Tumour growth and immune evasion as targets for a new strategy in advanced cancer

Andrea Nicolini\textsuperscript{1a}, Paola Ferrari\textsuperscript{1}, Giuseppe Rossi\textsuperscript{2b}, Angelo Carpi\textsuperscript{3a}

\textsuperscript{1}Department of Oncology, Transplantations and New Technologies in Medicine, University of Pisa, Italy; \textsuperscript{2}Unit of Epidemiology and Biostatistics, Institute of Clinical Physiology, National Council of Research, Pisa, Italy; \textsuperscript{3}Department of Clinical and Experimental Medicine, University of Pisa, Italy

Corresponding author:
Andrea Nicolini
Via Roma 67, Pisa 56126 Italy
+39 339 8724215
andrea.nicolini@med.unipi.it

\textsuperscript{a}Retired
\textsuperscript{b}Deceased

Running head: A new anticancer strategy

Keywords: breast cancer, immune evasion, tumour growth, cancer therapy

Word count: 11084
Abstract

It has become clearer that advanced cancer, especially advanced breast cancer, is an entirely displayed pathological system that is much more complex than previously considered. However, the direct relationship between tumour growth and immune evasion can represent a general rule governing the pathological cancer system from the initial cancer cells to when the system is entirely displayed. Accordingly, a refined pathobiological model and a novel therapeutic strategy are proposed. The novel therapeutic strategy is based on therapeutically induced conditions (undetectable tumour burden and/or a prolonged tumour ‘resting state’), which enable an efficacious immune response in advanced breast and other types of solid cancers.
Introduction

As testified by ancient writers, human beings have been suffering from cancer since always (http://www.bordet.be/en/presentation/history/cancer_e/concur1.htm). It is likely that environmental pollution and prolonged aging concomitant with some radical lifestyle changes (Ferlay et al. 2012; Howell et al. 2014) are among the main reasons for the increasing prevalence of cancer in the modern era. In its advanced stages, cancer is often an incurable disease and represents a serious threat to human life. In 2012, the International Agency for Research on Cancer reported 14.1 million new cancer diagnoses, 8.2 million cancer deaths and 32.6 million cancer diagnoses of <5 years worldwide. The cancer death rate ranges 69–173 per 100,000 men and 65–119 per 100,000 women (Ferlay et al. 2012). Thus, cancer has now acquired a major social relevance. Among women, breast cancer is the most common cancer in most regions of the world, with an estimated incidence of 246,000 new cases (29% of all cancer cases) and 40,450 deaths (14% of all cases) in 2016 in the United States (Siegel et al. 2016). This paper reviews some recent experimental and clinical data to propose an innovative therapeutic strategy for advanced breast and other cancers based on the relationship between tumour growth and immune evasion.

The biological cancer hallmarks and the current model

In 2011, Hanahan and Weinberg published an update (Hanahan and Weinberg 2011) of their milestone article on the principal hallmarks of cancer (Hanahan and Weinberg 2000). The authors reported that the principal biological capabilities acquired by cancer cells are sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming energy and metabolism and evading immune destruction. In this updated model, genomic instability and inflammation are the basis of all the hallmarks. The network sustaining cancer growth and progression is represented as an overall integrated circuitry comprising a few interconnected subcircuits. In turn, each sub-circuit comprises multiple interconnected pathological molecular pathways fostering different hallmark capabilities. Here, we focus on advances in the role of tumour growth and immune evasion in tumour progression and diffusion.
Tumour growth

Sustaining proliferative signalling. The growth-promoting signals in cancer cells are mainly induced by growth factors that bind cell surface receptors with intracellular tyrosine kinase domains. In addition, growth factors acquire the capability to carry mitogenic signals in some different ways. In particular, autocrine or paracrine proliferative stimulations and downstream molecular pathways, either constitutively activated or activated following somatic mutations, are common (Davies and Samuels 2010; Nicolini et al 2015). Many other mechanisms of tumour growth promotion (Aziz et al 2015; Gao J et al 2015; Wang C et al 2015; SPN et al 2015; Rohatgi et al 2015) have been reported. Some of these studies (Koval et al 2016; Van Geldermalsen et al 2016; Song et al 2015; Wang YL et al 2015) included triple-negative cancer subtype.

Evading growth suppressors. In recent years, a few tumour suppressor genes and the inhibition of anti-proliferative mechanisms have been identified (Ma et al 2015; Kochupurakkal et al 2015). The constitutive activation of the interferon gamma (IFNγ)/signal transducers and activators of transcription (STAT) 1/interferon-regulatory factor (IRF)-1 axis [T helper (Th) 1 phenotype] correlates with good prognosis and predicts better response to anti-cancer therapy (Ascierto et al 2011). IRF-1 regulates the transcription of a set of target genes that play principal roles in tumour immune surveillance and immune system development. The mechanisms by which IRF-1 mediates tumour suppression are not clear; however, several IRF-1 target genes that inhibit growth by cell cycle arrest and promote apoptosis have been identified (Dou et al 2014). Many reports have suggested a relevant role of IFNγ/STAT1/IRF-1 axis in the endocrine resistance of oestrogen receptor (ER)-positive breast cancer cells (Clarke et al 2009; Ning et al 2010; Schwartz JL et al 2011; Schwartz-Roberts et al 2015). Yang et al. (2017) recently showed that the suppression of the immune functions of T cells in the tumour microenvironment (TME) is another mechanism by which oestrogen drives cancer progression. Accordingly, in two experimental studies, IRF-1- and IFNγ-mediated apoptosis was induced by the anti-oestrogens tamoxifen (Bowie et al 2004) and fulvestrant (Bouker et al 2004). These observations also suggest changes in gene expression from heterogeneous tumour samples. In breast cancer, additional mechanisms regarding the evasion of tumour growth suppressors (You et al 2016; Xu YM et al 2015; Hu and Xie 2015) have been described.
Resisting cell death. In principle, cell death, mainly by apoptosis or necrosis, is thought to be a main natural hindrance to cancer development. Apoptosis plays a fundamental role in the homeostasis of healthy tissues. In the last decades, it has been fully elucidated how apoptosis is triggered in response to different physiological stimuli. The apoptotic machinery is governed by upstream regulators and downstream effectors, and the regulators include two major circuits: the extrinsic and intrinsic apoptotic programs (Cory and Adams 2002; Kiraz 2016). Recently, several mechanisms that can affect apoptosis in breast cancer cells have been described (Shrestha et al 2016; Sayeed et al 2013; Cao et al 2016; Zhou X et al 2015; Gao SP et al 2015; Armstrong et al 2015; Han Z et al 2015; Liu et al 2015; Li et al 2015; Saqcena et al 2015; Farrugia et al 2015).

Enabling replicative immortality. Other investigational findings on senescence (El Hasana et al 2015) and autophagy (Artero-Castro et al 2015) have been reported. Autophagy is a ‘self-eating’ process initiated by cancer cells in response to various stresses. Both autophagy upregulation and downregulation have been found in cancer, suggesting its dual oncogenic and tumour-suppressing roles during malignant transformation (Marinkovic et al 2018). However, in the last decades, accumulating evidence by experimental studies have indicated the relevance of autophagy in cancer progression and diffusion. These studies have elucidated further mechanisms of autophagy in human ER-α+ (Wang S et al 2017; Hou et al 2017; Galindo-Moreno et al 2017; Leignadier et al 2017) or ER-α+ and ER-α− (Han H et al 2017; Zhou H et al 2017; Lin Y et al 2017; Zhou X et al 2017) breast cancer cells. In addition, they found that cytotoxic (chemo or endocrine) treatment (Kondo et al 2005; Chen SY et al 2011) and severe hypoxia (Rouschop 2010) are two major stresses that could be evaded by autophagy. In fact, in both cases, autophagy allows the cancer cell to survive and become refractory to chemo-endocrine therapy and chemoradiotherapy. Other translational research studies (Tavera-Mendoza et al 2017; Ueno T et al 2016; Han Q et al 2017; Zhou J et al 2017) have been conducted to identify novel prognostic biomarkers or key targets for developing new therapeutic agents. Some clinical trials using the autophagy inhibitors chloroquine (CQ) or hydroxychloroquine (HCQ) are ongoing. Two of them (NCT02333890 and NCT01023477) are evaluating CQ efficacy in decreasing tumour growth prior to surgical intervention, and one (NCT01446016) is evaluating CQ efficacy when given in combination with taxane in metastatic setting in patients who had previously failed to respond to anthracycline chemotherapy. In two trials (NCT03032406 and NCT03400254), HCQ alone or with everolimus or gedatolisib has been administered for preventing recurrent breast cancer. In another trial (NCT00765765), HCQ has been administered in a metastatic setting in combination with ixabepilone vs ixabepilone alone, with the decrease in tumour growth and response rate being evaluated as the main end-points. All these trials
have recruited breast cancer patients independent of hormone receptor status. A further ongoing trial enrolling ER+ patients (NCT02414776) is evaluating the response rate following the addition of HCQ in patients showing progress with hormonal therapy.

**Immune evasion**

Mechanisms of immune suppression or immune escape.

Indeed, it is now a consolidated concept that during tumour development, a chronic inflammatory microenvironment reduces the anti-tumoural immune response and favours the escape of tumour from immune elimination (Bui and Schreiber 2007; Clevers 2004). Inflammatory immune cells include tumour-associated macrophages (TAMs), cytotoxic T (CD8) lymphocytes (CTLs), Th (CD4) lymphocytes, natural killer (NK) cells, regulatory T (Treg) cells and myeloid-derived suppressor cells (MDSCs). Among them, Treg cells, MDSCs and macrophages are mainly involved in the immunosuppressive action (Vasaturo et al 2015) via the secretion of key molecules, such as transforming growth factor beta (TGF-β), prostaglandin E2, indoleamine 2,3-dioxygenase and interleukin (IL)-10 (Capietto et al 2011). The abundance of Tregs, MDSCs and TAMs in the stroma also helps cancer cells to escape immune surveillance and is associated with worse prognosis (Mantovani et al 2006; Greten et al 2011; Bergenfelz et al 2015; Li F et al 2018), whereas CTLs are associated with a good prognosis (Tosolini et al 2011). Signals derived from cancer cells and the stroma determine the TAM phenotype from M1, which stimulates immunoprotective inflammatory responses, and M2, which has an immunosuppressive action. M2 phenotype is found in most tumours where the TAMs induce angiogenesis, tumour growth and metastasis by secreting soluble mediators, cytokines and chemokines and by directly interacting with cancer stem cells (CSCs) (Hao et al 2012). Soluble mediators, mainly growth factors, cytokines and chemokines, in addition to host immune cells are also produced by cancer-associated fibroblasts (CAFs) or by tumour cells themselves. Several growth factors, namely TGF-β, insulin-like growth factor 2 (IGF-2) and vascular endothelial growth factor (VEGF), cytokines, namely IL-1, IL-4, IL-6, IL-8, IL-10 and tumour-necrosis factor alpha, chemokines, namely chemokine (C-X-C motif) ligand 1 and C-C motif chemokine receptor 7, have been reported to be closely involved in tumour progression, invasion and immune escape (Gål et al 2017; Settarrahmane and Xu 2017; Eftekhari et al 2017) and are potential targets for anti-tumour therapies. Moreover, cancer cells not only express these soluble mediators but also frequently overexpress the related receptors to escape from the immune responses (Settarrahmane and Xu 2017). As is well known, tumour antigens must be presented in a human leucocyte antigen (HLA)-restricted way to be recognized by T-cell receptors. Impaired
HLA-I or HLA-II expression prevent the activation of cytotoxic immune cells or affect the antigen-presenting capability of antigen-presenting cells. In addition, aberrant HLA-G expression by cancer cells inhibit the activity of all immune cells. These HLA-associated immune evasion mechanisms occur early and frequently in most cancer types (Rodriguez 2017; McGranahan et al 2017). Nod-like receptor family caspase recruitment domain-containing 5 (NLRC5) has been found to be a crucial transcriptional co-activator of major histocompatibility complex (MHC) class I gene expression. NLRC5 expression strongly correlates with genes in the MHC class-I antigen presentation pathway, including transporter associated with antigen processing (TAP) 1. In different types of cancer, epigenetic and genetic alterations are most prevalent in NLRC5 among all the MHC class I-related genes and are associated with impaired expression of the MHC I pathway components and immune evasion (Yoshihama et al 2016). Accordingly, TAP1 downregulation has been shown to elicit immune escape in colorectal cancer (Ling et al 2017). Further described mechanisms of immune evasion in breast and other cancer cells involve increased programmed death-ligand 1 (PD-L1) expression (Martinez et al 2017; Coelho et al 2017), stabilized PD-L1 mRNA (Glodde and Holzel 2017) and altered PD-L1 function (Maj et al 2017) by different molecular pathways. Nuclear factor (NF)-kb and increased PD-L1 expression are involved in immune evasion and the progression of triple-negative breast cancer (TNBC) (Maeda et al 2018).

The expression of the enzyme arginase 1 (ARG 1) as a key mediator of immune suppression (Steggerda et al 2017) and the loss-of-function Janus-activated kinase (JAK) 1 mutations suggestive of immune suppression (Albacker et al 2017) have been also reported in multiple cancer types. Moreover, other recent studies have reported on immune evasion (Khaled et al 2013; Zhang YX et al 2015; Zelenay et al 2015; Hix et al 2011; Gameiro et al 2016; Heng et al 2016; Loi et al 2016; Zhang M et al 2015; Olesch et al 2015; Tao et al 2014; Loumagne et al 2014; Lim et al 2016; Virtanen et al 2014; Markosyan et al 2013).

**Probable reasons for the discrepancy between genetic and biological advances and clinical outcome**

Despite the vast acquired biological knowledge, advanced breast cancer remains a disease with poor prognosis. Currently, endocrine therapy, chemotherapy and, more recently, the so-called ‘targeted therapies’ are common medical treatments for the advanced disease stages. Despite the availability of new biological drugs and a more rational use of therapies, the clinical outcome remains poor. Thus, the life-expectancy of patients with advanced disease is dismal, and the median survival of a mixed population of metastatic breast cancer patients has not substantially improved in the last

The genetic and epigenetic heterogeneities. To date, cDNA and mRNA sequencing has allowed several ‘genetic signatures’ and molecular profiles to be defined, which usually differ in different tumours and different samples of the same tumour (spatial intratumour heterogeneity) (Verigos and Magklara 2015; Yachida et al 2010). Each of these multigene sets is different in terms of the number and, at least in part, the genes involved. Moreover, although the most relevant genes for predicting patient outcome are those involved in cell proliferation, only Oncotype DX (Paik et al 2004) and Mammaprint (van de Vijver et al 2002) can be routinely recommended for predicting response to a specific type of therapy (Duffy et al 2017). cDNA heterogeneity is increased further by epigenetic alterations and their ability to regulate gene expression (silencing oncogenes or activating repressor genes). The relevance of the contribution of epigenetic alterations to the genetic cancer heterogeneity became clear when hypo/hypermethylation of DNA and micro RNA (miRNA) function were investigated. Many patterns of methylation changes (hypo or hyper) have been described associated with different canonical pathways (Rodenhiser et al 2008) or sometimes corresponding to different known genetic signatures. Many up- or downregulated miRNAs, numerous oncogenic and tumour-suppressive miRNAs involved in relevant molecular pathways, such as proliferation and survival, cell migration and metastasis, CSC phenotypes and epithelial-to-mesenchymal transition (EMT) processes, have been reported (Bertoli et al 2015; Wang J et al 2015). Currently, breast cancer classification into molecular sub-types considers the genetic characteristics of tumour. If the epigenetic alterations were also taken into account, many more sub-types could be generated. This inconsistency indicates the existence of further different molecular subtypes of breast cancer and even more complex classifications than are currently known.

The genomic instability and the plasticity of phenotypes. Genomic instability is a prominent property of cancer cells that allows them to accumulate random mutations over time to acquire and better orchestrate their hallmark capabilities. The accumulation of mutations occurs due to the naturally developing genetic aberrations combined with those following the selective pressure of anticancer treatments (Zardavas et al 2015). Tumour stroma is also involved in genomic instability. In a study of 51 breast cancer gene (BRCA)1/2-related cancers and 134 sporadic breast cancers, the accumulation of genomic instability in the tumour stroma corresponded to that in the neoplastic epithelium (Weber et al 2006). In another study, human orthologs of genes identified in the stromal reaction to tumour progression in a mouse model were also expressed in several human cancers (Bacac et al 2006). These and other findings indicated that genomic instability induces stromal alterations capable of promoting neoplastic transformation and stimulating tumour progression. In
addition, genomic instability is favoured by the compromised surveillance system that normally detects and resolves defects in DNA or forces genetically damaged cells into senescence or apoptosis (Jackson and Bartek 2009; Kastan 2008). Recently, genetic differences have been shown between a primary breast tumour and its associated metastatic lesions, which developed over time (Zardavas et al 2015). Moreover, sequencing data from cell populations as well as from single cells have shown three classes of mutations, namely a) clonal mutations observed in the population sample and in most single tumour cells, b) subclonal mutations found only in single cells and not in the population and c) de novo mutations observed in one tumour cell only (Wang Y et al 2014). These findings indicate that there is significant tumour heterogeneity, even at the single-cell level, and suggest that different tumour subclones are the result of the accumulation of different point mutations over time (Wang et al 2014; Wang Y and Navin 2015). Thus, genomic instability of stroma and cancer cells accounts mainly for temporal intra-tumour heterogeneity and describes tumour evolution. Biological plasticity is another important feature that significantly contributes to temporal tumour heterogeneity during cancer progression. Therefore, an initially more genetically homogeneous population of cells within a tumour becomes phenotypically heterogeneous due to the presence of cells in distinct states of differentiation following phenotypic variability, at least in part. The main example of this biological plasticity is the phenotypic variability implicit in CSCs where the activation of an EMT (Kalluri and Weinberg 2009) or endothelial-to-mesenchymal transition (Potenta et al 2008; Mihira et al 2012) program converts epithelial or resident fibroblasts or endothelial cancer cells into mammary cancer cells (MCCs) or CAFs. On the other hand, programs that convert endothelial cells to mesenchymal cells or mesenchymal cells to endothelial cells have been documented within stroma (Medici et al 2010). Although all of these programs and the contextual signals tend to promote an invasive tumour phenotype, in the absence of exposure to these signals, cancer cells may also revert to a non-invasive state through a process termed mesenchymal–epithelial transition (MET). This process is associated with cancer progression and metastasis. At the site of metastases, mesenchymal tumour cells must undergo MET as metastases recapitulate the pathology of the corresponding primary tumours. Thus, ‘the cellular plasticity, the ability to undergo EMT and, subsequently, MET in the appropriate microenvironment, is a key feature of a successful metastatic cell’ (Hugo et al 2007). Moreover, cells do not complete these transformation programs and frequently acquire a few traits of the new phenotype while continuing to express residual traits of the old phenotype. This contributes to increased temporal tumour heterogeneity. Incomplete knowledge of the mechanisms and the ‘contextual signalling’ that affect pathological molecular pathways sustaining the cancer hallmarks. The deficiencies in the knowledge and the
complexity of the mechanisms sustaining tumour growth are well known to the investigators and
are also clearly mentioned in the updated article by Hanahan and Weinberg (Hanahan and Weinberg
2011). In a recent review (Smithson et al 2016), it has been suggested that ‘signalling represents the
language of the cell, where molecules (words) and cellular context (syntax) serve as units of
informational content’; in addition, the authors stated that ‘when we study signalling pathways in
normal cells or in the setting of cancer, we often fail to consider how the cellular language
conferring by these pathways is influenced by context, that is, the different extracellular signals
present in the immediate milieu, the various adaptive responses that limit and promote intracellular
signal transduction, the innate properties of distinct cell types responding to these cues, and the
impact of epigenetic/genomic changes on the ultimate consequence of these informational signals’.
They concluded that ‘a deeper appreciation of contextual signalling may improve our understanding
of the basic principles that govern development’. Recently, other authors have stated that ‘a direct
approach of inhibiting single oncogenic proteins misses the dynamic network context governing the
network signal processing’ (Fey et al 2016). Overall, these deductions demonstrate that the
comprehension of cancer is a work in progress.

Chemo-, hormone and targeted therapies: main limits. Locally confined primary cancer is
commonly called ‘early’ or ‘advanced cancer’ according to whether, at the time of diagnosis, it
corresponds to the initial or successive stages of the ongoing internationally recognized
clinicopathological classifications. Regional involvement (regional lymph nodes or regions around
the primary cancer) makes any locally confined primary cancer an advanced cancer. When organs
that are distant from the site of primary cancer are involved, cancer is called metastatic, and even
metastatic disease constitutes advanced cancer. Although surgery and radiotherapy function loco-
regionally, conventional chemo-, hormone and targeted therapies are directed to cancer cells
wherever they are present in the body tissues. Therefore, they are usually administered to patients
with advanced cancer to prevent (adjuvant therapy) or treat (salvage therapy) metastatic disease.
However, the development of acquired resistance and toxicity are two limiting aspects common to
all therapies, although they generally differ according to the type of drug. In hormone-sensitive
patients, hormone therapy is very rarely interrupted by heavy toxicity (Nicolini et al 2016). It is
likely that the absent or mild side effects and a more prolonged efficacy reflect the limited number
of normal tissues involved in addition to cancer cells (Couse and Korach 1999) as well as the more
terminal inhibition of multiple transduction signalling pathways activating the targeted intracellular
biological processes respectively. Usually, because of the lack of significant efficacy when
administered alone, the targeted therapies are given in combination with chemotherapy or hormone
therapy. Nevertheless, patients receiving targeted therapies often exhibit moderate or heavy toxicity
likely attributable, as for chemotherapy, to the unselected target cells. In these patients, the mean short duration of the efficacy likely reflects the higher number of mechanisms potentially responsible for the development of resistance (Granata et al. 2016; Roskoski 2014; Fey et al. 2016). Recently, a plethora of these mechanisms of resistance has been investigated in melanoma skin cancer (Wellbrok and Arozarena 2016). Table 1 summarizes the principal probable reasons of the discrepancy between the advances in biological knowledge and the persistently poor outcomes of advanced breast cancer.

**Advanced breast cancer: prognostic relevance of tumour growth and immune evasion**

In advanced breast cancer, the ‘driver genes’ or recurring ‘significantly mutated genes’ involved in the ‘genetic signatures’ that are specific for the molecular subtypes are known, in addition to some principal pathways and molecular cascades that they activate or inhibit (Stephens et al. 2012; Cancer Genome Atlas Network 2012). However, it appears that a lot of information regarding the regulation and post-translational modifications of the altered genes, the multiple signalling cascades they activate, their positive and negative loops and their interconnections is still unknown (Le Romancer et al. 2011). In addition, the way in which a single molecular pathway and sub-circuit contribute to the final hallmarks is unknown. The complexity of the system suggests that the overall integrated network on which each tumour is based is by far unknown, thereby facilitating the development of resistance to any conventional therapy. Thus, the complexity of the pathobiological model of advanced cancer, which has been uncovering following the progress in genetics and molecular biology, can be compared to the knowledge of the universe following the Hubble advent. This complexity accounts for the relatively poor clinical outcome. In fact, not temporally planned, not appropriately directed and/or not appropriately synergized targeting of one or a few molecular signalling pathways is unlikely to affect the outcome of such complex and entirely displayed pathological systems of any advanced cancer, especially advanced breast cancer. Despite this, we think that the relationship between tumour growth and immune evasion can offer new therapeutic opportunities via an efficacious immune manipulation.

Tumour growth and prognosis. Several findings have highlighted the clinical relevance of proliferation signalling in breast cancer. In particular, few studies have shown its significant relationship with patient outcome. Luminal A, which is the most common molecular breast cancer subtype with a favourable prognosis, exhibits low expression of cell proliferation-related genes compared with luminal B, which is characterized by a more aggressive phenotype and high
expression of these genes (Eroles et al 2012; Galanina et al 2011). A high expression of cell proliferation-related genes is common in basal-like breast cancer (BLBC) or TNBC, associated with the worst prognosis among the different molecular subtypes (Perou et al 2000). A PARADIGM analysis of basal-like versus luminal tumours demonstrated that hyperactivated FOXM1 is a transcriptional driver of this enhanced proliferation signature. Basal-like cancers have 80% of tumour suppressor protein (TP53) mutations; furthermore, the loss of retinoblastoma-associated protein (RB) 1 and BRCA1 genes and high phosphatidylinositol-3 kinase/protein kinase B (AKT) pathway activities are common features of this molecular subtype (Cancer Genome Atlas Network 2012). Moreover, a basal-specific trans module enriched for transcriptional changes involving cell cycle, DNA damage repair and apoptosis and reflecting the high mitotic index typically associated with basal-like cancers has been described (Curtis et al 2012). In two large-scale studies, a high ratio of the homeobox 13 to IL-17B receptor (IL-17BR) expression correlated with poor clinical outcome in resected node-negative ER-positive breast cancer patients receiving adjuvant tamoxifen. Interestingly, IL-17BR plays a role in recurrences, either by the induction of anti-tumour immunity or by mediating the response to growth factors involved in breast epithelial tumour proliferation (Goetz et al 2006; Erlander et al 2005; Goetz et al 2008). In another study (Paik et al 2004), the expression of 16 cancer-related and five reference genes were used to calculate a recurrence score (RS) for predicting the outcome of tamoxifen-treated, node-negative breast cancer patients. In a multivariate Cox model, the RS was significantly predictive of distant recurrence and overall survival (OS). In this study, the 16 selected genes were grouped on the basis of function, correlated expression or both. Two of the four groups, termed the proliferation and human epidermal growth factor receptor (HER)-2 groups, included five (KI67, STK15, survivin, CCNB1 and MYBL2) and two (GRB7, and Her2) of the 16 selected genes, respectively. Therefore, approximately half of the genes used to calculate the RS were directly related to tumour growth. In a successive investigation, the same RS was prognostic for tamoxifen-treated node-positive breast cancer patients and predicted a significant response to chemotherapy in patients with a high RS (Albain et al 2010).

Immune signatures and prognosis. Recently, well-described immune signatures have been reported in many studies on gene expression. Specifically, some prognostic immune signatures have been developed for HER-2+ ER-α−, TNBC or BLBC. This is of particular relevance as these breast cancers are among the molecular subtypes that are the most aggressive and resistant to therapy. In the studies on HER-2+ ER-α− breast cancers (Liu JC et al 2012; Liu JC et al 2017), the developed 17-gene immune signature, in addition to the high prognostic value, allowed the identification of patients who would benefit from combination therapy with trastuzumab and immunomodulatory
drugs. A biological network-driven gene selection in TNBC (Bonsang-Kitzis et al 2015) identified a stromal six metagene signature named immunity 1, immunity 2, proliferation/DNA damage, androgen receptor-like, Matrix/Invasion 1 and Matrix 2 clusters with the immunity two metagene having a high positive prognostic value. In a study on BLBC (Martinez-Canales et al 2017), 16 genes associated with immune function and upregulated in BLBC compared with their expression normal breast tissue were linked with improved clinical outcome. In particular, the association of upregulated HLA)T cell immunoreceptor with IG and ITIM (TIGIT) domains and HLA-C/HLA-F/TIGIT genes showed the most favourable outcome. In other two studies, the immune signature predicted benefit from trastuzumab in adjuvant (Perez et al 2015) or neoadjuvant (Varadan et al 2016) settings. Interestingly, in these and other studies (Levy et al 2016; Heimes et al 2017; Kim et al 2017), when immunological signature was associated with immune function and immune response, it directly correlated with a better clinical outcome. Moreover, epigenetic alterations, in addition to the genetic alterations, of immune genes with prognostic impact have been increasingly reported in different types of cancers, including breast cancer. In an investigational study (Xu Z et al 2016), all 10 B7 family members were amplified in breast cancer. In particular, B7 mRNA levels were upregulated in a cohort of 1,098 patients with different types of breast cancer and in 82 patients with TNBC. Promoter methylation analysis showed an epigenetic basis for the deregulation of certain B7 family genes, and only B7-H6 amplification was significantly associated with worse OS. In a further experimental study (Jeschke et al 2017), DNA methylation markers were profiled to identify a methylation of tumour-infiltrating lymphocyte (MeTIL) signature. The MeTIL signature measured TIL distribution in a sensitive way and predicted improved survival and response to chemotherapy in breast cancer better than the histopathological evaluation of TILs or gene expression-based immune markers. Tables 2A-B summarize the prognostic role of proliferation and immune signatures.

Relationships between tumour growth and immune evasion

Experimental studies. An increasing number of recent experimental studies have reported proliferation and tumour growth to be closely linked to immune evasion in breast cancer. A few of them are briefly described here. In one experimental study, tumour growth was found to be largely COX-dependent through immune evasion, thus supporting COX activity as a driver of immune suppression (Zelenay et al 2015). This observation has been confirmed by another experimental investigation, where mammary carcinoma cell-derived COX 2 was found to suppress tumour immune surveillance by enhancing intra-tumoral immune checkpoint activity. In the same study,
the examined v-erb-b2 avian erythroblastic leukaemia viral oncogene homolog 2 (ErbB2) transgenic mice that were deficient in mammary epithelial cell COX-2 (COX-2 MEC-KO mice) showed the decreased expression of Ki 67, a proliferation marker, and contained more CD4+ Th cells and CD8+ cytotoxic immune cells compared with wild-type mice, indicating enhanced immune surveillance. Moreover, in ErbB2-transformed mouse breast cancer cells, where lentiviral shRNA delivery was used to knock down COX-2, growth was strongly suppressed (Markosyan et al 2013). The results of another study (Loi et al 2016) suggested that Ras–mytogen-activated protein kinase (MAPK) pathway activation induces immune evasion in TNBC. In particular, genetic or transcriptomic alterations in Ras–MAPK signalling were significantly correlated with lower TILs. Moreover, MEK inhibition upregulated cell surface MHC expression and PD-L1 in TNBC cells both in vivo and in vitro. In a different study (Zhang H et al 2015), hypoxia-inducible factor 1 directly upregulated the transcription of CD47 expression in breast cancer cells, promoting the evasion of phagocytosis by macrophages and the maintenance of CSCs. In another study (Olesch et al 2015), microsomal prostaglandin E synthase (mPGES-1/2) in human and mouse models of breast cancer was shown to favour immune evasion. In addition, mPGES-1 inhibition increased CD80 expression by tumour-associated phagocytes, which triggered cytotoxic T cell activation and restricted tumour growth. A downregulation of miR-148a, which is closely involved in cancer cell proliferation, has been reported in both ER-positive breast cancer and TNBC. An experimental investigation (Tao et al 2014) has validated the hypothesis that E2 downregulates miR-148a through C protein-coupled oestrogen receptor-1 (GPER) and that E2 also affects the expression of HLA-G, which is an miR-148a target gene. Therefore, a new mechanism based on the ability of oestrogenic GPER signalling to trigger HLA-G expression through the inhibition of miR-148a, which supports immune evasion in breast cancer, has been elucidated. Epidermal growth factor receptor (EGFR) signalling is often dysregulated in TNBC and is also associated with increased glycolysis. A study focused on these aspects (Lim et al 2016) showed that the increased aerobic glycolysis induced by EGFR signalling in TNBC promotes cell proliferation and tumour growth accompanied by immune escape. In a MMTV-HER2/neu mouse mammary tumour initiating cells (TICs) model, a 17-gene HER2-TIC-enriched signature (HTICS) predicted the clinical outcome in multiple independent HER2+ cohorts. Four of the eight upregulated genes in HTICS were involved directly in cell cycle progression, DNA replication and mitosis. The upregulation of these genes was concomitant with the downregulation of genes involved in immune response, thus favouring immune evasion (Liu JC et al 2012).
Clinical studies in metastatic breast cancer with an undetectable or detectable-non-growing tumour burden following conventional therapy.

In the last decade, we have reported very promising results (Nicolini and Carpi 2005; Nicolini et al 2005; Nicolini et al 2007; Nicolini et al 2008) and the possible rationale (Nicolini et al 2006; Nicolini and Carpi 2009) of an open pilot clinical study using a new schedule of conventional anti-oestrogen therapy combined with immune stimulation. We have more times published both results (Nicolini and Carpi 2005; Nicolini et al 2014a) and their interpretation (Nicolini et al 2014b; Nicolini et al 2015; Nicolini et al 2016). Progression-free survival (PFS) and median OS times since the diagnosis of distant metastases in 31 endocrine-dependent breast cancer patients were 33 and 94 months, respectively. In 24 of these patients with high levels of hormone dependency [55% of ER-positive progesterone (Pgr)-positive], the median OS was 98.5 months and the delayed median PFS was 45 months compared with those in the remaining seven subjects with lower hormone dependency (20% of ER+ Pgr+), in whom the median OS was 37 months and the median PFS was 20 months. It is noteworthy that 16% of the patients have survived for more than 10 years in complete remission. The following mechanistic interpretation has been proposed: ‘within the TME, stromal cells, infiltrating lymphocytes and tumour cells foster tumour growth and immune evasion through a complex network of autocrine and paracrine loops mediated by cytokines and growth factors’. In an anti-oestrogen-responsive metastatic disease, a stable or decreased tumour burden and a lower genetic instability due to quiescent state (G0-G1 state) of tumour cells are also likely to reduce immune evasion by the downregulation of immune escape and immune inhibition. This favors the immune attack stimulated by the sequential administration of IFN-β and IL-2, which, by synergizing with anti-oestrogen therapy, can delay hormone resistance and clinically result into a prolonged response or stable disease. The larger the tumour working portion, the higher its biological aggressiveness. In those with shorter median survival and a likely lower hormone dependence, an expected greater and more aggressive portion of the tumour burden worked with a higher production of cytokines and growth factors. This can explain the earlier occurrence and perhaps more effective immune inhibition during the progression of metastatic disease in those surviving for <5 years. On the other hand, in those surviving for >5 years and with higher levels of hormone dependence, an expected smaller and probably less aggressive portion of the tumour burden worked with lower production of cytokines and growth factors. This may have permitted the immune system to work more effectively for a longer time. Moreover, during the clinical benefit, we showed that ‘laboratory evidence of the effect of immunotherapy as well as that hormone
resistance occurs at the progression of the disease concomitantly with a laboratory pattern compatible with immune inhibition’ (Nicolini et al 2007; Nicolini et al 2016). We have recently published the last updating on the issue (Nicolini et al 2018). In another pilot study, a maintenance immunotherapy with low-dose IL2 and 13-cis retinoic acid was administered to 100 consecutive metastatic breast cancer patients with a clinical benefit (complete response (CR)+ partial response (PR)+ stable disease (SD)) from 6–8 courses of induction chemotherapy. There were 68 ER+ and/or Pgr+ patients, all of whom, after the induction of chemotherapy, received endocrine-therapy with luteinizing hormone-releasing hormone (LHRH) analogues or letrozole in addition to the maintenance immunotherapy according to whether they were pre- or post-menopausal. In the 100 patients, the median PFS and OS were 37.1 and 57.5 months, respectively. PFS and OS were 44.7 and 64.5, respectively, in the 68 patients with ER+ tumours compared with 32.7 and 51.4, respectively, in the 23 patients with ER− tumours (Recchia et al 2008). In this study, the authors highlighted ‘a sustained improvement in lymphocytes, NKs and CD4+/CD8+ ratio with respect to baseline values’. In an earlier pilot study from the same research group, a maintenance immunotherapy with IFN-β, retinyl palmitate and tamoxifen until progression was administered to 23 metastatic breast cancer patients who had achieved a clinical response (11 CR and 12 PR) following six courses of induction and two successive courses of consolidation chemotherapy. All the 23 patients were unresponsive to the hormonal therapy. The PFS and OS were 31.4 and 44 months, respectively, in the 23 responders (CR+PR). The OS was 66 months (with 9-year survival rate of 34%) in the 11 complete responders and 17 months in the non-responders (seven with SD and six with progressive disease) (Recchia et al 1998). The reported median OS in ER+ Her2-negative metastatic breast cancer is 25 months (Savci-Heijink et al 2015) to 30.6 months (Zielinski et al 2016). In these last two pilot studies, the median OS was longer than expected in similar populations and was even longer in the two subsets of ER+ and complete responders (64.5 and 66 months, respectively). However, in these two subsets, the median OS was shorter than just reported (94 months). Indeed, in our pilot study, all the patients were selected because they showed a clinical benefit during anti-oestrogen therapy before receiving the additional immune therapy (Nicolini and Carpi 2005). In the first of the last two pilot studies, all the 68 ER+ patients received maintenance immunotherapy without any previous clinical evaluation of response to the hormone therapy. In the latter, all 23 of the recruited patients were unresponsive to the hormone therapy. In these two last pilot studies, the authors clearly refer to the well-documented immune modulation in addition to the anti-proliferative action by IFNs and retinoids. In particular, the capacity of retinoids to increase the number of IL-2 receptors and peripheral blood lymphoid cells expressing the surface markers of Th cells (Prabhala et al 1991) is mentioned. Moreover, the function of retinoids, which is to facilitate
the differentiation of immature myeloid suppressor cells (Gr1+ CD115−), is reported (Huang et al 2006). The biological activity of retinoids is mediated by nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which are ligand-activated transcription factors and are basally present in breast cancer cells. More recently, the relevant role of retinoids for breast cancer chemoprevention and treatment because of their ability to induce cell differentiation and growth suppression (Garattini et al 2014; Seo et al 2015) has been highlighted. Although ER+ breast tumours are also RAR-sensitive and are mainly activated by all trans-retinoic acid and 13-cis-retinoic acid (Garattini et al 2014), RXRs are critical for the growth of ER− breast cancer cells (Uray and Brown 2011) and are targeted by a special class of retinoids called rexinoids (Uray and Brown 2011; Seo et al 2015). Table 3 summarizes the data from these three studies. Another study (Greenberg et al 1996) has reported the long-term outcome of 1581 metastatic breast cancer patients from 18 successive front-line trials conducted from 1973 to 1982 at the University of Texas, MD Anderson Cancer Center. All the patients received induction-phase and maintenance chemotherapy that was usually continued for 2 years. The analysis identified 26 (1.6%) patients who were potentially cured among the 1581 evaluated individuals. In fact, they remained in first complete remission after a median duration of 191 months. All 26 of these patients participated along with 263 subjects who had achieved complete remission on anthracycline–cyclophosphamide-based front-line chemotherapy; comparison of the 26 patients with the overall 263 complete responders and total patient populations showed that they had an initially lower tumour burden. In the above-described studies, a longer than expected clinical benefit and OS were observed in patients with or without an immune modulation and/or active immune stimulation following response to the conventional anti-proliferative treatment: anti-oestrogens or conventional cytotoxic chemotherapy. In all of these studies, a low tumour burden was associated with a better clinical outcome; in some of these studies, laboratory data showed that immune therapy stimulated the immune response.

Clinical studies in lung, ovarian and colorectal cancers. Here, we summarize clinical studies in advanced cancers other than breast cancer, which contribute to the understanding of the relationships of tumour burden with clinical outcome and likely with immune surveillance (Table 4). The studies can be clustered into two groups. The first group includes studies on a population of patients showing a clinical benefit (CR+PR+SD) following conventional chemotherapy. The other group includes studies on patients with an undetectable residual metastatic disease following radical resection.
Patients with an undetectable or detectable-non-growing metastatic tumour burden following conventional chemotherapy. In this sub-group, three studies conducted by Recchia et al. can be included. In two phase II studies on advanced ovarian and advanced non-small-cell lung cancer, IL-2 and 13-cis retinoic acid, respectively, were given as maintenance therapy to patients responsive to and with measurable metastatic disease after conventional chemotherapy. In the advanced ovarian cancer trial, 96% of the patients receiving immune maintenance treatment were responders, 64% of whom showed CR (Recchia et al 2005); in the other study (Recchia et al 2006), 53% of the patients were the responders, only 6% of whom showed CR. In both studies, the remaining patients had stable disease. The above-described immune maintenance treatment was cyclically self-administered by educated patients and 2 months was considered to represent a single cycle of therapy. The PFS and OS were the secondary end-points for comparison with historical data from controls who were chosen to perfectly match the patients in the study group. The PFS and OS curves showed a statistically significant improvement in IL-2/13-cis retinoic acid-treated patients. For example, in the advanced ovarian cancer trial, the median PFS and OS were 50.5 and 102.5 months, respectively, in the study group compared with 15.5 and 29.6 months, respectively, in the controls. In the advanced non-small-cell lung cancer trial, the median PFS and OS were 16.5 and 23.4 months, respectively, in the study group compared with 8.4 and 11.8 months, respectively, in the controls. Another investigation using the same schedule of immunotherapy was conducted by the same research group in metastatic colorectal cancer patients who had a clinical benefit (CR+PR+SD) from induction chemotherapy (Recchia et al 2006). In this study, 25% were complete responders. After a median follow-up of 36 months, the median PFS was 27.8 months in the 40 recruited patients of the study group and 12.5 months in the 80 controls. The median OS was 52.9 in the study group and 20.2 in the controls. In all the three pilot trials, the number of total lymphocytes, NK cells, CD4+/CD8+ ratio and VEGF were determined in the peripheral blood of both the studied patients and controls. At baseline in all three studies, none of the evaluated immunological parameters in the two treatment groups (studied patients and controls) were statistically different. In the ovarian cancer study, a progressive increase in the lymphocyte count in IL-2-treated patients and a progressive decrease in the controls was observed, and the difference after 1 year became statistically significant in both the lung and colorectal cancer studies (p < 0.01 and p < 0.0001, respectively). Similarly, the NK cells increased in IL-2-treated patients compared with that in the controls, and after 1 and 2 years (ovarian cancer study) or 1 year (lung and colorectal cancer studies) the difference became statistically significant (p = 0.03 and p = 0.0007 for ovarian cancer; p = 0.04 and p < 0.0001 for lung and colorectal cancer, respectively). Again, in all the three studies, the CD4+/CD8+ ratio values increased in IL2-treated patients and decreased in
controls; after 1 and 2 years (ovarian cancer study) or 1 year (lung and colorectal cancer studies), the difference became statistically significant within the same group before and after maintenance immunotherapy and between them (IL-2-treated vs. controls). Finally, in all the three studies, the baseline VEGF values of IL-2-treated patients showed a statistically significant decrease after 1 year (lung cancer study, p = 0.0002). In the other two studies, the same significant decrease was maintained at 1 and 2 years (ovarian cancer study, p < 0.0049; colorectal cancer study, p < 0.0001).

Unlike in responding patients, in those progressing during conventional chemotherapy immunotherapy is more likely unsuccessful. In fact, in one of two recently published clinical trials conducted in advanced non-small cell lung cancer using immune checkpoint (CTL antigen 4, PD-1 and PD L1) inhibitors, a 30% increase in PFS (3.5 vs. 2.8 months) has been reported compared with the conventional chemotherapy (Brahmer et al 2015); in the other, without PFS improvement, there was a 30% increase in the OS (12.2 vs. 9.4 months) (Borghaei et al 2015). In the same target population of advanced non-small cell lung cancer in the previously mentioned experimental trial conducted by Recchia et al. (Recchia et al 2006), the significant PFS and OS improvement observed in patients treated with IL-2 and 13-cis retinoic acid maintenance therapy was 100% (8.4 vs. 16.5 months and 11.8 vs. 23.4 months, respectively) compared with that in the controls. However, in the pilot study by Recchia et al., the patients were recruited in clinical benefit, whereas in those conducted with the new immunological drugs, the patients were recruited when they were in progression following previous conventional therapy. Thus, this different condition at the time of recruitment may have substantially affected the outcome. Overall, these findings suggest that an appropriate immune maintenance therapy can significantly improve the clinical outcome of patients with a detectable-non-growing metastatic tumour burden. This improvement seems to be correlated with the proportion of complete responders to conventional chemotherapy, i.e., with the tumour burden.

Patients with gastrointestinal (GI) cancer and an undetectable residual metastatic disease following radical surgery. In a pilot study conducted by a group (Nicolini et al 2010) in patients with GI cancers who were apparently disease-free after primary surgery and had a high risk of relapse due to residual undetectable metastases, an almost double 5-year disease-free survival (DFS; 80.4%) and OS (87.1%) was reported compared with the expected. In this study, starting from the first year after conventional adjuvant chemotherapy till the fifth year, patients received 2-3 cycles of additional adjuvant chemotherapy using infusional 5- fluorouracil (FU) plus leucovorin. Moreover, it has been reported that 22%-27% of colorectal cancer patients are 10-year survivors following radical resection of synchronous or metachronous liver metastases without any adjuvant
chemotherapy (Fong et al 1999; Scheele et al 1990). Conventional adjuvant or neoadjuvant chemotherapy commonly with infusional 5-FU plus leucovorin significantly increased the 5-year DFS rate from 27%–42% to 37%–46% (Portier et al 2006; Mitry et al 2008; Liu et al 2016). It is noteworthy that the extension of primary tumour and liver recurrences were among the most significant predictors of worse prognosis in all the trials and that only about 20% of colorectal cancer patients are chemosensitive to infusional 5-FU plus leucovorin (Nicolini et al 1998). In patients with an undetectable minimal residual disease, an induced or spontaneous recovery of the immune surveillance can be predicted. An undetectable or detectable-non-growing tumour burden following conventional chemotherapy and an undetectable minimal residual metastatic disease following radical surgery are more suitable conditions for immune manipulation. The former condition, which likely occurred in our study and other mentioned pilot studies, benefited from an actively induced immune stimulation or immune maintenance therapy that improved the clinical outcome. Interestingly, in this condition, a prolonged ‘resting state’ (G0-G1 state) was likely due to the hormone therapy allowing more efficacious immune manipulation and better clinical outcome.

In addition to the already mentioned immune-modulating properties of retinoids and preclinical evidence (Moon et al 1983; Sporn and Roberts 1983) of their key role in controlling normal cellular proliferation and differentiation, it is well known that IL-2 is the principal growth factor for lymphocytes (Nicolini et al 2006). In addition, there is clinical evidence (The Nordic Myeloma Study Group 1996; Frasci et al 1993; Lippman et al 1992) supporting the efficacy of IFN therapy combined with conventional chemotherapy or retinoids in the settings of locally advanced or minimal residual disease of breast and other types of cancer. In the latter case, the reduction by surgical removal and/or a conventional anti-proliferative therapy of a previously well-detectable and extended cancer to minimally undetectable residual metastatic disease could have favoured the spontaneous recovery of the immune-surveillance. This maintained a small fraction of these patients in the disease-free and potentially healthy state. Lower residual metastatic tumour burden, chemosensitivity and other not yet well-understood reasons likely led to the selection of this small fraction of patients. This suggests that tumour burden and proliferation directly correlate with immune evasion and with the complexity of the activated network that sustains each cancer. The concept of a link between tumour burden and immune tolerance is also gaining acceptance within the scientific community (Clifton et al 2015; Migali et al 2016; Cimino-Mathews et al 2015).
A refined pathobiological model and a novel therapeutic strategy

In prolonged ‘resting state’ (G0-G1 state) non-growing condition during anti-oestrogen therapy (Doisneau-Sixou et al 2003; Osborne 1994; Wolf et al 1994) or in the ‘minimal residual metastatic disease’ molecular pathways promoting invasion and diffusion, angiogenesis and reprogramming energy and metabolism are likely downregulated, without clinical relevance. This is consistent with the finding that the rate of definitely cured patients after adjuvant therapy and/or primary operation is inversely correlated with tumour size at diagnosis. Accordingly, angiogenesis and metastatic processes are strictly linked to the progression of cancer and the shift from an oxidative to glycolytic metabolism, mainly through the ‘Warburg effect’, is favoured by hypoxia concomitant with tumour growth. Therefore, following these and the previously reported data and concepts about the relationship between tumour growth and immune evasion, we propose to refine the pathobiological model by Hanahan and Weinberg, as shown in Figure 1. By this model, long-term active anti-proliferative therapies and minimal residual disease are the conditions mostly favouring an efficacious immune manipulation.

Endocrine-dependent cancers

Recently, the genetic background of proliferation-promoting and -inhibiting action of oestrogens and anti-oestrogens, respectively, have been evaluated in depth, and the immunosuppressive function of sex hormones has been largely documented. These findings are summarized below.

ER-α-regulated genes in MCF-7 human breast cancer cell lines increase tumour growth. ER-α is a transcription factor that regulates many genes that play important roles in physiology and are involved also in the development and progression of breast cancer. MCF-7 cells have shown that although ER-α interacts with thousands of genomic regions, E2-responsive genes range from 100 to 1500 (Charpentier et al 2000; Coser et al 2003; Frasor et al 2003; Carroll et al 2006; Kininis et al 2007; Lin et al 2007). In previous study, Frasor et al. (Frasor et al 2003) summarized their findings and stated that ‘many genes whose expression is altered by E2 are associated with specific cell signalling pathways and regulatory factor-receptor loops. These include a general upregulation of positive proliferation regulators and the downregulation of negative proliferation regulators, which together may contribute to the overall stimulation of proliferation and suppression of apoptosis’.

Welboren used ChIP-Seq to map ER-α-binding sites and to profile changes in RNA polymerase II occupancy in MCF-7 cells in response to E2, tamoxifen or fulvestrant (Welboren et al 2009).
Overall, 1256 genes and five different clusters of genes were identified. In particular, many genes encoded proteins binding the nucleus and RNA binding the mitochondrion. Moreover, E2 induced the downregulation of pro-apoptotic genes Bad, Bak, Bik, and cyclin A and of genes involved in cell cycle arrest or proliferation, such as cyclin G2, a negative regulator of the cell cycle that maintains cells in a quiescent state. Cyclin D1 and IGF-binding protein 4 were the other regulated genes governing cell proliferation and growth. In another more recent study (Hah et al 2011), the authors demonstrated ‘a potent effect of E2 signalling on the protein biosynthetic machinery, which fits well with the known mitogenic effects of E2 on MCF-7 cells’ and highlighted that ‘E2 signalling has strong, immediate and likely direct effects on transcription by all three RNA polymerases’. In a research study (Kininis et al 2007) aimed at exploring the global mechanisms of oestrogen-regulated transcription, the authors reported that ‘many of these direct E2 target genes exhibit interesting modes of regulation and biological activities, some of which may be relevant to onset and proliferation of breast cancers (e.g. UGT2B15, CYP1B1 and PRUSE)’. In a review article following the above report, the same authors (Hah and Kraus 2014) concluded that ‘the most immediate effects of oestrogen signalling on the genome results in the regulation of mRNAs encoding proteins involved in transcription, nucleic acid metabolism, and G protein-coupled and cell surface signalling. Thus, oestrogen signalling propagates the hormone-dependent transcriptional response, leading to secondary and sustained effects. Over the long term, oestrogen signalling upregulates the protein biosynthesis machinery. This is likely how the oestrogen signalling pathway prepares the cell for translation of the mRNAs that are newly synthesized in response to oestrogen signalling. The immediate and sustained effects of oestrogen signalling underlie the mitogenic effects of oestrogen signalling in breast cancers’ (Figure 2).

Tamoxifen inhibits most ER-α-mediated proliferation genes. In a study conducted by Frasor et al. (Frasor et al 2004), the effects of different SERMs were investigated in MCF-7 cells. Based on the results, the authors stated that ‘it is apparent that many of the genes on which the SERMs act as antagonists could affect cell proliferation’ and that ‘their ability to block the E2 stimulation of cell proliferation suggests that the genes they antagonize are those that are essential for the stimulatory effect of E2 on cell proliferation’. They concluded that ‘it is of interest that several of these genes have potential tumour suppressor or anti-proliferative activities in breast cancer cells and could contribute to the beneficial effects of trans-hydroxy-tamoxifen’. A successive investigation by the same author focused on genes not or minimally regulated by E2 and preferentially regulated by tamoxifen in ER-α-positive MCF-7 human breast cancer cells (Frasor et al 2006). Among the 64 genes preferentially regulated by tamoxifen (50 up- and 14 downregulated) were PKIA, an inhibitor
of cyclic AMP-dependent protein kinase A activity; PTPRG, a receptor-type protein tyrosine phosphatase; and SOCS1, an inhibitor of JAK/STAT signalling; PTPRG and SOCS1 have potential tumour suppressor roles. All have the capacity ‘to alter different cellular signalling pathways and, thus, responsiveness of breast cancer cells to other hormones, growth factors, or cytokines. In addition, IEX1 has been shown to have growth-inhibitory effects suggestive of a beneficial effect of tamoxifen.’ Two tamoxifen upregulated genes, namely YWHAZ and LOC441453, showed significant association with disease recurrence. YWHAZ likely plays a relevant role in insulin receptor and epidermal growth factor receptor signalling and in cell cycle regulation. Overall, the findings reported in the two studies by Frasor were confirmed in another study by Welboren et al. (Welboren et al 2009). In this successive research, most of the E2-upregulated genes were antagonized by tamoxifen, which mainly showed agonistic behaviour on E2-downregulated genes. In addition, a group of genes was the only target of tamoxifen. Table 5 summarizes the gene ontology of tamoxifen ER-α-mediated genes assessed in some principal studies. The studies performed recently and mentioned above show that genes that are mostly affected by oestrogens and anti-oestrogens are proliferation genes.

Sex hormones and the immune response. Reportedly, testosterone has a general suppressive effect on the immune function (Roved et al 2017). In particular, testosterone has dampening effects on many innate immune cells (monocytes, macrophages, dendritic cells (DCs), granulocytes, NK cells, platelets and endothelial cells) and in DCs, it may also downregulate the expression of MHC class II receptors and co-stimulatory molecules (Hepworth et al 2010; Koh et al 2009). Regarding adaptive immunity, its action on type 1 (Th1) response is uncertain, whereas a significant decrease in type 2 and Th17-induced immune responses due to the suppression of functions associated with Th2 and Th17 differentiation has been reported (Hepworth et al 2010; Kissick et al 2014; Yao et al 2013). Th1 cells mostly activate macrophages and CD8+ CTLs, whereas Th2 cells mainly stimulate B cells to produce antibodies and Th17 cells to produce inflammatory cytokines (particularly IL-17 and IL-22) (Murphy and Weaver 2016). Regarding estrogens and adaptive immunity, Foo et al. in a meta-analysis of 38 studies (Foo et al 2017) found that oestrogens induce positive effects on humoral immunity but a significantly decreased effect on cell mediated immunity. In particular, oestrogens shift adaptive immune responses in favour of type 2 immune responses (Faas et al 2000), whereas type 1 and Th-17 responses are suppressed (Chen RY et al 2015; Chen LC et al 2015; Tyagi et al 2012; Wang C et al 2009). Data suggesting the promoting or inhibiting role of Th17 and IL-17 on tumorigenesis have been reported. Some findings (Alinejad et al 2017) have suggested that through activation of the ERK1/ERK2, NF-kb and BCL-2 pathways, the IL-17B/IL-
17RB system promotes inflammation, breast cancer progression as well as resistance to chemotherapy drugs (Alinejad et al 2016). Conversely, some findings have shown that IL-17 significantly induce MDSC differentiation, inhibit their proliferation and trigger apoptosis through the JAK/STAT3 pathway in vitro (Ma et al 2018), whereas other findings (Kryczek et al 2009; Benchetrit et al 2002) have supported an anti-tumour effect against certain tumours. A recent review (Rothenberger et al 2018) focusing on the role of the oestrogen pathway in the TME has confirmed that oestrogen promotes immune suppression through the modulation of pro-tumour responses independent of direct activity on tumour cells (Figure 2). In particular, data have suggested that oestrogens in the TME ‘shift the balance in favour of Th2 responses, production of tumour-promoting cytokines (IL-6, IL-4, TNF-α and IL-17A) and M2 TAM infiltration compared to the Th1 responses, associated Th1 cytokines (IL-12 and IFNγ) and M1 TAM infiltration’. Moreover, oestrogens are likely ‘to promote tumour immune evasion through the proliferation of Treg cell and MDSC populations, augmented tumour cell PD-L1 expression and inhibition of CD8+ T cell- and NK cell-induced apoptosis. In addition, CAFs may support the TME by providing paracrine sources of oestrogens and IL-6’. All these studies have clarified that many genes and, consequently, multiple molecular pathways whether directly or indirectly involved in breast cancer proliferation are induced by oestrogens and inhibited by anti-oestrogens. This, in addition to the relevant role of anti-oestrogens in inhibiting an immune-suppressive TME, promoted by oestrogens, makes them ideal candidates in the battle against cancer either alone or in combination with other drugs (Rothenberger et al 2018).

Locally advanced or metastatic disease. A successful immune manipulation is more probable in patients with metastatic disease in clinical benefit during anti-oestrogen therapy. In fact, anti-oestrogens, by directly acting on multiple genes or indirectly inhibiting proliferation, promote a prolonged ‘resting state’ (G0-G1 state) (Doisneau-Sixou et al 2003; Osborne 1994; Wolf et al 1994) concomitant with a non-growing tumour (clinical benefit) or a decrease in tumour burden to ‘minimal residual metastatic disease’ (CR). The probable concomitant downregulation of the multiple mechanisms responsible for immune tolerance permit an active immune modulation/stimulation, which, as reported above in the work of our group and other authors, significantly prolong the PFS and/or OS. Thus, first, in ER-positive metastatic breast cancers, the same schedules of immune-modulatory/stimulatory treatments combined with anti-oestrogens should be validated in large prospective randomized trials. Moreover, in the same population of ER-positive patients but with locally advanced cancer, they should be investigated as adjuvant treatments. The combination of hormone therapy with immunotherapy could be considered for the
same duration for which conventional anti-oestrogens are currently recommended (5–7 years). It
can be inferred that by replacing conventional anti-oestrogens with anti-androgens, the same
schedules of hormone immune therapies proposed in endocrine-dependent breast cancer could be
evaluated in metastatic and locally advanced hormone-dependent prostate cancers.

Endocrine-independent cancers at high risk of relapse

Locally advanced. In endocrine-dependent cancers with de novo or acquired hormone resistance
and other types of high risk endocrine-independent solid cancers (gastrointestinal, lung and ovary),
it is currently unproven and unlikely to obtain a prolonged ‘resting state’ of resistant cancer cells. In
these patients, conventional anti-proliferative drugs and/or therapeutic interventions (surgical and/or
radiological) should aim to decrease the tumour burden as much as possible. In patients with locally
advanced cancer and postoperative minimally residual disease, the analysis of proliferation markers
Ki 67 and p120 have shown that approximately 16% of disseminated tumour cells are in an active
cell cycle, whereas the majority remain arrested in G0 phase (Pantel et al 1993). Tumour dormancy
and the concomitant multi-drug resistance in the chemosensitivity test can explain the frequent
inefficacy of adjuvant chemotherapy. In dormant cells, a gradual proliferation can be triggered by
the changes in their microenvironment and/or the acquisition of additional genetic ‘hits’ (Kostler et
al 2000). Thus, in these patients, in the initial 6–8 months after conventional adjuvant chemotherapy
and/or radical resection, regular administration of a few cycles (3-4) of anti-proliferative drugs with
concomitant immune-modulating properties, such as taxanes or anti-metabolites (5-FU,
capcitabine), at low doses every 8–12 months for a few years can be considered (Nicolini et al
2010). In fact, it is likely that at least a few months are needed for residual resistant cancer cells to
grow and activate the multiple concomitant mechanisms necessary to mount a significant immune
tolerance in these patients. Thus, the main aim of this additional adjuvant chemotherapy is to
interrupt the probable ‘works in progress’. In fact, this procedure is expected to gradually switch off
the mechanisms triggering the proliferation of residual resistant cancer cells and the concomitant
immune evasion. Paclitaxel administered once a week for 2 weeks every 3 weeks or 5-FU infusion
administered for 5 days every 28 days (1 cycle) blocks MDSC expansion in addition to the well-
known anti-proliferative effect (Sevko et al 2012). Low dose of paclitaxel also has been reported to
simultaneously inhibit Treg cells (Sevko et al 2012) and exert immune-stimulatory effects on DCs
at ultra-low doses (Shurin et al 2009). On the other hand, 5-FU can counter one or more ways of
immune attack evasion by tumour cells, such as downregulation of MHC class I antigens, loss of
Fas expression and shedding of tumour antigens (Khallouf et al 2012). Potentially synergizing
immune drugs, such as IFNs, IL-2 and retinoids, can be included. These therapeutic schedules could significantly delay or decrease recurrences, as shown in GI cancers (Nicolini et al 2010).

Metastasis in clinical benefit during conventional therapy. In cases of resistant/non-endocrine-dependent breast and other types of solid cancers in clinical benefit (CR+PR+SD) following conventional therapy, immune maintenance therapy (immunomodulatory/stimulating drugs) can be attempted to delay the regrowth of tumour. In these cases, provided that immune tolerance is directly correlated with tumour burden (Migali et al 2016), more therapeutic effect is expected when immune manipulation follows CR or PR rather than SD (Recchia et al 2005; Recchia et al 2006; Recchia et al 2007). Table 6 summarizes these proposals.

A virtual equilibrium line between tumour burden and the immune system

In patients with undetectable or prolonged quiescent/non-growing state of residual cancer cells, the efficacy of the immune response (cure of the disease or delay of the occurrence of resistance to therapy) can be determined by the level at which a virtual equilibrium line is positioned by the opposite activities of cancer and immune cells. In fact, under these conditions, the tumour burden is balanced between the actions from residual resistant cancer cells and the spontaneously or actively modulated/stimulated immune response. The pressure exerted by the resistant proliferating cancer cells can be successfully or not counterbalanced by the immune response. When a complete response occurs, it is likely that the spontaneously induced or actively stimulated immune system prevails so that most or all of the residual resistant cancer cells are eliminated and the equilibrium line is positioned at level 0. When a partial response occurs, the immune system is likely to inhibit the formation of or eliminate any new cancer cells and only some of the residual resistant cancer cells; therefore, the line is positioned at level $>0<1$. When a stable disease occurs, a perfect equilibrium is established and the immune system inhibits the formation of or eliminates any new cancer cells, but not residual resistant cancer cells, and the line remains at level 1. In case of a disease progression, the activated immune system likely eliminates new cancer cells less than they are derived from the residual resistant cancer cells, and the equilibrium line is positioned at level $>1$. Any factor that affects the immune activation, the amount of residual resistant cancer cells and their phenotype can change the position of the equilibrium line (Figure 3).
Conclusions

In this article, the recent major advances acquired in molecular biology of cancer were the premise to discuss the apparent discrepancy in the poor prognosis of advanced breast cancer. In reality, it has become increasingly clear that advanced cancer, especially advanced breast cancer, is an entirely displayed pathological system that is much more complex than previously considered. Thus, the understanding of cancer is a work in continuous progress. General rules governing the entirely displayed pathological cancer system, if existing, should be identified. Experimental and clinical studies on breast cancer and other types of cancer support the notion of a close relationship between tumour growth and immune evasion. Based on these findings, we propose a novel therapeutic strategy and a refined pathobiological model for advanced breast and other solid cancers. The novel therapeutic strategy is based on therapeutically induced conditions (undetectable tumour burden or a prolonged tumour ‘resting state’), which enable an efficacious immune response in advanced breast and other types of solid cancers.

Acknowledgements

To Emilio Cardaci, Informatic service, Department of Medical Area, University of Pisa, Dr. Sara Russo, graduated in Architecture and Luigi Nicolini, for the technical assistance in the preparation of Figure 1. Authors also are thankful to Professor Vikas P. Sukhatme from Harvard Medical School, Boston, MA, for useful discussions.

Declaration of interest

Authors declare no conflict of interest.

Funding

No funding.
References


Bouker KB, Skaar TC, Fernandez DR, O'Brien KA, Riggins RB, Cao D, Clarke R 2014 Interferon regulatory factor-1 mediates the proapoptotic but not cell cycle arrest effects of the steroidal antiestrogen ICI 182,780 (faslodex, fulvestrant). Cancer Res. 64(11):4030-9.


Clevers H 2004 At the crossroads of inflammation and cancer. Cell 118(6) 671-674.


Couse JF, Korach KS 1999 Estrogen receptor null mice: what have we learned and where will they lead us? Endocr Rev 20(3) 358-417.


Davies MA, Samuels Y 2010 Analysis of the genome to personalize therapy for melanoma. Oncogene 29(41) 5545-5555.


Gameiro SR, Malamas AS, Tsang KY, Ferrone S, Hodge JW 2016 Inhibitors of histone deacetylase I reverse the immune evasion phenotype to enhance T-cell mediated lysis of prostate and breast carcinoma cells. Oncotarget 7(7) 7390-7402.


Huang B, Pan PY, Li Q, Sato AI, Levy DE, Bromberg J, Divino CM, Chen SH 2006 Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. Cancer Res 66(2) 1123-1131.


Kiraz, Y 2016 Major apoptotic mechanisms and genes involved in apoptosis. Tumour Biol 8471-8486.


Ma M, Huang W, Kong D 2018 IL-17 inhibits the accumulation of myeloid-derived suppressor cells in breast cancer via activating STAT3. Int Immunopharmacol 59:148-156.


Nicolini A, Carpi A, Ferrari P, Sagripanti A, Anselmi L 1998 A multistep therapy with subcutaneous low dose recombinant interleukin-2, 5-fluorouracil and leucovorin prolongs the


Recombinant leukocyte A interferon in advanced breast cancer. Results of a phase II efficacy trial.

al 2016 Critical Role of AMPK/FoxO3A Axis in Globular Adiponectin-Induced Cell Cycle Arrest
and Apoptosis in Cancer Cells. J Cell Physiol 231(2) 357-369.

Shurin GV, Tourkova IL, Kaneno R, Shurin MR 2009 Chemotherapeutic agents in noncytotoxic
concentrations increase antigen presentation by dendritic cells via an IL-12-dependent mechanism.
J Immunol 183(1) 137-144.


Smithson LJ, Anastasaki C, Chen R, Toonen JA, Williams SB, Gutmann DH 2016 Contextual

by RNAi-mediation inhibits proliferation and growth in MDA-MB-231 breast cancer cells: an in

Sporn MB, Roberts AB 1983 Role of retinoids in differentiation and carcinogenesis. Cancer Res
43(7) 3034-3040.

Steggerda SM, Bennett MK, Chen J, Emberley E, Huang T, Janes JR, Li W, MacKinnon AL,
Makkouk A, Marguier G, et al 2017 Inhibition of arginase by CB-1158 blocks myeloid cell-

Stephens PJ, Tarpey PS, Davies H, Van Loo P, Greenman C, Wedge DC, Nik-Zainal S, Martin S,
Varela I, Bignell GR, et al 2012 The landscape of cancer genes and mutational processes in breast

Tao S, He H, Chen Q, Yue W 2014 GPER mediated estradiol reduces miR-148a to promote HLA-G
expression in breast cancer. Biochem Biophys Res Commun 451(1) 74-78.


Figure legends

Figure 1. 
A refined patho-biological model for advanced breast cancer, relationship of tumor growth with immune evasion. 
(A-B) Any sphere represents an activated subcircuit sustained by a pathological molecular network (short arrows) converging to the hallmarks (long arrows). The different activated pathological molecular networks at least in part overlap each other (in shadow areas); the square represents the signaling (grid) from a supportive tumor microenvironment cross-talking with the subcircuits; both pentagon and triangle represent active genomic instability and inflammation (additional integrated hallmarks). (A1-B1) Any sphere represent a downregulated subcircuit; the different pathological molecular networks at least in part overlap each other (in shadow areas); the square represents the down regulated cross-talk of the microenvironment (grid) with the subcircuits; both pentagon and triangle represent the downregulated genomic instability and inflammation.

Figure 2. Effect of ER on breast cancer cells and tumor microenvironment (TME). MDSC: myeloid-derived suppressor cell (also see text).

Figure 3. Different levels of a virtual equilibrium line (VEL) in tumors with undetectable burden or with prolonged «resting state» of residual cancer cells following conventional therapy. A) CR: immune system strongly prevails and eliminates all or most Q/RRCCs; B) PR: immune system prevails and eliminates any new formed cancer cell but only some Q/RRCCs; C) SD: a perfect equilibrium occurs and immune system eliminates any new formed cancer cell but no Q/RRCCs; D) PD: immune system eliminates less new cancer cells than they are formed by Q/RRCCs (also see text). aCD4/CD8TL = activated CD4/CD8 T lymphocyte; aNK = activated natural killer cell; VEL = virtual equilibrium line; Q/RRCCs = quiescent/resistant residual cancer cells
Figure 1

297x209mm (300 x 300 DPI)
**Estrogen (E2)**

*E2-alpha mediated action on breast cancer cells*

**Immediate transcription**
- mRNAs encoding factors of G protein coupled signalling pathways and the transcription machinery

**Successive transcription**
- rRNAs (POL-I)
  - (45S transcription unit - 28S, 5.8S and 18S rRNA)
- mRNAs encoding elements of the protein synthesis machinery (POL-II)
- tRNAs (POL-III)

**Action**
- Secondary signalling and transcriptional responses
- Ribosome biogenesis; protein synthesis and processing

**Result**
- Cell growth and proliferation
- Immune response
- Th2 shifted response
- Immune suppression
- Treg and MDSC increase

*E2 in the TME*
A. Immune system

aCD4/CD8 TL (○) aNK (○) VEL level = 0 (CR)

No or undetectable Tumor burden

B. Immune system

aCD4/CD8 TL (○) aNK (○) VEL level > 0 < 1 (PR)

Q/RRCCs

C. Immune system

aCD4/CD8 TL (○) aNK (○) VEL level = 1 (SD)

Q/RRCCs

D. Immune system

aCD4/CD8 TL (○) aNK (○) VEL level > 1 (PD)

Q/RRCCs
Table 1. Probable main reasons of the discrepancy between the biological advances in knowledge and persistent relatively poor outcome of advanced breast cancer from therapies

<table>
<thead>
<tr>
<th>System</th>
<th>Problem</th>
<th>Result</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td><em>Spatial heterogeneity</em>: many genetic and epigenetic alterations that differ within the same tumor and from one to another. The contribution of the epigenetic alterations is unexpectedly relevant</td>
<td>Any tumor has the own “genetic signature” that differs from any other</td>
<td>Verigos and Magklara 2015, Yachida et al 2010</td>
</tr>
<tr>
<td></td>
<td><em>Temporal heterogeneity</em>: genomic instability and biologic plasticity are relevant properties of cancer cells</td>
<td>Any tumor can change its phenotype during progression</td>
<td>Wang Y et al 2014</td>
</tr>
<tr>
<td>Microenvironment</td>
<td><em>The stroma contribution</em>: the cross-talk between stroma and cancer cells</td>
<td>Further increase in the complexity of the overall network of the molecular pathways</td>
<td>Nicolini et al 2009, Hanahanan and Weinberg 2011</td>
</tr>
<tr>
<td></td>
<td>The “contextual signaling”: different extracellular signals present in the immediate milieu;</td>
<td>Different adaptive responses of cancer cells</td>
<td>Smithson et al 2016</td>
</tr>
<tr>
<td>Conventional</td>
<td>Time-limited efficacy</td>
<td>Toxicity, early arising of resistance</td>
<td>Collins 2014</td>
</tr>
<tr>
<td>therapies</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2A. Prognostic clinical relevance of proliferation signatures in breast cancer

<table>
<thead>
<tr>
<th>BC subtype/subgroup</th>
<th>Expression</th>
<th>Prognosis</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A vs Luminal B</td>
<td>Lower in Luminal A versus Luminal B</td>
<td>Better</td>
<td>Eroles et al 2012</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Galanina et al 2011</td>
</tr>
<tr>
<td>Basal-like/TNBC</td>
<td>High</td>
<td>Worst</td>
<td>Perou et al 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cancer genome atlas network 2012</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Curtis et al 2012</td>
</tr>
<tr>
<td>N- ER+</td>
<td>High HOXB13 to IL-17BR ratio</td>
<td>Poor</td>
<td>Goetz et al 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Goetz et al 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Erlander et al 2005</td>
</tr>
<tr>
<td>TAM treated N- or N+</td>
<td>High recurrence score with 16 selected</td>
<td>Poor</td>
<td>Paik et al 2004</td>
</tr>
<tr>
<td></td>
<td>genes, 7 of them being proliferation</td>
<td></td>
<td>Albain et al 2010</td>
</tr>
<tr>
<td></td>
<td>(5) or HER2 (2) related genes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TNBC: triple negative breast cancer; N- or N+: axillary lymph-node negative or positive; ER+: ER alpha positive; TAM: tamoxifen; HOXB13: homeobox13; IL-17BR: interleukin-17 B receptor (also see text).
Table 2B. Immune signatures and prognosis in breast cancer

<table>
<thead>
<tr>
<th>BC subtype/subgroup</th>
<th>Type of immune signature</th>
<th>Prognosis</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER2+</td>
<td>Immune index increase after brief exposure to trastuzumab</td>
<td>Better response (to neoadjuvant CT + H)</td>
<td>Varadan et al 2016</td>
</tr>
<tr>
<td>Early HER2+</td>
<td>9 or more of 14 immune function gene enriched tumors</td>
<td>Benefit (from adjuvant H)</td>
<td>Perez et al 2015</td>
</tr>
<tr>
<td>HER2+ REalpha-</td>
<td>17-gene HER2-TIC-enriched signature (HTICS+)*</td>
<td>Benefit (from adjuvant CT + H)</td>
<td>Liu JC et al 2012</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Liu JC et al 2017</td>
</tr>
<tr>
<td>BLBC</td>
<td>HLA-F/TIGIT and HLA-C/HLA-F/TIGIT upregulated genes</td>
<td>Significantly better DFS and OS</td>
<td>Martinez-Canales et al 2017</td>
</tr>
<tr>
<td>TNBC</td>
<td>Weak immunity two metagene expression</td>
<td>Poor DSS</td>
<td>Bonsang-Kitzis et al 2015</td>
</tr>
<tr>
<td>TNBC, Luminal, HER2+</td>
<td>High Metil score</td>
<td>Better outcome</td>
<td>Jeschke et al 2017</td>
</tr>
</tbody>
</table>

*This signature includes genes related to cell proliferation, immune response and cell migration; BLBC: basal-like breast cancer; TNBC: triple negative breast cancer; TIC: tumor-initiating cells; HLA: human leukocyte antigen; TIGIT: T-cell immunoreceptor with IG and ITIM domains; Metil: methylation of tumor infiltrating lymphocytes; CT: chemotherapy; H: trastuzumab; RFS: relapse free survival; OS: overall survival; DSS: disease-specific survival (also see text).
Table 3. Clinical outcome in metastatic breast cancer patients treated with hormone therapy and immune manipulation

<table>
<thead>
<tr>
<th>Recruited patients</th>
<th>Condition</th>
<th>N</th>
<th>Type of study</th>
<th>Control Group</th>
<th>Hormone therapy and immune manipulation</th>
<th>Reference (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>On clinical benefit during tamoxifen as first line therapy (induction time)</td>
<td>31</td>
<td>Pilot Phase II</td>
<td>Historical</td>
<td>Tamoxifen plus interferon-beta and interleukin-2 sequence</td>
<td>Nicolini and Carpi 2005 Nicolini et al 2014 (a)</td>
</tr>
<tr>
<td></td>
<td>Responders (CR+PR) to conventional CT</td>
<td>100</td>
<td>Pilot Phase II</td>
<td>Literature data</td>
<td>LHRH analog (premenopausals) or letrozole (postmenopausals) plus interleukin-2 and retinoic acid</td>
<td>Coates et al 2003 Gennari et al 2006 Alba et al 2007 Chung et al 1996</td>
</tr>
</tbody>
</table>

CT: chemotherapy; CR: complete response; PR: partial response; SD: stable disease; Clinical benefit (CR+PR+SD); PFS: progression-free survival; OS: overall survival.
### Table 4. Clinical relevance of tumor burden for an active or probably induced immune manipulation in cancer other than breast

<table>
<thead>
<tr>
<th>Tumor burden</th>
<th>Cancer type</th>
<th>Pts, n (%)</th>
<th>Immune-manipulation</th>
<th>Clinical outcome (mo)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undetectable (CR or radical surgery) or detectable (PR, SD) not growing disease following conventional CT</td>
<td>*Ovary</td>
<td>CR, 88 (64) PR, 14 (32) SD, 2 (4)</td>
<td>IL-2 plus 13-cis-RA</td>
<td>PFS/DFS (median; mo) &amp; OS (median; mo)</td>
<td>Recchia et al 2005</td>
</tr>
<tr>
<td></td>
<td>*Lung (non small)</td>
<td>CR, 3 (6) PR, 17 (34) SD, 18 (36)</td>
<td>IL-2 plus 13-cis-RA</td>
<td></td>
<td>Recchia et al 2006</td>
</tr>
<tr>
<td></td>
<td>*Colon</td>
<td>CR, 10 (25) PR, 11 (27) SD, 19 (48)</td>
<td>IL-2 plus 13-cis-RA</td>
<td></td>
<td>Recchia et al 2006</td>
</tr>
<tr>
<td></td>
<td>*GI (mixed)</td>
<td>19 (100)</td>
<td>Few cycles of additional conventional CT regularly given</td>
<td>80.4% 5-year vs 31.8% expected</td>
<td>87.1% 5-year vs 40.1 expected</td>
</tr>
<tr>
<td>Undetectable (m. r. d.) following radical surgery with or without adjuvant CT</td>
<td>Colorectal</td>
<td>1001 (100)</td>
<td>No active immune-manipulation</td>
<td>NA</td>
<td>Fong et al 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>173 (100)</td>
<td></td>
<td>NA</td>
<td>Scheele et al 1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td>86 with CT (100) 85 without CT (100)</td>
<td></td>
<td>DFS and 5-year DFS 24.4 and 33.5% vs 17.6 and 26.5% (controls) p=0.028</td>
<td>Portier et al 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>138 with CT (100) 140 without CT (100)</td>
<td></td>
<td>PFS and 5-year DFS 27.9 and 36.7% vs 18.8 and 27.7% (controls) p=0.058</td>
<td>Mitry et al 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6254** (100) (meta-analysis)</td>
<td></td>
<td>5-year DFS ranging from 13% to 46%</td>
<td>Liu W et al 2016</td>
</tr>
</tbody>
</table>

CT: chemotherapy; *pilot study; HT: hormone therapy; NA: not available; PFS: progression free survival; DFS: disease free survival; OS: overall survival; m. r. d.: minimal residual disease; CR: complete response; PR: partial response; SD: stable disease; GI: gastrointestinal; IL-2: interleukin-2; 13-cis-RA: 13-cis-retinoic acid; **neoadjuvant CT prior to hepatic resection (also see text). ***log-rank test
<table>
<thead>
<tr>
<th>Experimental model</th>
<th>SERMs</th>
<th>ER-alpha mediated genes (n)</th>
<th>Some main tumor growth related genes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>E2 regulated (n)</td>
<td>Clusters</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Not or minimally E2 regulated (n)</td>
<td>Cluster C1</td>
<td>Cluster C2</td>
</tr>
<tr>
<td>TOT</td>
<td>Full or partial antagonist Cluster A</td>
<td>Partial agonist/antagonist or full agonist Cluster B</td>
<td>AREG (E2 up)</td>
<td>RAB30 (Ral, TOT or ICI up)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18 E2 up 16 E2 down</td>
<td>51 TOT up</td>
<td>INHBB (E2 down)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18 E2 up 16 E2 down</td>
<td>24 TOT up</td>
<td>CCNA2 (Ral, TOT or ICI down)</td>
</tr>
<tr>
<td>TAM</td>
<td>NE</td>
<td>NE</td>
<td>50 TAM up</td>
<td>PTPRG (TAM up)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>548 E2 up TAM down</td>
<td>-</td>
<td>RAB30 (TAM up)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>172 E2 up TAM down</td>
<td>-</td>
<td>SOCS1 (TAM up)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>179 E2 down not or partially TAM downregulated</td>
<td>-</td>
<td>*IER3 (TAM up)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SERMs: selective estrogen receptor modulators; E2, TAM, TOT up: estradiol, tamoxifen, or transhydroxytamoxifen upregulated; E2, TAM, TOT down: estradiol, tamoxifen, or transhydroxytamoxifen downregulated; GO: gene ontology; Ral: raloxifene; ICI: ICI182.780; AREG: amphiregulin; SDF1: stromal cell-derived factor-1 (also known as chemokine ligand 12); TGFbeta2: transforming growth factor beta2; INHBB: inhibin beta B; RB1CC1: RB1-inducible coiled-coil1; RAB30: member of RAS oncogene family; TPM1: tropomyosin 1(alpha); IER3: immediate early response 3; CCNA2: cyclinA2; CDKN2C: cyclin dependent kinase inhibitor 2C; CDK8: cyclin dependent kinase 8.; PTPRG: protein tyrosine phosphatase receptor type G; SOCS1: suppressor of cytokine signaling 1; YWHAZ: 14-3-3zeta; *IER3 and IEX1: the same gene has been reported in the two A and C different categories (Frasor et al., 2004, Frasor et al., 2006)(also see text).
### Table 6. Proposal of a novel therapeutic strategy for advanced breast and other cancers treatment

<table>
<thead>
<tr>
<th>Setting</th>
<th>Target population</th>
<th>Therapeutical interventions</th>
<th>Main aim</th>
<th>Cancer type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adjuvant at high risk or metastatic</td>
<td>*Endocrine responsive</td>
<td>Large prospective randomized multicenter trials with hormone-immunotherapy</td>
<td>Significant PFS and/or OS increase</td>
<td>Breast and other solid tumors</td>
<td>Nicolini et al 2014 (a)</td>
</tr>
<tr>
<td>Adjuvant at high risk</td>
<td>Endocrine resistant/independent likely with m. r. d.</td>
<td>**3-4 cycles of taxanes or antimetabolites (5-FU, capecitabine) with or without partially synergizing immune drugs regularly given every 8-12 monts, for 5 years</td>
<td>Significant delay or decrease of the recurrence rate</td>
<td>Breast and Prostate</td>
<td>Nicolini et al 2010 Recchia et al 1998 Recchia et al 2006 Recchia et al 2007</td>
</tr>
<tr>
<td>Metastatic in CR, PR or SD following conventional CT</td>
<td>Endocrine resistant/independent with detectable metastatic disease</td>
<td>Immunomodulatory and/or immunostimulating drugs</td>
<td>To delay metastatic progression</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*In prostate cancer, anti-androgens replace anti-estrogens; **taxanes or antimetabolites (5-FU, capecitabine) should be chosen in relation to cancer type (breast and other solid cancers) according with current therapeutic recommendations; m. r. d.: minimal residual disease. CR: complete response; PR: partial response; SD: stable disease; PFS: progression-free survival; OS: overall survival.*