There was similarity between frequencies of ER$^+$, progesterone receptor (PR)$^+$, HER2$^+$, Ki-67$^+$ tumor cells and the matched pathological immune-histo-chemistry scores; besides, most tumor cells expressed the epithelial cell adhesion molecules and CD45$^+$ cells were immune cells. Endothelial cells (CD31$^+$) and fibroblasts (FAP$^{+/-}$SMA$^+$) occurred with lower frequency, while fibroblast subsets[16] and adipocytes were termed as "other". As expected, in TNBC and HER2$^+$ samples more immune cells were observed. Overall, the analysis showed 84 distinct cell phenotypes which were grouped in 45 epithelial plus ten CD4$^+$ and ten CD8$^+$ T cell clusters in addition to 19 myeloid clusters. The 45 epithelial clusters, based on marker expression, were classified into seven luminal groups, L1–L7, and two basal groups, B1 and B2. Fifty-five per cent of tumor-derived cells were luminal cells taking part of groups L1 and L2, while luminal progenitor cells phenotypes with elevated values of EpCAM and CD49f and low ERα[17] characterized group L3. Phenotypes in group L4 had high levels of ERα, progesterone receptor B (PRB), and androgen receptors (AR) as well as other factors or receptors likely responsible for tumor cell growth and diffusion.[18] In group L5 there were overexpression of tyrosine kinase receptors and enhanced values of ERα, the methyl-transferase EZH2, its target H3K27me3, and the anti-apoptotic factors survivin and BCL-2. K7, K8, K18, ERα, HER2 were expressed in phenotypes of group L6 together with decreased CD49f and elevated E-cadherin and CD24. Phenotypes in group L7 showed low ERα and HER2 and expressed HLA-DR, a surface receptor implicated in tumor immune response. Ki-67$^+$ luminal tumor cells were observed in all luminal clusters although in group L7 they were more often. All basal phenotypes did not express K7, K8, K18, ERα, and HER2, while enhanced expression of Ki-67, EGFR, and p53 occurred in basal-like tumor cells. The great heterogeneity was further documented by a different distribution of L1–L7 phenotypes in the tumor subtypes. ERα^+ cells ranged between 2% and 91% and ERα^+ AR$^+$ cells ranged from 0% to 44% (median, 1.7%). A basal phenotype (0% to 5%, range; 0.35%, median), was present in 130 of 135 luminal tumor samples and cells of group B2 prevailed in TNBC. The epithelial-mesenchymal transitions (EMT) phenotype in tumor cells occurred in TNBC and in a few luminal A and B tumors. All subtypes but luminal A and grade 3 tumors compared with non-tumor close tissue had elevated rate of proliferating cells. In the same study,[2] phenotypic abnormality, individuality and richness of tumor cells were examined. Phenotypic abnormality was defined according to how much tumor cell phenotype differed from non-tumor epithelial cells. Cells with abnormal phenotypes were well represented in high-grade tumors, mainly ER$^-$ tumors, in a subset of ER$^+$ tumors, and in those subtypes having poor prognosis. The individuality based on an individuality score according to whether cells of a sample looked more likely to cells of the same sample or to those from different samples, score close to 1 or 0, respectively. Tumor individuality and phenotypic abnormality had inverse correlation, while high-grade and luminal B and luminal B-HER2$^+$ tumors or TN subtype had more evident tumor individuality. Individuality in ER$^+$ tumors correlated with the proportion of ERα^+ cells. The rate of each epithelial cell cluster per sample defined tumor richness and clusters above 1% was unveiled. Tumor richness inversely correlated with individuality and in 62 (43%) of 144 tumor samples, at least 50% of tumor cells were represented by a single cluster, likely suggesting a single cancer cell clone which expanded. Finally, at least 50% of all cells in one or more tumors come from 82% of the 45 epithelial cell clusters.

2.2. Immune cells and macrophages phenotypes

In the same report by Wagner et al.,[2] the frequencies of tumor-associated immune cells largely varied in different patients and T cells and myeloid cells prevailed. In particular, regarding the former, a phenotypic continuum from CD4$^+$ to CD8$^+$ lineage was shown.[19,20] PD-1 and other co-inhibitory receptors and activation markers were differently expressed in CD8$^+$ and CD4$^+$ T cell clusters with increased PD-1 and other receptors co-expression likely representing enhanced T cell inhibition.[21] Most PD1$^+$ T cells were CD8$^+$; however the mean PD-1 expression level was more elevated in CD4$^+$ than in CD8$^+$ T cells. Eighteen percent luminal B tumors were PD-1$^+$ in more than 10% of T cells, unlike luminal A tumors where this percentage was only 7%. As expected, a higher Treg rate in ER$^-$ and in luminal B tumors was found. Tumor associated macrophage (TAM) populations resulted in 19 myeloid clusters and five categories. In 80% of tumors, PD-L1 expression was observed in at least 10% of myeloid cells[22] and the PD-L1$^+$ TAMs had different phenotypes. This heterogeneity suggests a CD38 link to PD-L1 and confirm that pro- and anti-inflammatory markers are co-expressed in myeloid cells associated with tumors.[19,23]

2.3. A different hierarchical classification

In another similar report,[3] single cells and tumor and stromal regions of 281 different primary breast tumors were dissected. Fifty-nine phenotypes among the various cell populations were found. Consistent with a previous study,[2] some tumor phenotypes only occurred in single patients. After tumor single-cell phenotypes were hierarchically clustered; overall, 26 meta-clusters were defined. Tumor regions comprehended different luminal HR$^+$ cell phenotypes, but in a few cases hormone receptors (HRs) were also expressed without cytokeratins (CKs) and in meta-clusters 19 and 20 luminal CK7 joined with specific luminal subsets. HER2 expression occurred in many phenotypes, but it was not a defining feature of meta-clusters. Phenotypes lacking HR and HER2 expression which characterizes TNBC and over-expressing Ki-67, p53, EGFR and the hypoxia marker CAIX were found in meta-clusters 15–18 and PhenoGraph clusters within meta-clusters 19 and 22. Cells from many different meta-clusters occurred in any breast cancer subtype suggesting that the common pathological classification does not entirely encompass the between and within-patient cellular heterogeneity.[2,24,25] Higher order interactions between one or more cell phenotypes characterizing the function of a multi-cellular tissue was termed community. Twenty-three tumor communities (TCs) were defined, while when stromal cells were also considered, 30 microenvironment communities (MCs) were described. A single cellular meta-cluster commonly prevailed in TCs. Some MCs comprehended fibroblasts interacting with different tumor cells, while others showed sparse stroma content or showed a high-rate T cells, macrophages, and wide networks of T and B cells or endothelial cells. As expected, MCs with a high rate of fibroblasts showed much less interacting immune cells and immune exclusion likely due to tumor desmoplasia induced by fibroblasts.[26] By unsupervised clustering, 18 heterogeneous single-cell pathology (SCP) subgroups were identified. Then, in an independent cohort of 72 patients, reproducibility and spatial variability of SCP classifications were assessed. There was a correspondence of all cellular meta-clusters with SCP subgroups; therefore those present in the main cohort also occurred in the second cohort. Moderate inter-region heterogeneity was shown in most patients. SCP 2 and SCP 7 consisting of CK+ HR+ and epithelial^{low} cells, respectively, were more divergent regions. In about 40% of tumors, all regions had the same classification, and in 60% one or more regions were different from the whole tumor classification. In a third report,[4] 483 primary breast tumor samples were assessed. A total of 57 cellular phenotypes were identified. The 57 phenotypes were also grouped into 11 integrative clusters (Int Clust) based on driver copy

number alterations (CNAs). The main characteristics of multiple breast cancer samples and their different hierarchical classification in the three reports[2-4] are shown in Table 1.

3. Primary breast cancer sub-clonal heterogeneity through multi-region sequencing and mutational evolution

3.1. Subclonal heterogeneity

Yates et al. investigated the sub-clonal composition and the spatial evolution of 50 primary breast cancers, including 27 $ER^+/HER2^-$, 3 ER^+HER2^+, and 20 TNBC.[27] A total of 290 samples were collected through a multi-regional sampling. High heterogeneity was observed only in three of the analyzed cancers. Among the remaining patients, 23 showed no significant difference in the mutations detected in different tumor sub-regions, while all the others showed an intermediate level of heterogeneity. Age at the diagnosis and tumor size unlike histology, ER status, grade, lymphocyte infiltration or Ki67 score were the only factors showing a positive correlation with intra-tumor heterogeneity. The authors also evaluated the spatial distribution of the sub-clones in 12 cancers. Eight biopsies from the resected tumor were analyzed for each patient. All tumors showed at least one driving mutation or CNA that was present in each sampled region. Significant spatial heterogeneity in point mutations ($n = 8$) or CNA ($n = 2$) was shown by 10 out of 12 tumors, with at least one mutation confined within 1–3 adjacent regions, suggesting that most sub-clones were restrained to limited geographical areas. However, the large tumors (>3 cm), showed greater heterogeneity likely because high heterogeneous tumors grow at a higher rate or as clonal sweep is more unlikely in larger tumors. Additionally, four of the 12 sequenced cancers exhibited mutations in cancer-driving genes that were not detectable in five to seven of the eight samples analyzed for each tumor. To evaluate the characteristics of the sub-clones at the metastatic sites, the authors carried out whole genome sequencing (WGS) of two primary tumors and matched lung or lymph node metastasis. In both cases, the sub-clones from which the metastasis arose were derived from an early branch of the phylogenetic tree, suggesting that targeting sub-clonal actionable mutations originated after the branching event may not prevent the disease's spread. There was also evidence of tumours migrating to colonize separate clonally related foci in multifocal breast cancers. Clonal sweeps were observed within each focus, with private mutations showing high allelic frequency. Genetic aberrations in the 30 ER^+ primary breast cancer patients are reported in Table 2.

3.2. Mutational evolution

In their work, Yates et al.[27] reported that mutations in crucial genes, including *TP53, BRCA2, PTEN, PIK3CA,* and *CDKN2A*, could arise either early or late during the disease progression. Indeed, while mutations within these genes were sub-clonal in some patients, they were fully clonal in others. Additionally, parallel evolution of *PTEN* and *TP53* mutations, *FGFR2* amplification, and *RUNX1* gene rearrangement was observed in four patients. When the authors examined ten multi-regional sampled cancers, for each patient, the percentage of the sub-clonal structural variants was proportional to that of the sub-clonal point mutations, suggesting that none of these two events was preferentially clonal or subclonal. Moreover, the events present during the early stage of the disease continued throughout its evolution. Large tandem repeats also keep accumulating as the cancer develops. Similar findings were reported by Shah et al.[28] on the primary tumor and metastatic samples of an ER^+ lobular breast cancer. Among the 32 somatic non-synonymous coding mutations found in the metastatic sample, 11 were present in the primary tumor, 19 were exclusive of the metastatic sample, and two were undetermined. The authors also analyzed the frequencies of these mutations and found that, of the 11 shared mutations, 5 (within *ABCB11, HAUS3, SLC24A4, SNX4*, and *PALB2*) had a similar frequency in the primary and metastatic tumor unlike the remaining six mutations (within *KIF1C, USP28, MYH8, MORC1, KIAA1468,* and *RNASEH2A*) which were present at a low frequency in the primary tumor and increased in the metastatic samples. These observations highlighted a significant evolution of the mutational landscape of this patient with minor sub-clones expanding and new point mutations appearing over time. The authors also evaluated changes in the RNA processing events and found that RNA-editing enzymes, which can be governed by oestrogens,[29] may recode transcripts, thus accounting for a divergence of the proteome from the genome.[30-32] Indeed, in addition to mutations, alterations in key regulatory elements can be crucial in cancer progression and drug resistance. As to this, Patten et al. analyzed the epigenome of 47 primary (drug-naïve) and metastatic (drug-resistant) breast cancers.[33] They observed that metastatic progression was driven by the expansion of sub-clones characterized by the activation of given sets of enhancers that promoted the expression of FOXA1.[34] Interestingly, this suggested that therapy may promote the expansion of a drug-resistant population. Accordingly, aromatase inhibitors (AI) therapy can drive drug-specific resistance by parallel genetic evolution *in vivo*.[35] This was confirmed in a further *in vitro* study showing the emergence of resistance to an AI through drug-specific epigenetic reprogramming.[36] AI treatments can also shape the composition of the TME. Recently, Brechbuhl et al. identified two subtypes of tumour-adjacent stromal cells (TASC) characterized by the expression of the melanoma cell adhesion marker 1 (CD146; $TASC^{CD146}$) or the CUB domain-containing protein 1 (CDCP1; $TASC^{CDCP1}$)[37] and by a specific signature. Interestingly, during exemestane, an AI, the TME composition was affected, with a significant decrease in the $TASC^{CD146}$ and an increase in the $TASC^{CDCP1}$ populations. Overall, the findings suggest that, in addition to clonal selection,[38] AI can induce transcriptional changes in cancer and TME cells, driving the onset of drug-resistant phenotypes.

4. Genomic alterations according to cell phenotype or integrative clusters

4.1. Phenotypic heterogeneity in ER alpha positive breast cancer uncovered through epigenomic assessment

In the report by Raza Ali et al., the association between different cellular phenotypes found in breast cancer TME and mutations within the most common driver genes was investigated.[4] They found that *TP53* mutations were recurrent in phenotypes enriched in basal-like TNBC, including phenotype 51, 9, and 57. Conversely, *TP53* mutations negatively correlated with phenotypes typical of luminal A cancers, including phenotypes 31 and 48. Interestingly, mutations within *PIK3CA* showed a reverse pattern, as they were positively associated with phenotype 48 and negatively associated with phenotype 57. Two phenotypes, 28 and 31, enriched in luminal B tumors, were not associated with point mutations but rather with CNAs, including *CCND1* and *TUBD1* gain and *ATM* loss. The luminal A phenotype 48, was associated with the greatest number of point mutations, affecting, among the others, *PIK3CA, MEN1, CBFB, MAP3K1, MAP2K4, CTCT*, and *GATA3* genes. Interestingly, the hypoxia-associated phenotype 9 cells showed *CD274* gain (encoding PD-L1) and *B2M* (β2 microglobulin) heterozygous losses, suggesting a relationship of hypoxia with immune evasion.[39] In the stromal component, *TP53* mutations were frequent in the fibroblast phenotypes 30 and 37 and myofibroblast phenotype 32, with 30 and 32 being enriched in the basal-like cancers. *PTEN* loss was observed in phenotypes 30 and the myofibroblast phenotype 21. At a genomic-wide level, 16p gains in both the luminal-associated phenotypes 31 and 48 and losses of 11q in phenotype 31 alone occurred. Loss of 5q and gain of 10p were also found in basal-like phenotypes 9 and 57, respectively. Interestingly genomic instability was associated with specific highly proliferative epithelial, fibroblasts, macrophages, and T-cells phenotypes, suggesting that genomic unstable tumors have highly proliferative cells and specific stromal and immune populations.[4] Patten et al. in their report proposed that the H3K27ac

Table 1
Cell phenotypes in TME of multiple breast cancer samples and different hierarchical classifications of their complexity.

Breast cancer samples, N							Cell phenotype, N	Hierarchical classification				Reference
Total	LA	LB	LB HER2+	Normal-like	HER2+	TN		Clusters		Groups		
								Kind	N	Kind	N	
144*	54	71	6	–	1	6	84	Epithelial	Ep01-Ep45	LB	L1-L7 B1-B2	2
								$CD4^+$T	T01-T04; T08-T09; T13; T17-T18; T20	–		
								$CD8^+$ T	T5-T7; T10-T12; T14-T16; T19			
								Myeloid	M01-M19	Category (n = 5) C1: M06, M15 C2: M03, M11, M13 C3: M08, M09, M16 C4: M01, M02, M06, M14, M17 C5: M07, M10, M12		
281**	173	–	29	–	23	48	> 59	Metaclusters		Communities		Ssubgroups, N	
								Kind	N	Kind	N		
								Epithelial	14	TC	23	18	3
								T	3				
								B	1				
								T+B	1	MC	30		
								Stromal	4				
								Other	3				
483***	149	133	–	62	63	59	57	Integrative clusters (n = 11) based on driver CNAs		Intrinsic subtype (n)			4
								Epithelial phenotype Enrichment pattern	N				
								ER^+	2	LB (28)			
								ER^+	3	LA-LB (31), LA (48), HR^+ (53), $LHER2^+$ (46)			
								ER^+	4	LA-LB (31), LA (48), HR^+ (53)			
								$EREB^+$	5	HER^+ (16)			
								ER^+	6	LB (28), LA-LB (31), LA (48), HR^+ (53)			
								ER^+	7	LA-LB (31), LA (48)			
								ER^+	8	HR^+ (53)			
								B	10				

* Six and **eight cancer samples were not characterized.
*** Breast cancer subtype classification reported on 404 tumors.
Abbreviations: B, basal-like; CNAs: copy number aberrations; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; HR, hormone receptor; L, luminal; LA, luminal A; LB, luminal B; MC, microenvironment community; N, number; T, T cell; TC, tumor cell community; TN, triple negative.

Table 2
Genetic alterations in 30 primary ER+ breast cancers uncovered by multiregion targeted sequencing.[27]

Tumor sample	Molecular subtype	Involved genes	N	Mutation type			
				Subs/indel driver	CN change	Clonal, N	Subclonal, N
1	ER+ HER2−	TP53, STK11, HRAS (+8q, −17p, +1q), FGFR1, MYC, CCNE1	6	2	6	8	0
2		TP53, PIK3CA, PTEN, HRAS (+8q, −17p, −8p), MYC	5	3	4	6	1
3		TP53, AKT1, MAP3K1, HRAS (+8q, −16q), FGFR1	5	3	3	6	0
4		ARID1A, MAP2K4, HRAS (−16q, +1q), FGFR1, CCND1	5	2	4	6	0
5		TP53, PIK3CA, HRAS (+8q, −16q, −8p), CCND1	4	2	4	6	0
6		BRCA2, HRAS (+8q, +17p, −16q, +1q),	2	1	4	5	0
7		PIK3CA, CDH1, HRAS (+8q, −16q, −8p)	3	2	3	5	0
8		GATA3, PTEN, CDH1, HRAS (−16q), CCND1	5	3	2	5	0
9		TP53, BRCA2, CDKN2A, HRAS (−17p, −16q), upd (17p)	5	3	3	3	3
10		HRAS (+8q, −17p, −16q, +1q)	1	0	4	4	0
11		PIK3CA, HRAS (+8q, −17p, −16q)	2	1	3	4	0
12		GATA3, TBX3, HRAS (−16q, +1q)	3	2	2	4	0
13		HRAS (+8q, −17p, −16q, +1q)	1	0	4	4	0
14		HRAS (+8q, −17p, −16q)	1	0	3	3	0
15		PIK3CA	1	1	0	1	0
16		TP53	1	1	0	1	0
17		PIK3CA, GATA3, FGFR2, HRAS (+8q, −17p, −16q, +1q, −8p), CCND1	5	3	6	8	1
18		PTEN, SF3B1, CREBBP, HRAS (+8q, −17p, −16q, +1q), FGFR1	5	3	5	5	3
19		TP53, MAP2K4, ARID1B*, MLL2*, AKT1*, HRAS (−17p, −16q)	6	5	2	6	1
20		ARID1A, HRAS (+8q, −16q, +1q), FGFR1, AURKA	4	1	5	6	0
21		TP53, HRAS (+8q, −17p, +1q, −8p), CCNE1	3	1	5	6	0
22		HRAS (+8q,−17 p,−16 q,+1q) CCND1	2	0	5	4	1
23		TP53, BRCA2, RUNX1, HRAS (+8q,−8p)	4	3	2	4	1
24		TP53, HRAS (+8q,+1q), FGFR1, MYC	4	1	4	4	1
25		TP53, PTEN, HRAS (+8q,+1q),	3	2	2	3	1
26		HRAS (+8q,−17 p,−16 q), FGFR1	2	0	4	3	1
27		GATA3, HRAS (+8q,−8p)	2	1	2	1	2
28	ER+ HER2+	PIK3CA, HRAS (+8q), ERBB2	3	1	2	3	0
29		ARID1A, SMAD2, HRAS (−17 p,+1q, −8p), ERBB2	4	2	4	6	0
30		ARID1A, HRAS (−17 p,+1q), ERBB2	3	1	3	4	0

* Detected in all but subclonal in same samples.
Abbreviations: CN, copy number; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; Indel, insertions and deletions; Subs, substitutions.

ChIP-seq signal could be used to estimate the phenotypic heterogeneity in breast cancer,[33] showing that the signal was proportional to the number of contributing cells. Additionally, transcription factor YY1 was a crucial partner of ERα in regulating the expression of genes typical of the genetic signature of ERα+ breast cancer. Overall, this work proved that epigenetic analysis could be used to qualitatively characterize the phenotypic heterogeneity in breast cancer.

4.2. The singletons and systematic classification of TME

In the work by Wagner et al.,[2] when the tumors samples were grouped based on the clusters' frequencies, three groups (Tu1-Tu3) contained many tumors, four groups (Tu4-Tu7) contained 3–4 tumours, and 36 (24% of the total) singletons contained just one tumor. Group Tu1 comprised 42 tumors enriched in the epithelial phenotypes, T-cells phenotypes, and macrophages phenotypes. Most of these were also present in non-tumoral tissues except for the (PD-1^{int} CTLA-4− CD38−) T14 and M12. The Tu2 comprised nine tumours characterized by high frequencies of the highly proliferative epithelial phenotype Ep19. The presence of 44 tumors characterized the Tu3 group. Within this group, high frequencies were observed for the epithelial ERα+ Ep09 and Ep14 and the epithelial ERα− Ep17 and Ep18. When the authors clustered the singletons based on the characteristics of the immune microenvironment, three groups emerged: TIG1, TIG2, and TIG3, containing 6%, 32%, and 50% of the total singletons, respectively. TIG1 had high frequencies of TAMs and T cell phenotypes. High frequencies of PD-L1+ TAMs and PD-1^{int} CTLA-4− CD38− T-cells and low frequencies of exhausted T-cells were observed in TIG3. Conversely, in TIG2 high frequencies of immunosuppressive T0 T-cells, PD-L1+ TAMs and exhausted T-cells occurred. Additionally, compared with TIG1 and TIG3 cancers, TIG2 tumors showed higher phenotypical abnormality and individuality scores. This group included both ERα+ and ERα− breast cancer subgroups. ERα− cells ranged from 15% to 98% and were characterized by EMT phenotypes and HLA-DR+ epithelial phenotypes. On the other hand, the ERα+cells within the TIG2 were characterized by high levels of pro-survival BCL-2 and survivin and the co-expression of AR, HER2, and PRB. These data suggested that the singletons of the TIG2 were populated by cells potentially able to escape cancer therapies. Systematic classification in this and other two reports[2-4] is summed up in Table 1.

5. The complexity of TME in metastatic disease makes questionable the concept of precision medicine and targeted therapy and rather supports efforts to possibly define specific conditions favoring the response to therapy

Although early screenings and diagnosis as well as advances in therapy have substantially prolonged the overall survival (OS), metastatic breast cancer is incurable and most patients die within five years from diagnosis.[40] Since about two decades most basic research has been focused on identifying molecular signaling/pathway or genetic/epigenetic alteration sustaining the main cancer hallmarks; at the same time accordingly most clinical trials/studies are carried out to challenge new drugs addressing the new different targets. Usually, advanced disease is the initial setting for therapeutic investigation, and when successful, the drug is tested in the neo-adjuvant/adjuvant setting of the same population. These drugs usually directly inhibit pro-tumoral mechanisms,[41] as it is the case for PI3K-AKT-mTOR pathway inhibitors in TNBC, CDK 4/6 inhibitors (palbociclib, ribociclib, and abemaciclib) in HR+ HER2− breast cancer, monoclonal antibodies against HER2 alone or associated with chemotherapy or a drug in HER2+ breast cancer sub-type and polymerase ADP-ribose inhibitors (PARPi) again in TNBC. Instead, sometimes they inhibit mechanisms of immune suppression,[10] as it is the case for the ICIs in TNBC. So far in this therapeutic strategy, the occurrence of constitutive ("intrinsic" or "de novo") or acquired resistance as

with more conventional chemo- and endocrine-therapies and of adverse events (AEs) have clearly been the two main limitations. Moreover, as targeted therapies are commonly given in combination with conventional treatment, in most patients further AEs worsen the quality of life (QoL), and in significant percentages or in some instances, heavy AEs account for a definite or transient treatment interruption. Additionally, in most successful clinical trials the benefit in terms of progression free survival (PFS) or OS is few months or 1–2 years at the best. Indeed, the recent researches dissecting multiple breast cancer tumor samples have shown that the qualitative and quantitative composition of epithelial and immune cell phenotypes in each tumor sample is different from any other (Tables 1-2). Moreover, genetic aberrations and transcriptional changes increase over time due to tumor cell genetic instability and therapeutic pressure. Collectively, this clearly highlights the complexity of TME and of the cancer landscape and accounts for the common arising of drug-resistance that impedes a decisive impact on the progression of metastatic disease.[41-45] This makes questionable the intriguing message of a successful treatment provided with precision medicine and rather supports further therapeutic strategies such as efforts to possibly define specific conditions favoring the response to therapy.

6. The tumor immune microenvironment

6.1. Immune cells and TILs in breast cancer

The TIME encompasses many types of immune cells that are recalled by various chemokines and cytokines produced by tumor cells and immune cells themselves. In a comprehensive study[46] evaluating gene expression profiles of 7,270 unrelated tumor samples of metastasis-free breast cancer patients, 22 immune cell types were identified. Among them, CD8 cytotoxic T cells (CTLs), CD4 T-helper cells, Tregs, T cells gamma delta, B lymphocytes, and NK cells known as TILs are the most studied immune cells in the TME. In breast tumors, T cells, B cells, and NK cells rate is about 75%, 20%, and 5%, respectively.[47,48] The highest proportion of total TILs is shown in TNBC followed by HER2$^+$ with HR$^+$ breast cancers having much lower levels.[49] In clinical laboratories, TILs assessment is carried out by a continuous parameter on a single hematoxylin and eosin (H&E)-stained tumor section and a score is assigned according to established criteria.[50] TILs are also distinguished in intra-tumor TILs (iTILs) and stromal TILs (sTILs), the latter placed in the tumor stroma. Recent guidelines suggest to quantify only sTILs. In fact, although stromal and iTILs are generally correlated, iTILS are less abundant and more difficult to be identified on H&E sections.[51] Interestingly, a software-guided image evaluation of TILs is advised by the International Immuno-Oncology Biomarker Working Group as this approach is considered to likely improve inter-observer variability.[52]

6.2. The TIME and clinical outcome

Luminal A breast cancer has the best prognosis followed by Luminal B and HER2$^+$ with worse and mostly TNBC with the worst prognosis.[53,54] Accordingly, based on Ki67 index, TNBC, HER2$^+$, Luminal B, and Luminal A in decreasing order respectively, are the subtypes with higher biological aggressiveness.[55] In contrast with the biological aggressiveness, TILs are higher in TNBC and HER2$^+$ than in Luminal subtypes.[49,56] In addition, while TILs in TNBC and in HER2$^+$ molecular subtypes commonly directly correlate with disease-free survival (DFS) and/or OS[57-59] in Luminal subtypes, more often in Luminal A than in Luminal B, they showed no or an inverse correlation with DFS and/or OS.[58,60-62] A few investigations have reported on CD8$^+$ T cells, Tregs, their ratio, and NK cells correlations with survival.[58] In TNBC, intratumoral more than intra-stromal CD8$^+$ T cells directly correlated with relapse-free survival (RFS).[63] In a meta-analysis,[64] similar findings occurred in TNBC and ER$^-$ HER2$^+$ breast cancer patients. However, no significant correlation was found in luminal subtypes, and in another study,[65] no significant correlation also in HER2+ subtype was reported. In basal-like tumors, intraepithelial CD8$^+$CD103$^+$ T cells showed a direct correlation with RFS and OS.[66] CD103$^+$CD69$^+$ tissue-resident memory T (Trm) cells are 40% of CD8$^+$ TILs as mean in human breast tumors.[67] In TNBC samples from patients relapsing before 3 or more than 5 years after diagnosis, Trm cells were 20% or 60% respectively of CD8$^+$ TILs.[58] With regard to Tregs, in the HER2$^+$ subtype they inversely correlated with DFS and OS[46] and increased levels of CD4$^+$CD25$^+$FoxP3$^+$ Tregs more often have been reported to predict higher risk of relapse, lower RFS and OS, and capable to select HR$^+$ patients more likely relapsing after 5 years.[68] Conversely, a positive correlation of FoxP3$^+$ Tregs has been reported in basal-like and TNBC subtypes.[69-71] Tregs often infiltrate tumors with CD8$^+$ T cells and CD20$^+$ B cells; therefore, in some studies, Tregs have been reported as a CD8/Treg ratio[72,73] and a score associating CD8/FOXP3 ratio and pathological the American Joint Committee on Cancer (AJCC) staging identified 100% of patients with prolonged OS.[74] FoxP3$^+$ regulatory T cells were associated with poor prognosis in HR$^+$ breast cancer that lacked CD8$^+$ T cells infiltration; conversely, they were an indicator of better survival in ER$^-$, including HER2$^+$/ER$^-$ subtype, mainly in those with co-existent CD8$^+$ T cells.[75] In HER2$^+$ subtype, prolonged DFS joined with higher NK cells activated fraction. Accordingly, in the TNBC subtype, a higher rate of resting NK cells joined with worse DFS and OS.[46] In other studies tumor infiltrating NK cells predicted better survival likely due to NK cells recruited by CD155 over-expressed on breast cancer cells.[58,76] Table 3 summarizes these main findings. Overall, Tregs/CD8$^+$ or CD8$^+$/Treg ratio[77–81], among the other immunological prognostic parameters, seem to better reflect the clinical course.

6.3. Different conditions, factors and response to therapy contribute to the immune balance in TIME and define immunogenicity

Tumor immune cells cross-talk directly and through mediated mechanisms, contribute to the immune balance that usually favors an immunosuppressive microenvironment.[10] HR positivity inversely correlates with TILs level, Treg/Th2 ratio, and CD8$^+$ effector T cells as well as Tregs present at the tumor edge.[82] Accordingly, in other studies,[83,84] ER positivity, in addition to a Th2 immune TIME induced a lower expression of MHC II molecules in breast cancer cells. In TNBC and HER2$^+$ subtypes, high genomic instability allows immune system to better recognize foreign antigens while contemporaneously increases tumor cells proliferation and survival by enhancing the abnormal signaling pathways, like EGFR, MET, and PI3K.[49] Tumor growth and survival commonly join with enhanced immune inhibition.[85] IL-6, IL-17, or TGF-β, are immunosuppressive cytokines that in the TME attract TAMs, Tregs and myeloid-derived suppressor cells (MDSCs) that restrict CD8$^+$ T cell infiltration, proliferation, and activity within the tumor.[86-90] An immunosuppressive TME can also be promoted through PD-L1 over-expression, which inhibits T cell activity upon binding of tumor PD-L1 to the PD-1 or B7-1 receptors present in T and B cells.[91] Additionally, in peripheral blood of HR$^+$ metastatic breast cancer patients particularly in PD-1$^+$ T cells, TCR signaling, a marker of T cell activity, is diminished.[92] During trastuzumab and T-DM1 treatment an increased TILs have been found.[93,94] Inhibition of CTLA-4 expressing activated T cells can increase CD8$^+$ effector T cells through the inactivation of FoxP3$^+$CD4$^+$ Tregs.[95,96] Higher adaptive type 1 T-cell immune response following cytotoxic chemotherapy has also been reported. Particularly a few chemotherapeutic drugs including doxorubicin improve immune recognition of the tumor by promoting stress proteins release by dying cells and thereafter the IFN-gamma secretion, antigen presentation, and activation of T-cells.[97] In a study which compared 114 patients who had received anthracyclines with 1,062 who had not, there was enhanced type 1 immune response and the CD8$^+$ and IFN-gamma over-expression joined with improved pathological complete response (pCR).[98] Paclitaxel can also enhance tumor infiltrating type 1 T-cells by up-regulation of type 1 cytokines expression and lowering the intratumor Th2 T-cells.[99,100] Low-doses cyclophosphamide decreases Tregs

Table 3
Prognostic role of TILs, some more common immune subpopulations, $FOXP3^+/CD8^+$ or $CD8^+/FOXP3^+$ ratio in primary breast cancer subtypes.

Additional immunological prognostic information	Intrinsic breast cancer subtypes								References
	Luminal A		Luminal B		HER2 +		TNBC		
	%	Correlation with DFS and/or OS	%	Correlation with DFS and/or OS	%	Correlation with DFS and/or OS	%	Correlation with DFS and/or OS	
TILs, median	Low	No or inverse	Low[a]	No or inverse	Intermediate	Positive	High	Positive	57,60
$CD8^+$ T cells	Presence	No	Presence	No	Presence	Positive[b]	Presence	Positive	63,64
$CD4^+CD25^+FOXP3^+$ Tregs	Increase	Inverse	Increase	Inverse	Increase	Inverse	Increase	Positive	46,68-71
$FOXP3^+/CD8^+$ ratio	Low	Inverse	Low	Inverse	Low[c]	No	Low[c]	No	77
	High	Inverse	High	Inverse	High	No	High	No	78
$CD8^+/FOXP3^+$ ratio	High	Positive	High	Positive	High	Positive	High	Positive	79
	NR	NR	NR	NR	High	Positive	High	Positive	80,81
Activated NK cells	High	Positive	High	Positive n	High	Positive	High	No	46
Resting NK cells	High	No	High	No	High	Inverse	High	Inverse	46
NK cells combined with m-CD155 in BCC	High	Positive	High	Positive	High	Positive	High	Positive	58,76

[a] Little bit higher TILs rate in luminal B than in luminal A breast cancer is reported.
[b] Positive correlation is reported in $HR^-$and controversial in HR^+HER2^+ breast cancer (75).
[c] The sample size was relatively small ($n = 29$).
Abbreviations: BCC, breast cancer cells; DFS, disease-free survival; HER2, human epidermal growth factor receptor 2; HR, hormone receptor; It, intratumoral; NR, not reported; OS, overall survival; TILs, tumor infiltrating lymphocytes; TNBC, triple-negative breast cancer; Tregs, regulatory T cells.

without decreasing circulating Th1 immune response.[101] Carboplatin and cisplatin induce MHC class I over-expression on the tumor while decrease intra-tumor MDSCs and Tregs.[102] Finally, radiotherapy can promote both immune-suppression, by a higher proportion of radio-resistant Tregs, and immune-stimulation through an immunogenic cell death.[97,103] Overall, the functional and quantitative composition of the TIME which changes over time with tumor evolution and treatment define the immune balance and the tumor immunogenicity that is the propensity of TIME to favor the immune response.

7. The G0-G1 state and micro-metastatic disease likely favor immunogenicity

7.1. The G0-G1 state and micro-metastatic disease in breast cancer and other solid tumors

There are findings suggesting that the G0-G1 state induced by anti-estrogens and micro-metastatic disease favor the response to immune-therapy. The relevance of the immune system on carcinogenesis and tumor growth in the last decades, convinced many investigators to turn their efforts in manipulating cell mediated immunity against tumor. Despite ER^+ breast cancer is considered an immunologically "cold" subtype, we first conducted a pilot clinical trial[104,105] followed by a 2:1 control-case observational clinical study[106,107] with additional immunotherapy in metastatic ER^+ breast cancer patients in clinical benefit during conventional first-line anti-estrogens. In both studies, where patients also received cyclic interferon beta interleukin 2 (INF-beta IL-2) sequence, soon after the immune stimulation the main effector T and NK cells significantly increased in peripheral blood and a significant prolongation of PFS and OS was observed. Based on these and other experimental findings,[85,108,109] we highlighted the capability of anti-estrogens to induce a G0-G1 state (resting state) during clinical benefit, likely along with a strong reversion of the immune inhibition at the TME favoring the immune response.[110] Accordingly, in the study by Wagner J et al.,[2] regarding the relationship of tumor-associated immune cells with the induced immune suppressive TME it is stated that "estrogen signaling is a shaping force in the tumor ecosystem". Also, we have reported on successful clinical outcome obtained with immune-therapy in 5 cancer cases likely with minimal residual disease and in another one with prolonged low tumor burden.[111] Four of them who had removed primary cancer, were patients at high risk of clinical recurrence likely due to residual micro-metastatic disease either because they were in biochemical progression (1 patient) or because had been just radically operated for local recurrence (1 patient) or overt metastatic disease (2 patients). All these four patients successfully received cycles of inhibiting immune suppression immune-therapy alone (2 patients) or alternated with conventional chemo (1 patient) or endocrine-therapy (1 patient).[111] Consistent with these data, cancer drives extensive disruption of haematopoiesis recently has been reported.[112]

7.2. In metastatic setting endocrine therapy in ER^+ more than chemotherapy in $HER2^+$ and TNBC subtypes synergizes with immunotherapy

Unlike current thought, there are findings suggesting that TME in metastatic setting is more immune-permissive and/or less immune suppressive in ER^+ breast cancer patients treated with conventional hormone therapy (HT) than in $HER2^+$ and TNBC subtypes treated with conventional chemotherapy (CT). As to this, in a few studies carried out in TNBC (4 studies)[113–116] and in $HER2^+$ (6 studies)[117–122] subtypes, median PFS or time to progression (TTP) in patients receiving first-line conventional CT (7 studies)[113–119] or endocrine therapy (3 studies)[120–122] alone (control groups) were compared with those observed in similar patients who had also received immunotherapy with an ICI or trastuzumab respectively (studied groups). In 3 of the 6 studies receiving CT carried out in $HER2^+$ subtype, controls and studied groups included similar proportions of HR^- and HR^+ patients. Instead, in the 3 remaining studies receiving endocrine therapy there was similar proportion of $HER2^+$ but only HR^+ patients, in controls and studied groups. These last 3 studies increased to 5 when our 2 mentioned clinical trials carried out in HR^+ patients were included. As shown in Table 4, in the 7 TNBC (4) and $HER2^+$ (3) study groups, receiving CT plus immunotherapy (checkpoint inhibitors or trastuzumab), median PFS/TTP increase ranged from +0.3 to +4.1 and from −1.7 to +5.6 months respectively compared with the control groups. Overall, in these 7 studies median PFS/TTP increase was +3.2 months. Interestingly, in the 3 $HER2^+$ HR^+ study groups conducted in metastatic HER2+ patients with conventional endocrine therapy plus trastuzumab, median PFS increase ranged from 2.4 to 10.8 months and the median PFS/TTP increase was 9 months. Furthermore, in our 2 investigational clinical trials carried out in metastatic breast cancer where conventional anti-estrogens were combined with INF-beta IL-2 sequence, median PFS further prolonged to 22 and 15 months, respectively, compared with the control groups. Trastuzumab, by blocking HER2 signaling, inhibits PI3K pathway ultimately, favoring apopto-

Table 4
In metastatic breast cancer immunotherapy synergizes with conventional therapy more in ER$^+$ than in HER2$^+$ and TNBC mulecular subtypes.

Breast cancer subtype		IT		Therapy ET/CT	Pts, N	PFS/TTP*, median, months		DFS		References
		Kind	Mechanism			CT	CT + IT	months	%	
TNBC		ICIs (PE ot ATZ)	Inhibits PI3K pathway and triggers ADCC	CT (nabPTX or PTX or GEM+CBD)	201 vs 566	5.6[a] 5.6[b]	9.7[a] 7.6[b]	+4.1	73	113
				CT (PTX)	101[c] vs 191[c]	5.7	6	+0.3	3.2	114
				CT (PTX)	184[d] vs 185[d]	5	7.5	+2.5	50	115
				CT (nabPTX or PTX or GEM+CBD)	211 vs 425[b] 103 vs 220[a]	5.6 5.6	7.9 9.7	+2 + 4.1	36 73	116
HER2$^+$	HR$^+$/HR$^-$[e]	Anti HER2 (trastuzumab)	CD8$^+$ T cells proliferation and plasma cytokines increase	CT (DOX or E+CY or PTX)	138 vs 143 96 vs 92	6.1* 3*	7.8* 6.9*	−1.7 + 3.9	28 132	117
	HR$^+$/HR$^-$			CT (DTX)	94 vs 92	6.1*	11.7*	+5.6	92	118
	HR$^+$/HR$^-$			CT (PTX)	60 vs 63	6.8*	10*	3.2	47	119
	HR$^+$			ET	54 vs 52	4.8*	13.8	+9	187	120
	HR$^+$			ET (anastrozole)	104 vs 103	2.4	4.8	+2.4	100	121
	HR$^+$			ET (letrozole)	31 vs 26	3.3*	14.1*	+10.8	327	122
HR$^+$/HER2$^-$		Cyclic IFN-beta IL-2 sequence	CD8$^+$ T and NK cells increase	ET	Pts, N	E	ET + IT	–	–	–
				ET (TAM-TOR)	30 vs 29	16	38	+22	137	104,105
				ET (AI/TAM-TOR)	95 vs 42	18**	33	+15	83	106,107

[a] CPS 10 or more.
[b] CPS 1 or more.
[c] PDL1 (IC) > 1%.
[d] PDL1 ≥ 1%.
[e] About half patients both in control and study groups received hormone therapy.
* From Kaplan-Meier estimates.
** About one third of controls were given biological targeted drugs in addition to ET.
Abbreviations: ADCC, antibody-dependent cell mediated cytotoxicity; AI, aromatase inhibitor; ATZ, atezolizumab; CBD, carboplatin; CT, chemotherapy; CY, cyclophosphamide; DFS, disease–free survival; DOX, doxorubicin; DTX, docetaxel; ER, estrogen receptor; ET, endocrine therapy; E, epirubicin; GEM, gemcitabine; HER2, human epidermal growth factor receptor 2; HR, hormone receptor; ICI, immune checkpoint inhibitors; IFN, interferon; IL, interleukin; IT, immunotherapy; N, number; NabPTX, nab-paclitaxel; OS, overall survival; PE, pembrolizumab; PFS, progression free survival; PI3K, phosphatidylinositol-3-kinase; Pts, patients; PTX, paclitaxel; TAM, tamoxifen; TNBC, triple-negative breast cancer; TOR, toremifene; TTP, time to progression.

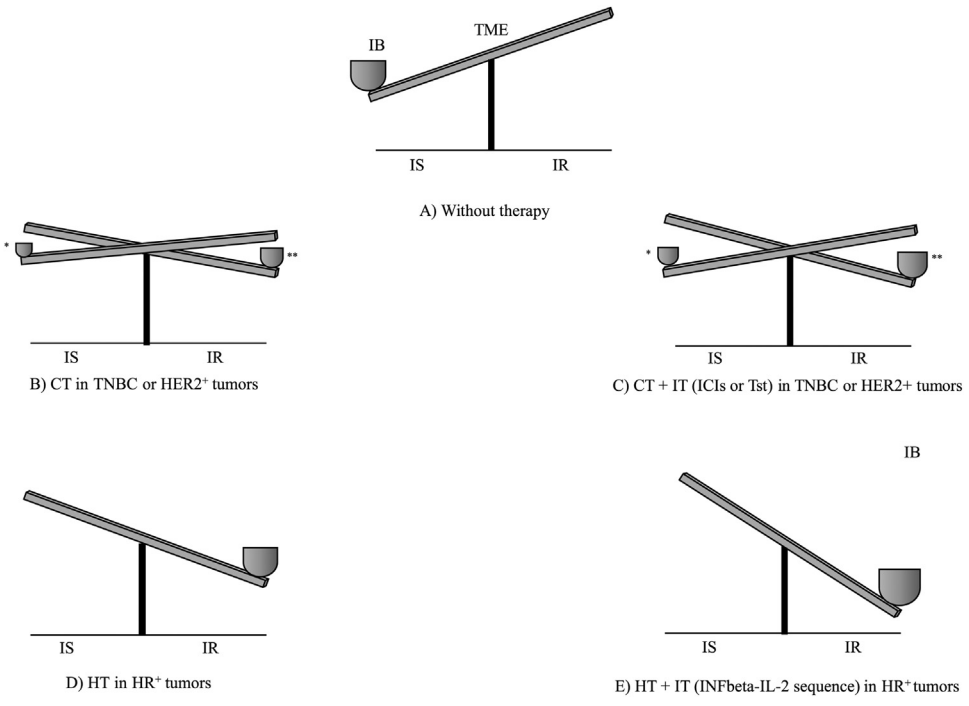

Fig. 1. Schematic representation of the proposed tumor immune microenvironment balance in patients receiving CT or HT without or with IT. (A) The IS significantly prevails. (B and C) The IB becomes slightly less immune suppressive or immune permissive and this effect increases when CT is given in association with IT and more during immunomodulatory** (anthracyclines, taxanes, CBDCA, etc.) than non-immunomodulatory* CT. (D and E) The IB becomes significantly immune permissive and this effect increases when HT is given in association with INFbeta-IL-2 sequence. In the illustration, the size of the scale plate correlates with the IB size. CT, chemotherapy; HER2, human epidermal growth factor receptor 2; HR, hormone receptor; HT, hormone therapy with antiestrogens; IB, immune balance; ICIs, immune checkpoint inhibitors; IFN, interferon; IL, interleukin; IR, immune response; IS, immune suppression; IT, immunotherapy; TME, tumor microenvironment; TNBC, triple negative breast cancer; Tst, trastuzumab.

sis and angiogenesis inhibition. Also, it is reported to trigger antibody-dependent cell mediated cytotoxicity (ADCC),[123] which is responsible for the activation of NK cells, macrophages, and dendritic cells, which on turn induce an adaptive immune response.[124,125] Thus, trastuzumab as PD-1/PD-L1 inhibitors promote an indirect immune activation unlike INF-beta IL-2 sequence that likely favored by the G0-G1 state induced by antiestrogens, actively boost an adaptive and innate immune response involving effector T and NK cells. The more immune-permissive and/or less immune suppressive TME induced by anti-estrogens in ER^+ more than conventional CT did in HR^+/HR^- $HER2^+$ and TNBC subtypes is consistent with the recent observation that both TNBC and $HER2^+$tumors have a lower intra-tumoral CD8/Treg ratio compared to $HR^+/HER2^-$ tumors, indicating higher levels of immune-suppression in the TME.[49,82] Thus, as shown in Fig. 1, endocrine therapy more than CT synergized with and stimulated immune response.

8. Conclusions

In each tumor, despite the low driver mutations,[24,25,126] an evolving different complexity occurs based on the structure and organization formed by the multiple intermingled epithelial and immune cell phenotypes in TME. Artificial intelligence can substantially improve the approach to treatment by better defining in any cancer a gene regulatory network with control hubs to be addressed as targets.[127,128] In addition to cancer detection and diagnosis, optimization of cancer treatment through identification of new therapeutic targets and drugs discovery are the principal potential applications of artificial intelligence and machine learning techniques. Over the last decades, an accelerated development of artificial intelligence algorithms occurred that can be divided into network-based biology analysis algorithm and machine learning-based (ML-based) biology analysis algorithm according to the data of biological network structure. While the former can be helpful to identify cancer targets by several different network approaches, the latter not only can efficiently handle a huge and complex amount of molecular data, but also can unveil feature or relationships in the biological networks so that such advanced biology analyses can identify precise target and allow drug discovery for cancer.[129,130] However, currently it remains very hard to unwind cancer complexity and successfully target the multiple key molecular pathways that foster tumor growth and progression. The advances in basic research have unveiled the intricacy of advanced breast cancer landscape and despite the many expectations, so far patients had limited benefit from targeted therapies strategy. Moreover, the few approved drugs which enter clinical practice come at high cost covered by the National Health Services. Besides this strategy likely reduced the interest of pharmacological factories to sponsor alternative research and researchers devoting their efforts to explore other routes/fields often do not obtain the needed collaboration. In the last two decades, immune-therapy as a powerful and harmless tool to fight cancer has gained increasing interest and there is currently large consensus that triple negative and $HER2^+$ due to higher TMB and an increased rate of TILs are two molecular subtypes more immunogenic than ER^+/luminal breast cancers. Indeed, perhaps the overall variable qualitative and quantitative composition of the TIME better defines the tumor immunogenicity that is also affected by treatment and tumor diffusion. In fact, the just mentioned consensus apparently contrasts with some clinical data here shown, as median PFS/TTP increase was 3.2 months in 7 studies conducted in metastatic TNBC (4) and in $HER2^+$ (3) patients who received conventional chemotherapy plus immunotherapy (checkpoint inhibitors or trastuzumab), compared with 9 months in 3 studies conducted in metastatic $HER2^+$ patients treated with conventional endocrine therapy plus trastuzumab. Instead, these data are consistent with findings reported in our two investigational studies and the recent observation that both TNBC and $HER2^+$ tumors have a lower intra-tumoral CD8/Treg ratio compared to $HR^+/HER2^-$ tumors, indicating higher levels of immune-suppression in the TME.[49,82] In addition, it has been found that anti-estrogens can revert[110] the immunosuppressive TME shaped by estrogen.[2] This could account for an improved synergism of immunotherapy when given in association with endocrine- rather than with chemotherapy including also when combined with immune modulating antiblastics. Thus, it is an easier achievement for current research to focus on specific conditions as the G0-G1 state induced by anti-estrogens or quiescence reported in polymorphonuclear leukocytes that mainly seems to favor endocrine and immunotherapy response in advanced breast and other cancers. Recently, our group and other researchers,[10,101-103] following advances on molecular biology and other experimental findings,[104,105] have appointed micro-metastatic disease as the ideal target for attaining a definite cure of cancer. We also have described a feasible, innovative protocol based on prolonged, intermittent given conventional chemo- or endocrine-therapy alternated with novel schedules of immunotherapy. We aspire for this cost-effective protocol to be progressively implemented and evaluated in multicenter prospective randomized clinical trials. The aim is to significantly enhance the rate of high-risk cancer patients achieving definitive cures. However, such trials may not align with the interests of those who take profit from clinically overt metastatic disease. So, it is not surprising that a majority of financial support continues to endorse a therapeutic strategy that primarily extends OS and/or PFS in metastatic setting. While targeted therapies have contributed to the discovery of new treatment options, along with novel biologic prognostic and predictive biomarkers, it is essential to recognize that other treatments designed to counteract metastatic disease have often been restricted. In conclusion, the growing complexity of TIME elucidated by basic research and the limited clinical outcome of precision medicine strategy arise questions more than provide answers within the scientific community.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

All authors contributed to writing the manuscript and designed and prepared the figure.

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