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Review

Triple-negative breast cancer: Present challenges and new perspectives

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ABSTRACT

Triple-negative breast cancers (TNBC), characterized by absence of estrogen receptor (ER), progesterone receptor (PR) and lack of overexpression of human epidermal growth factor receptor 2 (HER2), are typically associated with poor prognosis, due to aggressive tumor phenotype(s), only partial response to chemotherapy and present lack of clinically established targeted therapies. Advances in the design of individualized strategies for treatment of TNBC patients require further elucidation, by combined 'omics' approaches, of the molecular mechanisms underlying TNBC phenotypic heterogeneity, and the still poorly understood association of TNBC with BRCA1 mutations. An overview is here presented on

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Abbreviations: aCGH, array comparative genomic hybridization; CK, cytokeratin; CI, confidence interval; EGF, epidermal growth factor; EGFR (or HER1), epidermal growth factor receptor; EMT, epithelial–mesenchymal transition; ER, estrogen receptor; HER2 (or Her2/neu, or ErbB2), human epidermal growth factor receptor 2; HMMR, hyaluronan-mediated mobility receptor; HR-MAS, high resolution magic angle spinning; IHC, immunohistochemistry; LC, liquid chromatography; MNI, mode-of-action by network identification; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; MRSI, magnetic resonance spectroscopic imaging; ODE, ordinary differential equation; PARP, poly(ADP-ribose) polymerase; PCA, principal component analysis; PET, positron emission tomography; PR, progesterone receptor; Rb, retinoblastoma; ROC, receiver operating characteristic; TK, tyrosine kinase; TKI, tyrosine kinase inhibitor; TNBC, triple-negative breast cancer.

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BRCA1
BRCA2
Chemotherapy
Targeted therapies

TNBC profiling in terms of expression signatures, within the functional genomic breast tumor classification, and ongoing efforts toward identification of new therapy targets and bioimaging markers. Due to the complexity of aberrant molecular patterns involved in expression, pathological progression and biological/clinical heterogeneity, the search for novel TNBC biomarkers and therapy targets requires collection of multi-dimensional data sets, use of robust multivariate data analysis techniques and development of innovative systems biology approaches.

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1. Introduction

Triple-negative breast cancers (TNBC), defined as tumors that are negative for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), nowadays represent the focus of increasing interest at the clinical, biological and epidemiological level (Irvin and Carey, 2008; Reis-Filho and Tutt, 2008; Stockmans et al., 2008; Bauer et al., 2007; Dent et al., 2007), due to the aggressive behaviour of the tumor, poor prognosis and present lack of targeted therapies (Mersin et al., 2008; Kaplan et al., 2009; Tan and Swain, 2008). A better understanding of pathological mechanisms of TNBC onset and progression, including the still unclear association with BRCA1 mutations, and the causes of phenotypic heterogeneity may allow improvement in planning prevention and designing novel individualized treatments for this breast cancer subgroup (Goldhirsch et al., 2009).

The new approaches of personalized therapy make use of specific molecular signatures, biology markers and clinicopathological features in tumors and patients. For breast cancer, the first clinically used predictive prognostic markers, arising from elucidation of hormonal regulation, were ER/PR which led to the tailoring of endocrine/anti-hormonal therapy. The first cytogenetic predictor for breast cancer treatment has been the HER2 (HER2/neu, c-erbB2) gene amplification and protein overexpression. A monoclonal humanized antibody, trastuzumab (Herceptin®), is currently used in the treatment of breast cancer patients presenting with HER2 positivity. Recent scientific and technological advances nowadays provide a large inventory of candidate DNA, RNA, and protein biomarkers, as well as a range of metabolites and networks of cell signaling pathways (Swanton and Caldas, 2009; Bathen et al., 2007; Gast et al., 2009; Chen and Wang, 2009), all potential candidates for disease risk assessment, screening, diagnosis, prognosis, prediction of therapy response and selection of personalized therapy. Nevertheless, such predictors of TNBC prognosis and targeted therapy are presently ill-defined, making the true challenges of this disease still unmet. A critical step in the expansion of personalized therapy involves development of new molecular imaging tools, adjusted to monitor specific molecules and pathways involved in cancer associated signaling and metabolism. The emerging field of molecular imaging (He et al., 2003; Belkic, 2004; de Vries et al., 2007; Hospers et al., 2008) enables translational medicine from drug discovery via pre-clinical to clinical research and development and finally, to the clinical practice. Imaging biomarkers have proven utility in the spectrum from intact cells, to experimental tumors in small animals, to patients. Biomarkers are useful in longitudinal quantification of the course of malignancy and therapy response, as well as in early identification of cancer patients and in therapy decision. In conclusion, the success of biomarkers depends on our ability to reveal critical cancer related molecular events and the mechanisms of action of targeted therapy (Tan and Swain, 2008), on how effectively a specific biomarker is related to other biomarkers and to a specific disease condition, as well as on sensitivity and specificity of the available analytical and imaging tools (Dowsett and Dunbier, 2008).

In the search for TNBC biomarkers of diagnosis, prognosis and prediction of therapy response, high dimensional data sets can be generated from different modern 'omics' related analyses, such as microarrays in genomics, proteomics and MR-based metabolomics. The complexity of aberrant molecular patterns involved in expression, pathological progression and biological/clinical heterogeneity of the TNBC phenotype requires collection of multi-dimensional data sets, use of robust multivariate data analysis techniques (Bishop, 1995; Duda et al., 2001; Vapnik, 2002) and development of innovative systems biology approaches (Chuang et al., 2007; Goh et al., 2007; Pujana et al., 2007; Shen et al., 2009; Fitzgerald et al., 2006; Aebersold et al., 2009). The combination of these progressively more potent technological tools may open new perspectives to the fight against this challenging breast cancer subgroup with worse prognosis and still limited therapy options.

2. Clinical features of TNBC and limitations of current treatment options

2.1. *Incidence, recurrence and outcome*

According to current estimates, TNBC accounts for 10–17% of all breast carcinomas, depending on thresholds used to define ER and PR positivity and HER2 overexpression (Reis-Filho and Tutt, 2008). In different series and patient populations TNBC may range 6–28% of breast cancers (Haffty et al., 2006; Rakha et al., 2007; Dent et al., 2007; Kwan et al., 2009), but even higher incidence rates are reported for some ethnical groups such as African Americans and for younger patients (Stead et al., 2009; Trivers et al., 2009; Lund et al., 2009; Morris et al., 2007; Carey et al., 2006).

Despite its relatively small proportion among all breast cancers, TNBC is responsible for a relatively large proportion

of breast cancer deaths, due to its generally aggressive clinical course. In a retrospective study on a cohort of 1601 patients with breast cancer (Dent et al., 2007), a subgroup of 180 women with TNBC had a significantly lower mean age at diagnosis ($P < 0.0001$), increased likelihood of distant recurrence (hazard ratio *vs* other breast cancer phenotypes equal to 2.6; 95% confidence interval (CI) 2.0–3.5; $P < 0.0001$) and death (hazard ratio 3.2; 95% CI 2.3–4.5; $P < 0.001$) within five years of diagnosis, but not thereafter. Also, patients with TNBC were more likely to present with larger mean tumor size ($P < 0.0001$) and histological tumor grade ($P < 0.0001$). Although data on differential lymph node spread in TNBC and other breast cancer subgroups are still conflicting (Dent et al., 2007; Reis-Filho and Tutt, 2008; Rakha et al., 2008), an interesting feature was the substantial lack of correlation between nodal metastasis and tumor size among women with tumors smaller than 50 mm (Dent et al., 2007) (Fig. 1A). A similar trend was also found in BRCA1-associated tumors (Fig. 1B), as discussed in Section 3.2 (Foulkes et al., 2003a). The patterns of recurrence in TNBC were qualitatively different from the non-TNBC

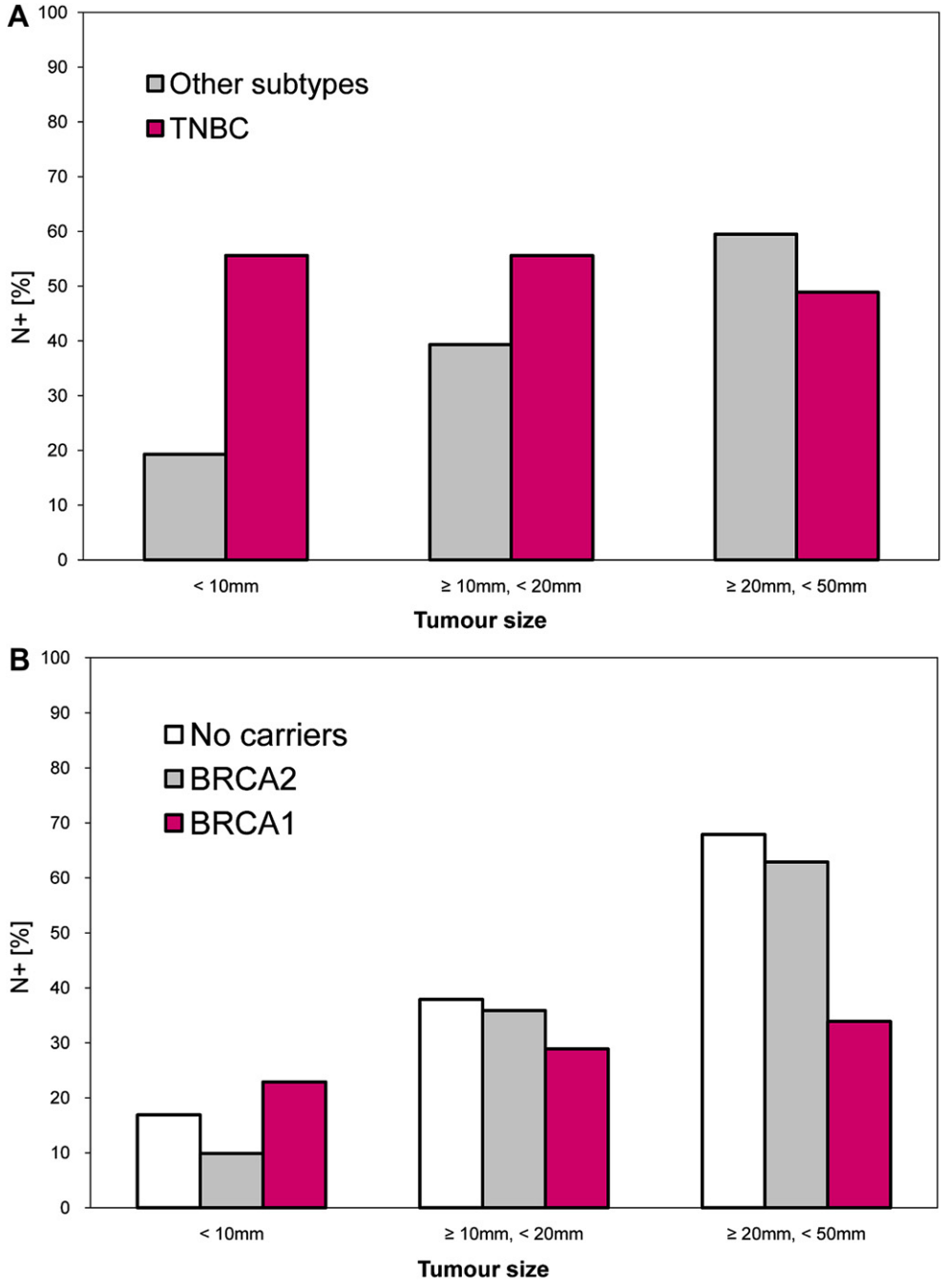


Figure 1 – Lack of correlation between tumor size and nodal status among (A) triple-negative breast cancers (TNBC) with tumor size smaller than 50 mm (χ^2 test for trend: $P = 0.47$ for TNBC; $P < 0.0001$ for other breast cancer subtypes) and (B) BRCA1-associated breast cancers (χ^2 test for trend: $P = 0.183$ for BRCA1-associated tumors; $P < 0.0001$ for BRCA2-associated breast tumors and for breast tumors in non-carriers). N^+ positive nodal status (at least one positive lymph node). Graphs adapted from Dent et al. (2007) and (Foulkes et al. 2003a).

group. In the former, the risk of distant recurrence peaked at approximately three years and declined rapidly thereafter, whereas in the other breast cancer types the recurrence risk seemed to be constant over time (Dent et al., 2007). TNBC has a preference for visceral metastases (Liedtke et al., 2008).

It should be emphasized that TNBC currently includes a heterogeneous group of tumors. Already by simple morphology, a group of patients with TNBC can be identified who have a more favourable outcome, for example patients with invasive adenoid cystic, apocrine and typical medullary tumors (Japaze et al., 2005; Azoulay et al., 2005; Orlando et al., 2005). Even within the relatively homogeneous group of patients with triple-negative invasive ductal carcinoma, patients with higher or lower risk may be identified, based on specific molecular markers (Viale et al., 2009).

2.2. *Current cytotoxic treatment options*

Several studies support the notion that primary TNBC is a chemosensitive disease. However, although TNBC is associated with enhanced pathological complete response to neoadjuvant chemotherapy (von Minckwitz et al., 2008), these cancers show worse survival due to higher relapse among those with residual disease after chemotherapy (Liedtke et al., 2008; Carey et al., 2007). In fact, despite the high sensitivity to chemotherapy, TNBC patients with truly chemosensitive disease still represent a minority among all TNBC patients. In the metastatic setting, patients progress quickly on first-, second-, and third-line palliative treatment (Kassam et al., 2009).

As triple-negative disease is often characterized by an impaired DNA repair process, cytotoxic agents inducing DNA damage may be of specific value. Recent small and non-randomized studies have shown promising results with cisplatinum, both in the neoadjuvant and metastatic setting (Byrski et al., 2009; Sirohi et al., 2008), although the clinical response to paclitaxel and cyclophosphamide may also be high (Rouzier et al., 2005). A new approach under clinical trial includes the use of ixabepilone, a microtubule inhibitor (Thomas et al., 2007; Baselga et al., 2009b).

Larger prospective clinical trials are needed to determine the optimal cytotoxic treatment for TNBC.

3. Molecular profiling of triple-negative breast cancers

3.1. *The relationship between TNBC and basal-like breast cancer*

Molecular profiling of human breast cancers by gene expression assays provided in the last decade new grounds to a clearer understanding of the heterogeneous nature of these tumors with promising trends for outcome prediction and development of individualized therapies (van't Veer et al., 2002; van de Vijver et al., 2002; Perou et al., 2000; Sørlie et al., 2003; Chang et al., 2005). The gene expression based classification proposed by Perou and Sørlie ten years ago originally defined six subtypes (Perou et al., 2000; Sørlie et al., 2003). Three of these were characterized by expression of ER and luminal epithelial cell related genes (Luminal A, Luminal B and Luminal C) while the remaining three groups, basal-like, ErbB2+ and normal-like, showed an expression phenotype more similar to myoepithelial/basal epithelial cells. The ErbB2+ tumors were in particular characterized by high expression of ErbB2 and genes located adjacent to the ErbB2 locus and the normal-like subgroup showed expression patterns similar to normal breast tissue samples. Although this seminal work was based on analyses on neoadjuvantly treated breast carcinomas, the main findings have since been validated in numerous independent cohorts and these intrinsic subtypes show different mutation patterns, prognosis and routes of progression (Sørlie et al., 2003; Calza et al., 2006; Hu et al., 2006; Langerod et al., 2007; Naume et al., 2007; Smid et al., 2008; Parker et al., 2009). Later, using array comparative genomic hybridization (aCGH), several investigators have found that genomic alterations seem to be more frequent in some of the intrinsic subclasses (Chin et al., 2006; Fridlyand et al., 2006; Bergamaschi et al., 2006). Of particular interest to this review, basal-like tumors frequently have complex rearrangements with higher numbers of gains and losses compared to luminal subtypes (Chin et al., 2006; Fridlyand et al., 2006; Bergamaschi et al., 2006), although there is evidence of a subgroup of basal-like tumors with low genomic instability (Chin et al., 2007). Such heterogeneity is recognized by gene expression classification as well (Kreike et al., 2007) and multiclonal basal-like tumors have been described (Navin et al., 2010). Recently a distinct type of rearrangements dominated by genomewide duplications was identified in a selection of basal-like tumors and cell-lines (Stephens et al., 2009), suggesting that these types of tumors represent a distinct type of breast carcinomas with a unique path of progression (Navin et al., 2010; Dalgin et al., 2007). In addition, the emerging knowledge of a cellular hierarchy in the breast supports the theory that basal-like tumors may have a distinct etiology (Villadsen et al., 2007; reviews in: Sims et al., 2007; Polyak, 2007).

Immunohistochemistry (IHC) is frequently used to explore the distribution of the molecular subtypes by using formalin-fixed, paraffin-embedded tissues from larger cohorts of breast cancer patients. The ultimate selection of surrogate markers is an ongoing debate and a consensus for an appropriate panel still has to be reached (Rakha et al., 2008). Triple negativity is often used to identify basal-like tumors (Kreike et al., 2007) although a supplement of additional markers has superior prognostic value (Cheang et al., 2008; Nielsen et al., 2004). Triple negativity as a selection criterion is highly sensitive of basal-like tumors, but not specific as both luminal, ErbB2+ and normal-like tumors (as identified by gene expression) can be steroid receptor negative and HER2 non-amplified (Naume et al., 2007). The heterogeneity of TNBC is acknowledged and includes both basal-like and non-basal-like tumors (Rakha et al., 2009; reviewed in Hurvitz and Finn, 2009). IHC-based studies use different markers to define their basal-related tumors and the lack of a systematic classification scheme makes comparison of results difficult. Acknowledging the differences between the two terms is important in clinical studies aiming to identify prognostic markers and targets for therapy for these important subgroups of breast carcinomas.

Morphologically basal-related tumors are typically of high histological grade with a high mitotic count and they frequently exhibit geographic tumor necrosis, central scar,

pushing margings and/or stromal lymphocyte enrichment (Fulford et al., 2006; Livasy et al., 2006; Rakha et al., 2006). Although most TNBCs are classified as ductal carcinomas, tumors of 'special types' such as medullary and adenoid cystic carcinoma fall into this category as well (Bertucci et al., 2006; Jacquemier et al., 2005; Vincent-Salomon et al., 2007; Weigelt et al., 2008). Many of the clinical features of the basal-like phenotype are similar to those of TNBC (see Section 2.1), including shorter relapse-free and overall survival times compared with other types of breast cancers, a tendency toward visceral versus bone metastasis (Rodriguez-Pinilla et al., 2006; Rakha et al., 2008), and over-representation in BRCA1 mutation carriers (Foulkes et al., 2003b; Haupt et al., 2010).

3.2. *The BRCA-associated triple-negative breast cancers*

Following identification, mapping and cloning of the two major breast cancer predisposing genes, BRCA1 (chromosome 7q21) and BRCA2 (chromosome 13q12) (Hall et al., 1990; Miki et al., 1994; Wooster et al., 1995), increasing attention has been focused on biological and molecular characteristics of breast cancers in BRCA- and non-BRCA1/2 (BRCAX) mutation carriers, in relation to carcinogenesis, disease progression and outcome (Breast Cancer Linkage Consortium, 1997; Lakhani et al., 1998, 2002; Verhoog et al., 1998; Moller et al., 2002; Narod and Foulkes, 2004; Robson et al., 2004; Brekelmans et al., 2006; Bonadona et al., 2007; Moller et al., 2007; Honrado et al., 2007; Melchor and Benitez, 2008).

Major clinical characteristics of breast cancer in BRCA1 mutation carriers, compared with age-matched patients unselected for family history were envisaged to be younger age at onset (Robson et al., 2004; Cornelis et al., 1995), frequent bilateral occurrence (Hall et al., 1990; Ford et al., 1994; Easton et al., 1995), high frequency of ductal histotype cancer, although with a relative excess of medullary and atypical medullary histotypes, higher overall grade and worse histoprognostic features (Breast Cancer Linkage Consortium, 1997; Lakhani et al., 1998, 2002; Verhoog et al., 1998; Jacquemier et al., 1995; Marcus et al., 1996; Eisinger et al., 1996). Histopathological characteristics frequently detected in BRCA1-associated breast cancers are higher mitotic counts, greater degree of nuclear polymorphism and less tubule formation. However, multifactorial analysis of histopathological differences between sporadic breast cancers and cancers involving BRCA1 and BRCA2 mutations (Lakhani et al., 1998) demonstrated that many of these features were linked with each other, the only factors independently associated with BRCA1 being high mitotic count, presence of lymphocytic infiltrate and presence of smooth noninfiltrative pushig border. A similar multifactorial analysis showed that reduction in tubule formation (together with higher overall grade) and presence of continuous pushing margins were also significantly associated with BRCA2-related breast cancer.

Combined immunohistochemical and molecular analyses of cancer-associated genes and encoded proteins carried out within a large collaborative study of the Breast Cancer Linkage Consortium (Breast Cancer Linkage Consortium, 1997; Lakhani et al., 1998, 2002) showed that breast cancers in patients with BRCA1 germline mutation are more often negative for ER, PR and HER2 and are more likely to be positive for p53 protein compared with controls. BRCA2 tumors did not show, instead, any significant difference in the expression of these proteins. In fact, only 10% of BRCA1-mutated cancers showed positive staining for ER compared with 66% of BRCA2 and control tumors; positivity to PR was 21% for BRCA1-cancers compared with 55–61% for BRCA2 and control cancers; and a low positivity (3%) was found for HER2 in both BRCA1 and BRCA2 tumors. Multiple logistic regression analysis of the immunohistochemical factors (ER-negative, PR-negative and HER2-negative) and morphological features that were significant predictors of BRCA1 status indicated that the ER status was the most significant risk factor (Lakhani et al., 2002). On the other hand, the expression of ER is known to inversely correlate with tumor grade (Henderson and Patek, 1998) and is reported as one of the most important prognostic and predictive markers for breast cancer (Osborne, 1998).

Unsupervised cluster analysis of non-BRCA1/2 breast cancer families (50 probands) demonstrated heterogeneity of BRCAX families (Honrado et al., 2007). In fact two main groups were identified, one of high grade and ER-negative (50%) and one of low-grade and ER-positive tumors (50%). These two groups were in turn subdivided into five subgroups: three among the high-grade and two among the low-grade groups; one overexpressing HER2 (18%); one with a basal-like phenotype (14%); one with a normal breast-like phenotype (18%); a luminal A subgroup (36%) and a luminal B subgroup (14%).

At the molecular level, BRCA1 and BRCA2 proteins are known to be involved in DNA repair (Scully, 2000), cell cycle checkpoint control through regulation of p53 activity (Liu and Kulesz-Martin, 2001) and maintenance of global chromosome stability (Venkitaraman, 2002).

A more complete molecular portrait has been recently consolidated for the majority of breast tumors in BRCA1-mutated patients. This general genomic and proteomic profile typically includes lack of (or low) expression of hormone receptors, HER2 and BCL2 and overexpression of p53, EGFR and basal cytokeratines (CK 5/6) (Lacroix and Leclercq, 2005; Honrado et al., 2006), associated at clinical level with high frequency of ductal type, high proliferation rate, high histological grade and manifested lymphocyte infiltration. The reported molecular features are also characteristic to basal-like carcinomas (Perou et al., 2000; Gorski et al., 2009), although overlap of tumor biological features between BRCA1-associated and basal-like cancer subtype is not complete (Dawson et al., 2009). Depletion of BRCA1 affects differentiation and enhances proliferation of mammary epithelial cells, as also confirmed by studies on animal models (Kubista et al., 2002; Furuta et al., 2005; Bradley and Medina, 1998).

Over-representation of the TNBC phenotype in BRCA1-associated tumors allows a rational retrospective interpretation of a series of similarities separately reported for the clinicopathological features of these two breast cancer subgroups. Of particular interest in this respect are the younger age of the first breast cancer event; high histopathological grade; some common morphological features (e.g. smooth noninfiltrative pushing border); and the above mentioned lack of correlation between tumor size and nodal status (Fig. 1).

Tumors arising from BRCA1 and BRCA2 mutation carriers appear to have specific pathological and gene expression

profiles (Honrado et al., 2006), while BRCAX tumors are increasingly believed to originate from multiple distinct genetic events (Lacroix and Leclercq, 2005). The use of microarray technology in the evaluation of the immunophenotypic features of hereditary breast cancer (Palacios et al., 2003) revealed distinct characteristics in BRCA1 and in non-BRCA1/2 tumors, whereas BRCA2 tumors presented intermediate patterns. No cases of HER2 amplification and/or overexpression were found by the authors, except in sporadic breast cancers.

The BRCA1 tumor suppressor gene and the HER2 oncogene are located in close proximity on the long arm of chromosome 17 (17q11–21), but the aggressive pathological features of BRCA1-associated tumors appear unrelated to amplification of the adjacent HER2 oncogene (Grushko et al., 2002).

The molecular mechanisms responsible for tissue–specific carcinogenesis in BRCA1-associated breast cancer are still to be clarified. The existence of common genomic features in the basal-like phenotype of BRCA1-associated breast cancers and that of normal breast stem cells recently suggested that BRCA1 may act as a human mammary stem cell fate regulator (Foulkes, 2004; Liu et al., 2008). In particular, the BRCA1 expression was found to be required for the differentiation of ER-negative stem/progenitor cells to ER-positive tumoral cells and was proposed to result in the accumulation of genetically unstable breast stem cells, providing prime targets for further carcinogenic events (Liu et al., 2008). On the other hand, the basal-like molecular subtype can also be found in sporadic, BRCA2- and BRCAX-associated cancers, although with a much lower frequency (10–20% compared with up to 90% in BRCA1-classified cancers). A critical role of the BRCA1 protein has also been shown in the development of these basal-like carcinomas (Melchor and Benitez, 2008 and ref. therein).

Attention has been focused on the possible molecular mechanisms underlying the very low or inexistent overexpression (and gene amplification) of HER2 (0–3%) in BRCA1/2-associated breast cancers (Lakhani et al., 2002; Palacios et al., 2003; Grushko et al., 2002) compared with that (15–20%) in BRCAX-associated tumors (Honrado et al., 2007). A possible co-deletion of HER2 and BRCA1 loci (Johannsson et al., 1997) would not explain the lack of HER2 overexpression in BRCA2-associated cancers. It has been postulated (Melchor and Benitez, 2008) that the defects in the DNA repair system associated with deleterious BRCA1 mutations may not be compatible with the proliferation stress of the aberrant signaling cascade triggered by HER2 tyrosine phosphorylation and therefore, under these conditions, HER2-overexpressing cancer cells are unlikely to survive.

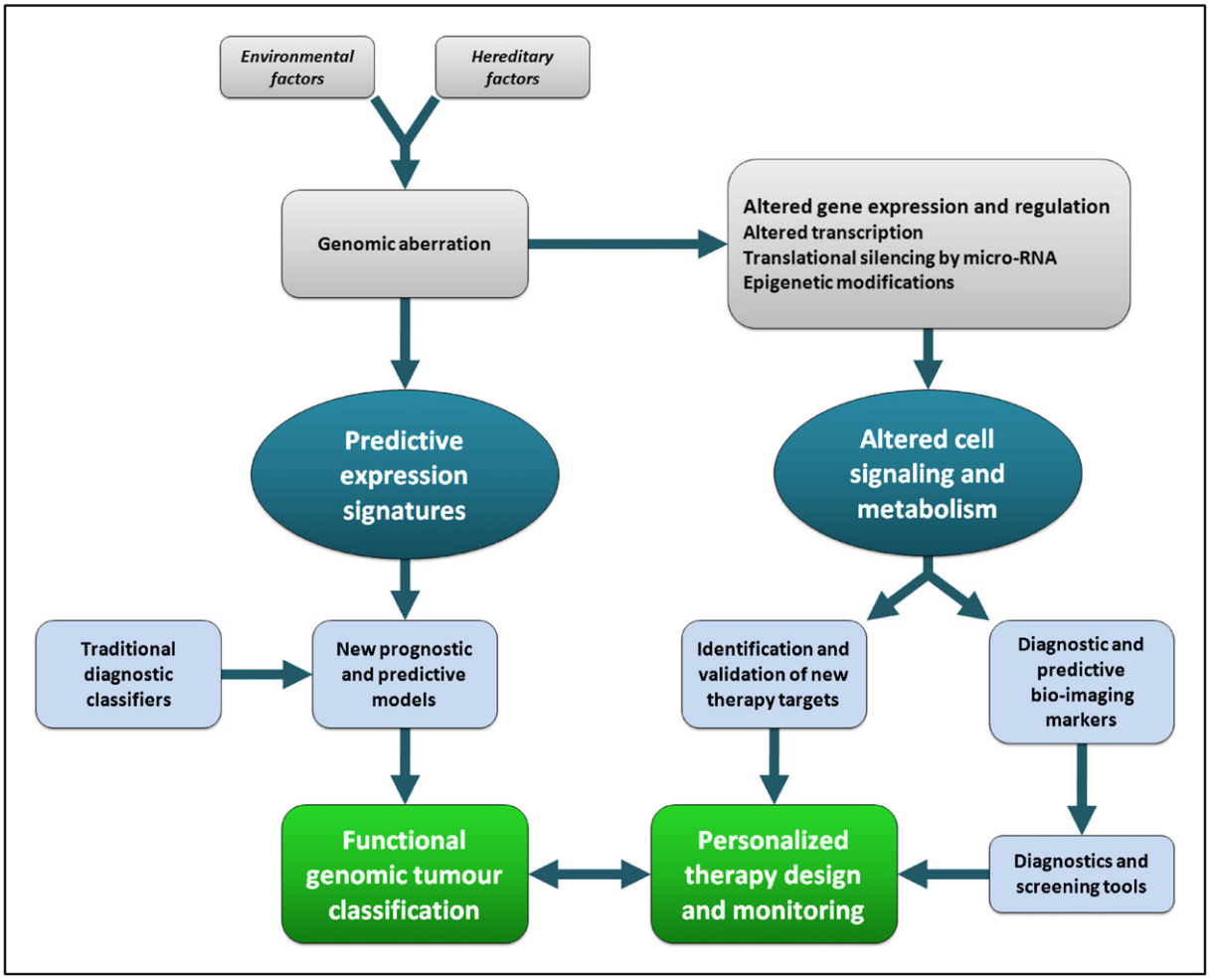


Figure 2 – Integration of predictive expression signatures and altered cell signaling patterns in the search for pathway-driven tumor therapeutics and bioimaging.

4. Imaging features

Examinations of women of 50 years and over (Dent et al., 2007) showed that patients with TNBC had a much lower proportion ($P = 0.0008$) of breast cancers first detected by mammography or ultrasound (19.5%) than patients with other breast cancers (36.5%). This result supported the conclusion that in a mammography screening programme offered to women over 50 years of age, TNBC often presents as an interval cancer, in agreement with a previous study (Collett et al., 2005). This feature, which may relate to differences in breast density, or to a rapid tumor growth in relation to the screening interval, warrants further investigations to optimize multimodality and periodicity of screening events in surveillance programs addressed to women belonging to populations at high risk of TNBC. Mammographic examinations on premenopausal women showed that a circumscribed mass (without spiculated margins) and absence of microcalcifications were most commonly presented features in TNBCs compared with cancers of other subtypes (Yang et al., 2008; Wang et al., 2008). The absence of spiculated masses and pleomorphic microcalcifications suggested that mammography may not be the ideal tool for early detection of TNBCs.

The use of dynamic contrast-enhanced magnetic resonance imaging (MRI) showed that 97% of TNBC lesions were of mass-type, with typical malignant signal enhancement kinetics (Chen et al., 2007). Correlations between MRI and pathological findings recently investigated in surgically confirmed TNBC, compared with ER-positive, PR-positive and HER2-negative breast cancers (Uematsu et al., 2009), showed that the former subtype was significantly associated with high histologic grade, unifocal lesions, smooth mass margin, rim enhancement, persistent enhancement pattern, and very high intratumoral signal intensity on T2-weighted MR images. The last feature was significantly associated with intratumoral necrosis, a characteristics typically associated with poor prognosis.

These radiological imaging methods, and their possible combination with functional imaging approaches, such as diffusion-weighted MRI (Bogner et al., 2009), may assist in TNBC prognosis and treatment planning.

A study on fluorodeoxy-glucose-positron emission tomography (FDG-PET) characteristics showed a sensitivity of 100% for detection and a higher FDG uptake in TNBC compared with ER-positive/PR-positive/HER-negative tumors, suggesting enhanced glycolysis in the former, more aggressive subgroup (Basu et al., 2008). Further studies should be devoted to better clarify the potential of PET approaches in evaluating prognosis and predicting therapy response in TNBC.

Finally, non invasive in vivo localized magnetic resonance spectroscopy (MRS) examinations and high resolution magic angle spinning (HR-MAS) MRS analyses on surgical specimens may offer new perspectives to the characterization of metabolic profiles of breast cancers, also in relation to lymphatic spread, grade and hormone receptor status (Bathen et al., 2007; Podo et al., 2007; Sitter et al., 2009; Giskeødegård et al., 2010).

Table 1 – Possible agents for TNBC targeted therapy.

TNCB biomarkers	Major affected signaling pathways or cellular process	Biological effects	Proposed TNBC therapeutic agents
EGFR (or HER1) tyrosine kinase	MAP kinase (RAS-RAF-MEK1/2 – ERK1/2)	Cell proliferation	*Cetuximab; Erlotinib*; *Gefitinib*
c-KIT	PI3K-AKT	Cell survival	*Imatinib*; *Sunitinib*; *Dasatinib*
TOP2A (topoisomerase II α)	DNA replication	Chemotherapy response	Antracyclins, Metoxantrone and etoposide
c-Myc	Gene transcription Cell-cycle control	Cell growth Angiogenesis Apoptosis	10058F4 small molecule compound (potential)
PARP (poly(ADP-ribose) polymerase) BRCA1–associated cancers	DNA repair	Apoptosis	*Olaparib*
VEGF/VEGFR (tumor endothelial cells)	Hypoxia-induced metabolic pathways	Angiogenesis	*Bevacizumab*; *Sunitinib*; *Sorafenib*
p53	Cell-cycle control	Proliferation/ apoptosis	Not available
Src kinase	STAT-pathway; PI3K; FAK	Cell proliferation; cell survival; angiogenesis; cell invasion, adhesion and migration	*Dasatinib*
TGFβ	T-reg activation	Repression of antitumor immunity	Anti-TGF-β Ab; anti-sense oligonucleotides; selective TK inhibitors
	Epithelial-mesenchimal transition (EMT)	Tumor cell motility, blood borne metastasis	
Alpha B-crystalline	MAPK/ERK	Anchorage independent cell growth; increased migration and invasion	ERK kinase inhibitors (potential)

5. Towards pathway-driven TNBC therapeutics

5.1. *Concepts and tools*

Classification of tumors by molecular profiles and increasing knowledge on altered regulation of gene expression at the translational, transcriptional and epigenetic levels (Fig. 2) allowed in the last decade the identification of possible novel markers and targets for pathway-driven therapeutics in tumors (Cleator et al., 2007; Tan and Swain, 2008; Dowsett and Dunbier, 2008; Swanton and Caldas, 2009; Bouchalova et al., 2009; Goldhirsch et al., 2009; Bosch et al., 2010).

Accumulation of defects in cellular regulation mechanisms is the underlying cause for disease heterogeneity and differential response to treatment, also within a subgroup like TNBC. This can be at the level of gene amplification, methylation, expression, mutations, or other as yet unknown regulation mechanisms. Activation of an oncogene can increase activity in downstream pathways without necessarily requiring overexpression of proteins in these pathways. Beyond the wide diversity in the combination of overexpressed pathways, the basal-like subtype is reported to be associated with increased activity of the HER1, RAS, CTNNB1, TP53 and E2F3 pathways (Bild et al., 2009). Other notable characteristics of TNBC include a high level of proliferative genes, including Ki-67, basal cytokeratins such as CK5 and CK17, caveolin-1, alpha B-crystallin, frequent p53 mutations, Retinoblastoma (Rb) pathway inactivation, high c-kit expression, reduced DNA repair capability, increased angiogenesis and TGF-regulated genes (Schneider et al., 2008).

The above mentioned TNBC biomarkers and characteristics are known to have relevant implications on cell signaling pathways, tumor metabolism, evasion of cell-cycle control, invasion and metastasis, as summarized below (Table 1).

Understanding the relations between these potential markers may help to appreciate the heterogeneity of these tumors and improve the response prediction to single agents and combination treatments.

The multiplicity of ongoing efforts to identify, characterize, and validate new biomarkers and therapy targets for TNBC (Bosch et al., 2010) shows the complexity of such a goal and the need for well designed and rigorous studies, at all levels of investigation, to resolve conflicting results, which halt or slow down the advancement of this field. Systems biology can be an important tool for elucidating crosstalk between pathways and understanding temporal effects of tumor behaviour.

5.2. *Targetable pathways and cellular processes*

Proliferation and death are uncommon features for non-tumorigenic, healthy cells and therefore these events are under tight control at many levels. Fluxes through pathways are intricately balanced by stimuli of different nature and checked by multiple feedback control mechanisms at cellular, tissue, organ and organism level. Backup mechanisms to assure proper functioning in case the first level of control goes awry are present in normal cells. Tumor cells have accumulated defects that allow them to bypass the normal control mechanisms that check proliferation. For tumor cells to grow and proliferate, they must evade checks on several cellular controls. The increased energy requirement of tumor cells causes metabolic stress at the level of nutrient and oxygen supply. The accumulation of gene amplifications, translocations and mutations must evade cellular control on DNA damage during cell cycle checkpoints. Whereas most cells can only survive with close contacts to their neighbours, metastatic tumor cells must overcome the apoptosis directed controls associated with anoikis. Within one tumor, large differences exist in local environment that may cause cells at the edge to proliferate, while cells that are deprived of nutrients and oxygen may undergo necrosis, therewith exposing their neighbours to stressful conditions. Histopathology can make this cellular diversity visible, but molecular techniques like gene expression profiling and proteomics determine the average of all these microenvironments in a selected tissue sample.

5.2.1. *Metabolism*

Rapidly growing tumors need a high supply of nutrients and oxygen. Tumor cells have developed several mechanisms to satisfy their needs in these respects, varying from assuring a good nutrient supply by stimulation of new blood vessels formation, to adjustments in glucose metabolism and methods to survive at low oxygen concentrations. Although debatable whether it is the only cause, hypoxia promotes via the Hypoxia-inducible factor 1 (HIF-1) a change in metabolic pathways in tumor cells (Marin-Hernandez et al., 2009). Tumor cells derive most of their energy from glycolysis rather than from oxidative phosphorylation, even though the yield in ATP per glucose is reduced from 36 for the aerobic oxidative phosphorylation to 2 for the anaerobic process. Furthermore, the formation of lactic acid poses challenges for pH homeostasis in the cell. Glutaminolysis is used to produce sufficient amounts of precursors for synthesis of small molecule building blocks to sustain proliferation, which are usually provided by the TCA cycle. This shift in metabolic pathways makes tumor cells less sensitive to hypoxia and reactive oxygen species.

The flux through metabolic pathways is regulated at different levels, ranging from de novo synthesis of proteins to activation of limited duration of proteins via covalent modification and allosteric activation or inhibition by substrates or products of a pathway. In regulation of the balance between anabolic and catabolic pathways, the protein kinases Akt, with constituents of the Akt pathway like mTor, and AMPK play crucial roles. Akt activity for example is stimulated by a multitude of growth factors via receptor tyrosine kinase induced signaling, transmitted by PI3K, and by small intermediate metabolites like retinoic acid and prostaglandin. AMPK senses a low energy state by the AMP/ATP ratio. Not only do Akt and AMPK directly regulate the balance between the pathways, they also affect levels of tumor suppressor protein p53 by either promoting its degradation or stimulating its synthesis. Discussing the subtilities of the pathways and their regulation is too farfetched for this review. The role of p53 in regulation of metabolism in tumor cells has been recently reviewed (Vousden and Ryan, 2009).

Whereas an abundant energy supply leads to cell proliferation, an insufficient energy supply leads to autophagy and

cell death. Autophagy is part of everyday normal cell growth and development eliminating malfunctioning proteins, reshaping membranes, recycling of receptors, etc. During nutrient starvation, autophagy can provide additional energy or building blocks by the breakdown of non-vital cell components, ensuring that vital processes can continue. mTor plays an important regulatory role in this process. Prolonged starvation will lead to cell death and necrosis, events often found in tumors. The products that result from necrosis, pose additional stress on neighbouring cells and lead to induction of the NFkB pathway.

5.2.2. *Evasion of cell-cycle control*

Under conditions of sufficient nutrient supply, proliferation can take place. In most non-tumorigenic cells, the G0 phase is the default state. Strong stimuli are required to leave this state. When it occurs, the steps of the cell cycle are tightly controlled. In many cancer cells, the control on the cell cycle progression is faulty, permitting cells with errors in DNA to proceed to the next step of the cycle and produce more genetic diversity. TNBC suffers from frequent mutations in the tumor suppressor protein p53 and in Rb pathway inactivation (Schneider et al., 2008), whereas mutations in c-Myc are relatively rare (Rodriguez-Pinilla et al., 2007).

The Rb pathway in the G1/S checkpoint is activated by phosphorylation of retinoblastoma by cyclin/CDK complexes, leading to the activation of transcription factor E2F. The activity of cyclin/CDK is stimulated by the proto-oncogene Myc, which in turn is activated by e.g. the HER1 (or EGFR) pathway. Myc regulates the expression of a multitude of proteins (Bouchalova et al., 2009).

Upregulation of CDNK2A, often observed in TNBC, inhibits the CDK2 activity and therewith promotes inactivation of p53, resulting in blockage of the senescence and apoptosis pathways. The increased expression of CDNK2A and related CDNK2's allows bypassing the checks in cell-cycle control in the Rb pathways and promotes progression into the G1/S phase (Schneider et al., 2008; Musgrove and Sutherland, 2009). BRCA1 and BRCA2 also have a function in cell-cycle control via their regulation of p53 activity (Liu and Kulesz-Martin, 2001). After amplification of DNA, the daughter strands must be separated. Topoisomerases, notably TOP2A, are essential in this process. TOP2A amplification is most frequent in HER2-overexpressing tumors (Durbecq et al., 2003) and although a triple-negative phenotype was associated with TOP2A expression, no amplification was found (Tan et al., 2008).

DNA damage repair is not only essential during cell division, but also is a continuous process. Cells are exposed to factors that can cause damage to their DNA. These defects must be repaired (Jackson and Bartek, 2009). When that is not possible, cells go into apoptosis. Exposing cells to chemotherapy or radiation therapy causes irreparable damage in most cells, but some are more resistant than others. BRCA1 and BRCA2 are involved in DNA repair. Cells lacking either of these proteins are more susceptible to poly(ADP-ribose) polymerase (PARP) inhibitors (see below).

5.2.3. *Invasion and metastasis*

Cells thrive only when they are in close contact with neighbouring cells and the extracellular matrix. When cells become detached from their natural environment, they die by anoikis. Tumor cells escape this fate when they undergo the epithelial mesenchymal transition (EMT) to acquire a mesenchymal-like phenotype and can become invasive. The essential features of EMT are the disruption of intercellular contacts and the enhancement of cell motility, thereby leading to the release of cells from the parent epithelial tissue. The EMT enables cells to penetrate vessel endothelium and enter the circulation to form distant metastasis (Guarino et al., 2007). The Basal B subgroup of breast cancer has enhanced invasive properties and a predominantly mesenchymal gene expression signature, distinct from subgroups with predominantly luminal (termed Luminal) or mixed basal/luminal (termed Basal A) features (Guarino et al., 2007). Epithelial mesenchymal transition has long been associated with breast cancer cell invasiveness (Neve et al., 2006). During EMT the abundance of the proteins N-cadherin, Vimentin, several metalloproteinases and transcription factors like Snail1 (Snail), Snail2 (Slug) increases, while decreased concentrations are observed for E-cadherin, Desmoplakin, Cytokeratin and Occludin. Several interconnected transduction pathways and a number of signaling molecules potentially involved have been identified. Growth factor driven receptor tyrosine kinases play an important role, but Wnt, NFkB, integrin and TGFβ signaling also contribute (Lee et al., 2006; Giampieri et al., 2009). Most of these pathways converge on Akt (Iliopoulos et al., 2009) and on the downregulation of the epithelial molecule E-cadherin, an event critical in tumor invasion and a 'master' programmer of EMT (Guarino et al., 2007).

EMT is accompanied by changes inside the cells, such as reorganisation of the actin cytoskeleton and regulation of focal adhesion (reviewed by Jiang et al., 2009).

5.2.4. *Integration of signals*

As illustrated above, different processes take place either simultaneously or sequentially in tumor cells. Signals from the environment can also lead to conflicting stimuli as input. The different routes converge on central signaling hubs. Coordination of the processes requires a tight orchestration of these events, with Akt, mTor, p53, HIF-1, NFkB and Myc being some of the leading conductors, though at different time scales.

Whereas the Akt/mTor kinases regulate rapid responses of short duration, proteins like p53, Myc and HIF influence the expression of large numbers of genes.

HIF-1alpha is involved in the activation of numerous cellular processes including resistance against apoptosis, overexpression of drug efflux membrane pumps, vascular remodeling and angiogenesis, as well as EMT and metastasis (Marin-Hernandez et al., 2009) and in immune reactions and inflammatory response (Hellwig-Burgel et al., 2005).

5.3. *TNBC biomarkers for assessment of prognosis and therapy targeting*

Parameters like the proliferating index, ploidity, presence of p53, cytokeratins, HER1, and numerous other molecular alterations, may also be useful for prognostic evaluation, for predicting therapeutic response and for guiding patient management.

Given the poor outcome of TNBC despite cytotoxic treatment, alternative approaches are currently explored for possible application at the clinical level (Table 1).

5.3.1. *HER1*

HER1 (EGFR) is a receptor tyrosine kinase, that belongs to the HER family of transmembrane receptors. HER1 gene is located on 7q12 and its protein product – 170-kD glycoprotein – plays an important role in cell proliferation, migration and protection against apoptosis mediated by subsequent activation of intracellular pathways. After binding of epidermal growth factor (EGF), the HER1 receptor can dimerize with other members of the HER family, and it has to create homo– or heterodimers to be functionally active (Hynes and Lane, 2005).

Increased HER1 expression is detected in about 40% of breast carcinomas. Particularly, HER1 expression is higher (up to 80%) in TNBC and metaplastic carcinoma (mostly basal-like), where it possibly substitutes ineffective, but otherwise major proliferation/survival pathways of breast cancer induced by expression and activation of HER2, ER and PR proteins. Currently, however, HER1 gene status is not used in clinical practice to guide therapy in breast cancer. HER1 protein could be targeted by monoclonal antibodies and/or synthetic tyrosine kinase inhibitors (TKIs). Monoclonal antibodies (cetuximab, panitumumab) are now clinically used in the treatment of colorectal cancer and head and neck carcinoma. TKIs are also important in the therapy of pancreatic and non-small cell lung cancer.

Given the subset of TNBC which overexpresses EGFR (Viale et al., 2009), targeting EGFR seems to be a rational approach. Although cetuximab monotherapy has little clinical activity, in combination with chemotherapy it may enhance tumor response (Corkery et al., 2009; Carey et al., 2008). HER1 targeted treatment with cetuximab in breast cancer has not produced satisfactory results probably because of the activation of downstream signaling pathways (Shiu et al., 2008) or because of inadequate patient selection. Evaluation of the results of studies testing TKIs (erlotinib, gefitinib) in breast cancer indicated that HER1 protein must be present in targeted tumor tissue to obtain valuable treatment results (Agrawal et al., 2005). Furthermore, it might be better to target more than one of these receptors simultaneously. Thus, HER1 assessment could reveal a particular group of breast cancer patients with probably good response to HER1 targeted therapy.

5.3.2. *TOP2A*

TOP2A gene is located on 17q21–22, encoding topoisomerase II alpha, which appears to be a molecular target for anthracyclines and hence is predictive of response to anthracycline therapy. Good response to anthracyclines is associated with TOP2A amplification, while deletion may be accompanied by resistance. (Burgess et al. (2008) identified in a nonselected series, TOP2A expression levels as major determinants of response to doxorubicin, which is a topoisomerase II inhibitor, and showed that suppression of TOP2A levels produces resistance to doxorubicin in vitro and in vivo.

Recent publications describe TOP2A amplification in 2.7–8.8% of HER2 non-amplified breast cancers (Knoop et al., 2005). Adjuvant anthracycline treatment of TNBC patients was shown to be associated with poor response in patients with low expression of TOP2A protein. Microarray expression analysis indicated that in a subgroup of TNBC there is a significant downregulation in PTEN and TOP2A which might partly explain observed differences in response to chemotherapy in TNBC (Weigelt et al., 2009). It should be noted, however, that patients with a pathologic complete response to anthracycline-based neoadjuvant chemotherapy had a good prognosis regardless of subtype (Carey et al., 2007).

5.3.3. *c-Myc*

c-Myc is a major transcription factor that encodes nuclear DNA binding proteins that regulate cell growth, transformation, angiogenesis, cell-cycle control and apoptosis; c-Myc is directly involved in regulating up to 15% of all human genes. c-Myc amplification is one of the most frequently detected aberrations in breast cancer. Amplification is clearly associated with poor prognosis. In ER-positive breast cancer cells c-Myc expression is under the regulation of estrogen, but in ER-negative/PR-negative cells c-Myc constitutive expression level is usually high. C-MYC protein may affect the response to chemotherapy, probably through DNA damage response regulation (Aulmann et al., 2006; Corzo et al., 2006). BRCA1 is linked to transcriptional regulation through interaction with Myc. Hence, the Myc role in BRCA1-associated breast cancer makes it an important target in basal-like/triple-negative breast cancers.

It was found that only 4% of basal-like carcinomas showed Myc amplification, compared to 8.75% and 10.7% of luminal and HER2 tumors, respectively (Rodriguez-Pinilla et al., 2007). Myc amplification displayed a significant association with shorter metastasis-free and overall survival and proved to be an independent prognostic factor in multivariate survival analysis. Thus, Myc amplification is not associated with "basal-like" phenotype and proved to be an independent prognostic factor for breast cancer patients treated with anthracycline-based chemotherapy.

Conflicting results were obtained using microarray technology and performing a detailed kinetic study of genes that respond to MYCN or MYCNDeltaMBII induction in primary human fibroblasts (Chandriani et al., 2009). An overlapping set of 398 genes was designated as "Core MYC Signature" and used for further analysis. Comparison to a panel of breast cancers revealed a strong concordance in gene expression between the Core MYC Signature and the basal-like breast tumor subtype. This concordance was supported by the higher average level of Myc expression in the same tumor samples. The Core MYC Signature has clinical relevance as this profile can be used to deduce an underlying genetic program that is likely to contribute to a clinical phenotype and may predict clinical responsiveness to drugs that are designed to disrupt Myc-mediated phenotypes.

5.3.4. *VEGF receptor*

Targeting angiogenesis by the monocloncal antibody bevacizumab, added to paclitaxel, was shown to be beneficial in terms of prolonged progression free survival (from 5.9 to 11.8 months) in a randomized phase III trial which included patients with both ER- and PR-positive, as well as ER- and PR-negative disease. The majority of patients in this study were HER2-negative and in the subset analysis ER-/PR-positive and negative patients had a similar benefit from bevacizumab.

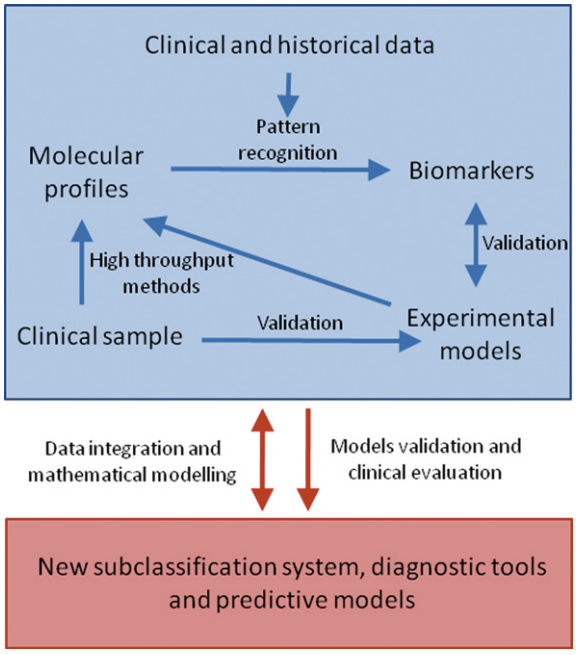


Figure 3 – The flow, analysis and integration of multi-dimensional molecular, biological and clinical data from different biomedical sources leading to a new classification system, diagnostic tools and predictive models in breast cancer.

In the neoadjuvant setting preliminary results from a non-randomized phase II study have been reported which added bevacizumab to cisplatinum. Effects were modest (complete pathological response in 15%) while toxicity was considerable (Ryan et al., 2009). Also, the effects of the small molecule TKI sunitinib, which targets angiogenesis by binding the intracellular domain of cell surface receptors, is modest in metastatic breast cancer and is achieved at the price of significant toxicity (Burstein et al., 2008). In a recently presented randomized phase II trial it was shown that the addition of the another TKI, sorafenib, to capecitabine increased progression free survival from 4.1 to 6.4 months compared to capecitabine monotherapy, again at the cost of considerable toxicity (Baselga et al., 2009a). Of note, these studies with TKIs have not been specifically designed for patients with TNBC.

5.3.5. *The role of PARP inhibition*

One of the promising new agents for the treatment of TNBC are poly(ADP-ribose) polymerase (PARP) inhibitors. A subset of PARPs is specifically involved in the detection of single strand breaks and the recruitment of base excision repair elements. In cells with alterations in BRCA function, as is often seen in TNBC, DNA repair processes are largely dependent on PARPs. Inhibition of PARP in these cells ultimately leads to cell death (McCabe et al., 2006; Farmer et al., 2005). Phase I data for the PARP inhibitor olaparib (AZD2281) suggest antitumor effectiveness in cancers associated with the BRCA1 or BRCA2 mutation (Fong et al., 2009). Preliminary results of a recent randomized phase II study with the PARP inhibitor BSI-201, combined with carboplatin and gemcitabine in metastatic TNBC, showed significantly improved clinical benefit rate, progression free survival and overall survival (O'Shaughnessy et al., 2009).

In conclusion, the development of targeted agents is urgently needed for patients with TNBC. Although promising agents are being developed, no targeted treatment is yet available for routine clinical practice.

6. The promise of multidimension technological approaches

The multiplicity of interacting related factors across the entire genome, the integration of pathways responsible for molecular and pathological factors, and the evolution of imaging approaches, require handling of redundant network interactions (Fig. 2) rather than simple linear systems, to design more robust prognostic and predictive models and treatment algorithms for TNBC and other breast cancer subtypes (Goldhirsch et al., 2009).

Powerful technologies allowing us to take a more comprehensive overview of these multiple events are represented by multivariate data analysis tools and integration of their results into a suitable systems biology approach.

6.1. *Multivariate analysis of multi-modal, multi-dimensional 'omics' data*

In the search for biomarkers for breast cancer diagnosis and recognition of subtypes, such as TNBC, for predictive markers of response to treatment, and in the attempt to understand the biological processes/pathways involved in breast cancer (which could also lead to new biomarkers and therapy targets), high dimensional data are frequently used. These data result from the different modern 'omics' related analyses (e.g. microarrays in genomics, mass spectrometry combined with some kind of separation, such as liquid chromatography (LC), proteomics or NMR-based metabolomics). Multivariate data analysis techniques are especially suited for this kind of high and multi-dimensional data sets, in order to improve medical knowledge discovery and integrate various sources of biomedical information (Fig. 3). However, analysis and integration of (bio)medical data remain a challenging task because of the highly complex nature and the large diversity of the data. Therefore, these approaches, which include data modeling, data visualization and data mining, require a multidisciplinary research environment, unifying expert knowledge from the field of medicine, biochemistry, bioinformatics, biology, chemistry, machine learning and statistics. Data analysis processes and, in particular, the integration of heterogeneous biomedical information provide improved prevention, diagnosis and treatment of disease (e.g. cancer), since the technology enables to better understand the underlying biological relations. Discovery of these relations results in the development of new screening techniques and strategies in the early diagnosis of cancer, including tools for the monitoring and interpretation of disease progression to expand the possibilities and effectiveness of already existing therapies. There is an increasing interest in using biological characteristics to subcategorize cancer within a histological class to provide an improved diagnostic system. For instance, breast tumors could be classified into subtypes, having distinct differences in gene expression patterns, by means of multivariate

analysis techniques (Perou et al., 2000, 1999). In addition, evidence has been reported that the incorporation of gene expression signatures into clinical risk stratification can refine prognosis, resulting in optimized therapeutic strategies for breast cancer (Acharya et al., 2008).

In the context of breast cancer all kinds of studies are performed using 'Omic' data. Most studies involved one type of data, mostly targeted at classification: healthy versus tumor, benign versus tumor, good prognosis versus bad prognosis, etc. Regarding for instance transcriptomic data, Chen et al. (2009) recently combined this data with prior knowledge from already known pathways in order to improve prediction (they applied supervised principal component analysis (PCA) to aggregate genes in so-called supergenes which are mostly related to outcome). Proteomics data is widely used in breast cancer recognition. An overview has been recently presented by Gast et al. (2009), based on measurements in various matrices like serum, plasma, nipple aspirate fluid, saliva, etc., although the data are multivariate and high dimensional advanced multivariate techniques are rarely used (see e.g. Belluco et al., 2007). An additional problem was the relative low number of samples involved. Furthermore, most of the emphasis was on classification and less on identification of the peaks involved. Metabolites are, in contrast to proteins, much easier to identify. Altered metabolic activities of cancer cells have been observed in many studies. Data in these studies result from mass spectrometry, and in vivo and ex-vivo MRS (e.g. HR-MAS MRS of breast cancer tissues). An overview of ex-vivo MRS studies is presented by Sitter et al. (2009). Again, also in these studies relative simple univariate statistical tests are used to differentiate benign from cancer tissues concentrating on choline related peaks. A broader overview of the applications and perspectives of metabolomics is presented by Claudino et al. (2007). Multivariate techniques (Partial Least Squares, Probabilistic Neural Networks, Support Vector Machines) are applied in e.g. Bathen et al. (2007) on HR-MAS spectra of breast cancer tissue biopsies, in Woo et al. (2009) using endogenous steroids and nucleosides profiling from GC–MS and LC-MS measurements of urine, and in Henneges et al. (2009) using a limited set of metabolites from LC-MS spectra of urine samples. Combining the data and information resulting from the various 'omics' techniques is expected to improve prediction and classification. An example of this kind of studies is presented in Chuang et al. (2007). The authors applied a protein-network-based approach to analyze the expression profiles of the two cohorts of breast cancer patients previously reported by two other groups. The protein–protein interaction network information was extracted from yeast data from literature. Still mostly statistical techniques were applied to combine the information, after which logistic regression was used to classify the patient data. An improved accuracy was achieved compared to the original articles.

Although having their value in the diagnosis, sub-classification and prognosis prediction of breast cancer, most of these studies provided just limited or partial additional insight on the complex biological processes involved in breast tumor formation, the biological differences underlying the various types of breast tumor and prognosis, and the processes influencing the response differences of the various therapies. Recent functional genomic and proteomic approaches include protein–protein, protein–DNA or other 'component–component' interaction mapping (interactome mapping), systematic phenotypic analyses (phenome mapping) and transcript or protein localization mapping (localizome mapping). 'Omic' approaches have already been applied to many biological processes, leading to large lists of genes, proteins and metabolites potentially involved in the corresponding biological processes. As stated earlier, an improvement is expected in combining the data and the results of the various single 'omics' approaches leading to an approach within Systems Biology called "top-down" modeling of the biological processes. Examples of studies more or less aiming at this goal are in Pujana et al. (2007), related to breast cancer and in Ergűn et al. (2007), related to prostate cancer, whereas Nam et al. (2009) is on the border between the application of various data sources to improve classification and the use of these various sources to obtain better insight. In Nam et al. (2009), gene expression data sets of breast cancer and normal subjects, transcriptional regulation information and metabolic pathway information from appropriate databases were combined. Using statistics the authors tried to find altered metabolism (altered pathways) which then lead to a set of potential biomarkers. The latter were then tested in urine samples of various types of breast cancer patients and healthy individuals. Six biomarkers appeared to be statistically different, but the area under the receiver operating characteristic (ROC) curve improved when the biomarkers were combined using multivariate classifiers. Beside this, the candidate biomarkers were tested for potential use as indicators of cancer progression. Pujana et al. (2007) started from four known breast cancer-associated genes and their products, BRCA1 and BRCA2 and ATM and CHEK2, called 'reference genes/proteins', and used data from gene transcript abundance measurements from samples of healthy human tissues or organs and three cell-lines, to find genes which potentially are functionally associated (using correlation). Next, these potential functional associations were investigated and ranked by integration with functional associations found in largely non-overlapping 'Omic' data sets by using interologous functional relationships from four species. This ultimately has led to genes/proteins which later were proven to have a functional relationship with the reference genes/proteins. Ergűn et al. (2007) used an approach called mode-of-action by network identification (MNI). The goal is to find transcript (genes) which are mostly inconsistent with 'normal' regulatory gene networks and which could be mediators of a disease. These 'normal' regulatory gene networks are first trained using a large set of microarray expression data from 13 projects spanning 7 cancer types by training a relative simple mathematical model. PCA was applied to reduce the massive dimensions of the data. Then using test data from three types of prostate cancer, those genes were identified which could potentially be mediators for each type. Shen et al. (2009) developed a technique which could be used in this context. They developed a latent variable model to allow multiple data types for the purpose of integrative clustering. Additional progress in Systems Biology is expected from bioinformatic and chemometric techniques, which can handle and intelligently explore and apply the complex multi-modal multi-dimensional 'omics' data.

An essential aspect of the use of multivariate analysis tools in clinical applications is setting up research studies at more than one medical center. A multi-center approach allows researchers to address a larger population, which is decisive for a better understanding of the disease and the underlying biological relations (Garcia-Gomez et al., 2009). Depending on the disease, the number of data is often limited. Large scale multi-center data collection overcomes the problem of sparse data, scattered over local centers' databases and potential bias due to single-center effects. Therefore, intelligent multivariate analysis should be applied to a centralized collection of multi-center data or to a distributed database, where each node represents a single center. Furthermore, analysis of multi-center data, including different types of data, requires the introduction of a common terminology, which is related to the design of ontologies. In addition, the data of the different centers have to be compatible, demanding a fixed protocol for data acquisition and acquisition conditions. In conclusion, results from the analysis of multi-center studies can potentially unravel the biological dynamics of disease and improve healthcare.

An illustration of the use of multivariate analysis in multi-center studies is the development of decision support tools within the context of the European projects IOTA, eTUMOUR and HealthAgents (http://www.etumour.net; http://www.healthagents.net; Van Holsbeke et al., 2007; Timmerman et al., 2005). The multi-center project IOTA focuses on the characterization of ovarian tumors. Ultrasound images and clinical data from 1066 patients were acquired during the period 1999–2002 (Van Holsbeke et al., 2007). Mathematical models to predict the risk of malignancy were developed and were internally and externally validated on nearly 2000 patients with adnexal tumors in 20 centers throughout the world during the periods 2002–2005 and 2005–2007, respectively (Timmerman et al., 2005). Currently, the IOTA project investigates the incorporation of proteomics data in the process of pattern analysis. The multidisciplinary eTUMOUR and HealthAgents projects aimed to develop and validate decision support tools for brain cancer diagnostics by combining multimodal multi-dimensional clinical data, 'Omic' data (genomics, metabolomics, transcriptomics) and bioimaging data (in vivo magnetic resonance spectroscopy (MRS), magnetic resonance spectroscopic imaging (MRSI), magnetic resonance imaging (MRI), which were acquired over multiple centers from all over the world and stored in a database system (Lluch-Ariet et al., 2008). Models for tumor diagnosis were developed from these data and included artificial neural networks, support vector machines, discriminant analysis and nearest neighbour classifiers (Bishop, 1995; Duda et al., 2001; Vapnik, 2002). Furthermore, these models were included in a graphical user interface to make them available for clinicians. The existing breast cancer consortia either focus on 'Omic' data only (such as METAcancer (http://www.metacancer-fp7.eu), MetaBre (http://www.metabre.org), TRANS-BIG (http://www.breastinternationalgroup.org/Research/TRANSBIG.aspx), or on imaging data only (such as MammoGrid (http://www.cems.uwe.ac.uk/cccs/project.php?name=mammogrid) using mammographic data). This overview enlightens the need for setting up similar European-wide or worldwide consortia for breast cancer diagnostics by combining all data, from the nano-level ('Omic' data) up to the macro-level (in vitro, in vivo) so as to further improve breast cancer diagnostics and prognosis.

6.2. *The power of systems biology approaches in cancer research*

Systems biology is an area that has both provided many promises to support understanding of cellular regulation and that requires new ways of producing and analyzing data. Experimental research and computational modeling of biological processes ranges from small regulatory networks to full-scale genomic models. Depending on the specific question, they are of different value. Clinically relevant studies need models that provide testable predictions.

Network models comprise interaction information of all compounds of a type such as proteins or genes. Model analysis may reveal insight into hidden relations. A respective study has, for example, linked breast cancer susceptibility with centrosome dysfunction (Pujana et al., 2007): by combining expression profiles with functional genomic and proteomic data, 118 genes were aligned with potential functions. Among those genes is the one coding for hyaluronan-mediated motility receptor, HMMR, and a functional association with the breast cancer-associated gene BRCA1 was predicted. Another large-scale study has systematically linked disorders and genes associated with the diseases (Goh et al., 2007).

Signaling pathways by which cells receive and process external information are a subject of intensive study in experimental and computational systems biology. Amongst the most carefully studied pathways are the EGFR (Samaga et al., 2009; Schoeberl et al., 2002), Wnt (Lee et al., 2003), Jak/STAT (Swameye et al., 2003), TGFβ (Zi and Klipp, 2007; Schmierer et al., 2008), and NFkB pathways. As example for the relevance of signaling pathways for breast cancer, it has been demonstrated recently that TGFβ signaling switches breast cancer cell motility (Giampieri et al., 2009). The models are frequently formulated as sets of ordinary differential equations (ODEs) describing the temporal evolution of the involved components and their activity. They serve various purposes, among them most notably (i) just to understand their architecture and the observed dynamics and (ii) to rationalize the interdependence of independently measured data such as ligand supply, phosphorylation states, protein interactions, and gene expression effects (e.g. Chen et al., 2009). Dynamic features such as the effect of positive or negative feedback (e.g., Bluthgen et al., 2009; Kholodenko, 2000), or crosstalk between different pathways (Borisov et al., 2009) have been studied extensively.

Cell cycle progression is highly regulated and failure to respond to checkpoints or external signals may be a first step to cancer. Mathematical modeling of cell cycle has initially focused on model organisms such as frog eggs or yeast (Chen et al., 2004, 2000), but also on mammalian cells (Alfieri et al., 2009). Again, these models first of all serve to test our understanding of the structure or wiring of cell cycle machinery, but they also analyze detailed questions such as the effect of mutants or of unreplicated DNA on cell cycle progression (Zwolak et al., 2009). While including more and more details of the protein machinery and other components into models, the

regulation of cell cycle by nutrition, signaling, or checkpoints becomes more and more predictable based on model simulations.

Development, stem cell differentiation and cell reprogramming have been described with mathematical models on different levels. The Davidson lab has delivered gene regulation networks of Xenopus eggs (Davidson et al., 2003) with ever increasing detailedness over last years, which contain activating and inhibiting influences of developmental genes on each other and also provide a link to regulatory signaling pathways such as Wnt pathway. Translating this static representation into a dynamic model is a challenge on its own, but appears feasible with upcoming data. A computational ODE model describes the dynamics of the core network governing stemcellness and cellular differentiation (Chickarmane and Peterson, 2008).

Target prediction is an important goal of systems biology approaches. Based on sufficiently well described networks and properties of the compounds' interaction, mathematical models can be useful to predict targets for treatment and test the outcome of different target positions, treatment strengths, target combinations or temporal combination scenarios (Fitzgerald et al., 2006; Schulz et al., 2009). This field is only in its infancy, since there is still a lack of sufficiently well understood networks, however there are already very promising examples among signaling pathway models, such as the study of the ErbB network with sensitivity analysis (Schoeberl et al., 2009), which identified ErbB3 as a key node in response to ligands. Boolean modeling (assigning activity values of 0 and 1 to nodes which are updated in time based on their inputs from other nodes) of the ErbB receptor regulated G1/S transition has revealed new potential targets in case of de novo trastuzumab resistance in breast cancer.

Chronotherapy is a field that is based on the fact that living beings exhibit various rhythms, such as cell cycle, circadian or annual rhythms. Circadian rhythms have been investigated, their components identified (Ueda et al., 2005) and their dynamics described with mathematical models (Brown et al., 2008). It has turned out that cancer treatment has different effects if supplied at different times of the day (Levi and Schibler, 2007).

Taken together, different directions of systems biology combining experimental and computational analysis have already made first promising contributions to understand aspects of cellular regulation and dysfunction. Further investigations need precise and reproducible data and sensible mathematical descriptions to produce predictive and helpful models in cancer research (Aebersold et al., 2009).

7. Conclusions

7.1. Challenges and limitations

A better understanding of pathological mechanisms of TNBC onset and progression, including the still unclear association with BRCA1 mutations, and the causes of TNBC phenotypic heterogeneity is expected to improve diagnosis, prognosis, treatment and prevention of these cancers. A major limitation to a robust delineation of differences between basal-like and triple-negative breast cancer subtypes is the present lack of consensus on immunohistochemical assays to be used in the clinical setting, and the still unclear relationships between traditional diagnostic classifiers and genomic aberrations.

7.2. Limitations of the present therapeutic options

Larger prospective clinical trials are needed to optimize chemotherapy treatments and predict the response of different TNBC subgroups to cytotoxic agents. Although promising agents are presently being developed and tested, no targeted treatment algorithms are as yet available for TNBC at the clinical level.

7.3. Perspectives

Improved awareness of the significance of gene expression signatures combined with a holistic characterization of cell signaling pathways' aberrations, may lead to more suitable prognostic and predictive models, to more adequate algorithms of therapy targeting, and improved molecular imaging approaches for earlier and non invasive tumor response monitoring. Well designed and rigorous studies are needed to resolve still conflicting or partial results.

A possible breakthrough in the field may derive from application of robust multivariate data analyses on well programmed collections of multi-modal, multi-dimensional data sets provided by the simultaneous use of different 'omics' at pre-clinical and clinical level, and processing of the results in the frame of a suitable Systems Biology approach.

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