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Vered Stearns
Editor

Novel Biomarkers in the Continuum of Breast Cancer

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About the Breast Cancer Research Foundation

The Breast Cancer Research Foundation (BCRF) is committed to being the end of breast cancer by advancing the world's most promising research. Since its founding by Evelyn H. Lauder in 1993, BCRF has funded investigators who have been deeply involved in every major breakthrough in breast cancer prevention, diagnosis, treatment and survivorship. In 2015, BCRF committed nearly $54 million to support 240 scientists at leading medical institutions internationally, making it one of the world's largest non-governmental funders of breast cancer research. By investing 91 cents of every dollar directly in its mission, BCRF remains one of the nation's most fiscally responsible nonprofits. It is the only breast cancer organization to hold an "A+" from CharityWatch and has been awarded Charity Navigator's highest rating of four stars 13 times since 2002. For more information, please visit: www.bcrfcure.org

Series Editors

Larry Norton, M.D.
Deputy Physician-in-Chief for Breast Cancer Programs; Medical Director, Evelyn H. Lauder Breast Center; Norna S. Sarofi m Chair in Clinical Oncology, Memorial Sloan Kettering Cancer Center

Dr. Norton is a board-certified medical oncologist with broad interests in cancer prevention, diagnosis, and treatment. In his clinical practice, he cares for women with breast cancer and is now Deputy Physician-in-Chief for Breast Cancer Programs at Memorial Sloan Kettering and Medical Director of the Evelyn H. Lauder Breast Center.

His research concerns the basic biology of cancer; the mathematics of tumor causation and growth; and the development of approaches to better diagnosis, prevention, and drug treatment of the disease. He is involved in many areas of research, including identifying the genes that predispose people to cancer or that cause cancer and developing new drugs, monoclonal antibodies that target growth factor receptors, and vaccines. A major milestone in his research career was the development of an approach to therapy called "dose density" or "sequential dose density," which maximizes the killing of cancer cells while minimizing toxicity.

Dr. Norton is currently the principal investigator of a program project grant from the National Cancer Institute (NCI) that is aimed at better understanding breast cancer in the laboratory and in bringing these advances into clinical practice. On

a national level, he was formerly the Chair of the Breast Committee of the NCI's Cancer and Leukemia Group B. He was President of the American Society of Clinical Oncology (ASCO) from 2001 to 2002 and was appointed by President Clinton to serve on the National Cancer Advisory Board (the board of directors of The NCI). He has served as Scientifi c Director of the Breast Cancer Research Foundation since its inception in 1993.

Among many awards over the course of his career, he was honored to receive ASCO's David A. Karnofsky Award and The McGuire Lectureship at the San Antonio Breast Cancer Symposium. He is an author of more than 350 articles and many book chapters; has served as a visiting professor throughout USA, Canada, South America, Europe, Israel, and Asia; and has also trained many cancer doctors and researchers.

Clifford A. Hudis, M.D.
Chief, Breast Medicine Service
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Dr. Hudis is Chief of the Breast Medicine Service and Attending Physician at Memorial Sloan Kettering Cancer Center (MSKCC) in New York City, where he is co-Leader of the Breast Disease Management Team and a Professor of Medicine at the Weill Cornell Medical College. He is the immediate Past President of the American Society of Clinical Oncology (ASCO), Chairman of BCRF Scientific Advisory Board, co-Chair of the Breast Committee of the Alliance (formerly the CALGB), and a member of the Steering Committee of the Translational Breast Cancer Research Consortium (TBCRC).

A 1983 graduate of the Medical College of Pennsylvania (a combined 6-year BA/MD program with Lehigh University), Dr. Hudis trained in Internal Medicine in Philadelphia before completing his fellowship in Medical Oncology at MSKCC. He joined the faculty in 1991.

Dr. Hudis's research includes the development of a wide range of novel drugs and the study of relevant correlative science endpoints in breast cancer. With his collaborators both at MSKCC and elsewhere, his personal research is focused on understanding the mechanisms that link diet, obesity, infl ammation, and breast cancer risk and outcomes. Building on their discoveries of low-grade infl amma-

tion in association with overweight and obesity, Dr. Hudis and his colleagues are studying interventions that may reduce the risk, and return, of breast cancer. Across his service at MSKCC, his team conducts studies of novel-targeted therapies in advanced disease, of new strategies in the adjuvant and neo-adjuvant settings, and of risk reduction.

Preface

Today more than ever, we recognize that breast cancer is a collection of many unique diseases with some common features. Advances in technologies have allowed researchers to simultaneously study alterations in thousands of genes or gene products that may be present in small amounts of tissue or in blood. Alterations in individual or multiple genes or gene products represent biomarkers that indicate ultimate outcomes (prognostic) or responses to treatment (predictive). Understanding how to apply biomarkers in clinical settings requires a rigorous developmental process. Validated biomarkers may represent distinct characteristics that indicate differential outcomes and that could influence treatment recommendations for individual patients.

This volume represents a collection of chapters centered on standard and emerging biomarkers in the continuum of breast cancer. Distinguished authors review markers of risk, markers of chemoprevention, markers that predict response to standard therapy such as endocrine agents or anti-HER2 agents, and markers capable of influencing treatment decisions that include pharmacogenetics, metabolomics, tumor heterogeneity, circulating tumor cells, and circulating DNA. The authors are all experts in their respective fields and, in this volume, they provide not only a review of the current status of the biomarker, but also their own perspectives on how biomarkers may be used in treatment and in future research directions. New technologies coupled with novel clinical-trial designs will allow us to advance the science of biomarker discovery and validation in a manner that is as rigorous as the process for developing and approving new medicines.

Vered Stearns MD

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I would like to thank the chapter authors for their generosity and dedication. I would also like to thank my colleagues at the Breast Cancer Research Foundation (BCRF) for inspiring me to lead this collection. In particular, I thank Dr. Larry Norton for his vision and for assembling a scientific powerhouse. Dr. Norton's stimulation of the scientific exchange of ideas and collaboration has undoubtedly led to some of the most cutting-edge advances in this field. I also thank Myra Biblowit, Margaret (Peg) Mastrianni, and the entire BCRF staff, a team whose accomplishments greatly exceed their numbers. Thank you for all that you do. I also want to acknowledge and express my most sincere thanks to the late Mrs. Evelyn Lauder for her dedication and focus on the cause of advancing breast cancer-related research, and to Mr. Leonard Lauder for ensuring that her legacy continues. I would like to thank the generous BCRF Donors for their support and commitment. The advances that BCRF researchers have made would not have been possible without you.

This work would also not have been possible without the guidance of my many mentors and colleagues over two decades, in particular Dr. Daniel Hayes and Dr. Nancy Davidson, without whom I would not be the investigator I am today. I also want to express my heartfelt appreciation to my patients and their loved ones: you have allowed me into your world, and you have inspired me to dedicate my scientific career to improving the outcomes of individuals diagnosed with breast cancer and those at risk for developing the disease. I thank Kristen Wagner-Smith for her tireless administrative support, and for always staying one step ahead. Most of all, my love and gratitude to my husband Max, and to my children, Shira, Keren and Eric, who have supported my academic pursuits with encouragement and warmth.

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Genomic Biomarkers for Breast Cancer Risk

Michael F. Walsh, Katherine L. Nathanson, Fergus J. Couch and Kenneth Offit

Abstract Clinical risk assessment for cancer predisposition includes a three-generation pedigree and physical examination to identify inherited syndromes. Additionally genetic and genomic biomarkers may identify individuals with a constitutional basis for their disease that may not be evident clinically. Genomic biomarker testing may detect molecular variations in single genes, panels of genes, or entire genomes. The strength of evidence for the association of a genomic biomarker with disease risk may be weak or strong. The factors contributing to clinical validity and utility of genomic biomarkers include functional laboratory analyses and genetic epidemiologic evidence. Genomic biomarkers may be further classified as low, moderate or highly penetrant based on the likelihood of disease. Genomic biomarkers for breast cancer are comprised of rare highly penetrant mutations of genes such as *BRCA1* or *BRCA2*, moderately penetrant mutations of genes such as *CHEK2*, as well as more common genomic variants, including single nucleotide polymorphisms, associated with modest effect sizes. When applied in the context of appropriate counseling and interpretation, identification of genomic biomarkers of inherited risk for breast cancer may decrease morbidity and mortality, allow for definitive prevention through assisted reproduction, and serve as a guide to targeted therapy.

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As inherited variation in DNA sequence has been shown to correlate with future disease risk, genomic tests constitute objective "biomarkers" of an individual's susceptibility to cancer [1]. A family history of breast cancer has long been thought to indicate the presence of inherited genetic events that predispose to this disease. Although familial breast cancer has been recognized since the nineteenth century, the detailed medical description of inherited breast (and ovarian cancer) in families took place in the 1970s [2, 3]. Subsequently, up to 15% of patients diagnosed with invasive breast cancer were shown to have at least one first-degree female relative (mother, sister, or daughter) with the disease. Testing for genetic biomarkers of risk has evolved over the past two decades to complement family history and physical findings. The most notable of these genetic biomarkers emerged from genetic analysis of families affected by multiple cases of early-onset (50 years of age) breast cancer, leading to the discovery of the breast cancer susceptibility genes, *BRCA1* and *BRCA2* [4–6]. The genetic mapping of *BRCA1* strongly suggested an inherited risk of breast cancer resulting from genetic alterations located on chromosome 17q21 [7]. The subsequent discovery of *BRCA1,* and later *BRCA2* [8, 9]*,* initiated widespread interest in hereditary breast cancer. These discoveries also galvanized resource allocation to investigators exploring translation of this information to improve clinical care for those with breast cancer susceptibility. In the late 1990s, mutations in *BRCA1/2* were established as the main contributors to familial breast cancer, and population specific frequencies of mutations in these genes were compiled [10–14]. In the 10 years following, the clinical utility and the benefits of clinical genetic biomarkers became evident, as genetic testing led to individualized risk reduction strategies including preventive surgeries, chemoprophylaxis and targeted therapies [15, 16].

Although genetic tests for cancer risk constitute "biomarkers" in a general sense, these genomic markers are distinct from non-genetic biomarkers in that they reflect the impact of modifiers of penetrance, population-specific differences in allele frequencies, and influence of gene-environment interactions. As genomic testing continues to evolve, biomarkers of various strength and significance are being routinely detected and gene-gene and gene-environment interactions are beginning to emerge [17–22]. Understanding the functional significance of genomic alterations is conceptually critical in assessing the potential utility of genetic variants as biomarkers. The type of alteration and the location of an aberration in a gene, i.e., a synonymous missense variant, a nonsense missense variant, a deletion/duplication, a translocation, or an inversion, all bear on the assessment of a gene test as a "biomarker" of inherited cancer risk. Thus, understanding the type of genetic change is as important as the fact that the gene is altered.

Novel biomarkers are being revealed by next generation sequencing and tend to be associated with low and moderate penetrance genomic loci [23]. As more is known, algorithms will be required to weigh multiple biomarkers simultaneously

and hence allow clinicians to most informatively provide recommendations pertaining to risk reduction surgeries, surveillance guidelines, family planning, apply novel therapies, and modify and dose-adjust existing therapies.

Genetics in Breast Cancer Predisposition

Although the ease of testing for different genetic biomarkers is appealing in the "information age," the ability to contextualize this information remains a challenge. Statements from the American Society of Clinical Oncology (ASCO) have stressed the process of offering predictive genetic testing and the elements pertaining to medical, social, and psychological consequences of positive, negative and yet to be determined results. Provided here is an updated algorithm of the contents of informed consent for genomic testing for inherited genetic changes (Table 1).

Genetic testing for mutations in *BRCA1, BRCA2,* and other breast cancer susceptibility genes has served as a model for the integration of genomics into the practice of personalized medicine, with proven efficacy required for enhanced screening and prevention strategies, and as markers for targeted therapy. The rapid pace of molecular sequencing still requires due diligence to assure that the basic tenets of genetic counseling are fulfilled. Historically, a clinical genetics visit entails rapport building, a detailed account of the family history in the form of a pedigree, documentation of medical history, a physical exam with specific focus on the presence or absence of syndrome stigmata (e.g. macrocephaly or skin findings which may be manifestations of alterations in specific breast cancer genes), review of genetic concepts, discussion of options for screening and early detection, an opportunity for questions, a link to supporting services and a plan for follow up. In cases whereby a genetic visit indicates testing, the basic elements of informed counseling remain the standard of care [24], although these may increasingly be conveyed and communicated in on-line via video conferencing as well as in-person contexts. In an era of increasing somatic genetic analysis of breast and other tumors for the purposes of "targeting" therapies, it will be important to distinguish whether the primary purpose of genomic analysis is to determine inherited susceptibilities, or whether this information may emerge as a secondary byproduct of tumor genomic analysis (Fig. 1).

The current number of individuals having been tested for mutations in *BRCA1/2* exceeds one million. Pathogenic mutations appear to account for ~30% of high-risk breast cancer families and explain ~15% of the breast cancer familial relative risk (the ratio of the risk of disease for a relative of an affected individual to that for the general population) (Fig. 1) [4–6, 25]. Contextualizing disease risk of inherited mutations and sequence variants in *BRCA1/2* can be complex, since the pathogenicity of sequence variants is uncertain, and requires annotation and curation using existing databases (e.g. the Breast Cancer Information Core; www.research.nhgri.nih.gov/bic).

Table 1 HUGO Gene ID, inheritance pattern, clinical manifestations and context dependent guidelines for highly penetrant breast cancer predisposition syndromes

Characterization of breast cancer predisposition syndromes						
Gene	Syndrome	Inheritance	Overt stigmata	General surveillance	Context specific risk reduction considerations	Penetrance
BRCA1/ BRCA2	Hereditary breast and ovarian	AD	No	Exam, Imaging (MRI, mammography)	Chemoprevention with tamoxifen, Mastectomy, TAH/BSO	High
TP53	Li Fraumeni	AD	No	Biochemical and endocrine testing, MRI whole body	+/− Mastectomy	High
PTEN	Cowden	AD	Yes	Clinical exam, MRI, colonoscopy, skin exams	+/− Mastectomy	High
STK11	Peutz-Jeghers	AD	Yes	Clinical exam, mammography, colonoscopy, skin exam	Polypectomy, Mastectomy, TAH/BSO	High
CDH1	Familial gastric cancer	AD	No	Colonoscopy, esophogealduodenaloscopy, mammography, MRI	Gastrectomy	High

AD autosomal dominant, *MRI* magnetic resonance imaging, *TAH/BSO* total abdominal hysterectomy bilateral salpingo-oophorectomy

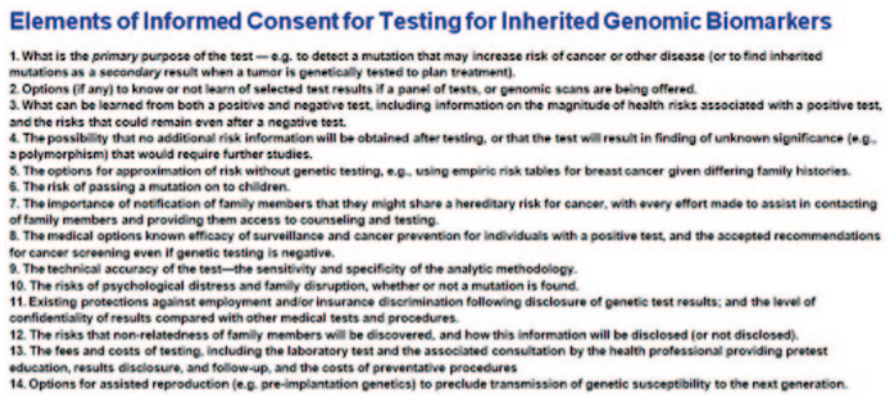

Fig. 1 Elements of informed consent

Syndromes of Breast Cancer Predisposition

Hereditary Breast and Ovarian Syndrome

BRCA1 and *BRCA2* are the predominant breast cancer susceptibility genes. Pre-test probability for *BRCA2* testing is higher for families with male and female breast cancer and for *BRCA1* testing in families with both breast and ovarian cancer [26]. 18,000 cases of breast cancer annually are associated with an obvious hereditary predisposition. Detection of breast cancer leads to a cure rate of more than 90% if detected at an early stage. All told more than 200,000 breast cancer survivors in the United States developed their primary cancers as a result of a constitutional (inherited) predisposition, highlighting the importance and rationale for genetic testing [27]. Estimates range from one in 150 to one in 800 individuals in the population who are genetically predisposed to developing breast cancer and in certain ethnic groups these estimates are as high as 1 in 40 [28, 29]. A woman carrying a mutation in *BRCA1* has a lifetime breast cancer risk as high as 70% by age 70 by epidemiologic analysis [29, 30–32]. In select families with a high frequency of early onset of breast or ovarian cancer risk, estimates further increase to as high as 90% lifetime breast cancer risk [33].

Highly Penetrant Breast Cancer Genes

BRCA1 and BRCA2

The *BRCA1 and BRCA2* genes function in DNA damage response and homologous recombination [34]. *BRCA1* is a large gene located on chromosome 17 and is made up of 24 exons, 22 of which are coding and two of which are non-coding. *BRCA2* spans greater than 70,000 bases and the gene is comprised of 27 exons (genenames.org).

Premature truncations of the BRCA1 and BRCA2 proteins by nonsense or frameshift alterations are the predominant genomic aberrations underlying susceptibility. Variants of uncertain significance were initially observed in up to a quarter of patients, however the frequency of these predominantly missense variants of unknown significance (VUS) dropped to between 2 and 5% as large databases of genetic variants and "high-risk" kindreds were created [35, 36]. With the uptake of commercial testing by new laboratories, and the expansion of testing criteria beyond "high-risk" kindreds, this percentage of VUS may again increase [37].

Over 2000 distinct rare variants, in the form of intronic changes, missense mutations, and small in-frame insertions and deletions, have been reported in *BRCA1* and *BRCA2* (Breast Cancer Information Core; www.research.nhgri.nih.gov/bic). The main domains of *BRCA1,* which are critical for DNA repair activity, are located in the RING finger and BRCT domains. In *BRCA2*, highly penetrant, pathogenic missense mutations reside mainly in the DNA binding domain [38, 39]. Large genomic

rearrangements or structural variations occur in *BRCA1* (14% of mutations) and *BRCA2* (2.6% of mutations). A reason for the relative increase in structural variations in *BRCA1* compared to *BRCA2* results from the large number of Alu repeats in the genomic region containing the *BRCA1* gene [40].

Population specific or "founder" mutations in *BRCA1/2* have been described. Some of the most common founder mutations occur in individuals of Ashkenazi (eastern European) Jewish ancestry, including two mutations in *BRCA1* (185delG and 5382insC) and one mutation in *BRCA2* (6174delT) [41–43]. A small number of patients in the Ashkenazi population with breast cancer have non-founder mutations in *BRCA1/2* (5% of all mutations) and thus reflex full gene sequencing may be required if founder mutations are non-revealing [42, 43]. The Ashkenazi Jewish founder mutations are the best studied and described; 3% of individuals in this population carry a founder mutation. Other examples of *BRCA1* founder mutations are reported in the Dutch and Hispanic populations. Again for these populations, targeted sequencing for specific *BRCA1/2* mutations is advised before reflex to full gene testing in cases of a negative result. Carriers ascertained from population studies demonstrate a lower penetrance of disease in comparison to those identified through kindred based studies, which is not surprising as a striking overt phenotype in the families prompted initial study.

Including follow up recommendations for screening and prevention for *BRCA1* mutation carriers remains as a standard of care given a ~57% probability of developing breast cancer and a 40% chance of developing ovarian cancer by age 70. *BRCA2* mutation carriers are estimated to have a 49% chance of breast cancer and an 18% chance of ovarian cancer [44]. Contributing factors to the development of cancer include environment, modifying genomic alterations and the specific type of constitutional aberration in *BRCA1/2*. Statistical evidence has emerged suggesting genotype-phenotype correlations with regard to ovarian cancer risk. The early literature correlated the location of mutations in *BRCA1/2* with specific phenotypes and gleaned that nonsense and frameshift mutations located in the central regions of either coding sequence, termed ovarian cancer cluster regions (OCCR), were associated with a greater risk of ovarian cancer than similar mutations in the proximal and distal regions of each gene [45, 46]. Among the greater than 22,000 *BRCA1/2* mutation carriers enrolled in Consortium of Investigators of Modifiers of *BRCA1/2* (CIMBA) group, the relative increases in ovarian cancer and decreases in breast cancer risk for mutations in the central region of each gene and higher risk of breast cancer for mutations in the 5′ and 3′ regions of each gene have been observed. Further variability in risk is also partly explained by common genetic modifiers of breast and ovarian cancer risk in *BRCA1/2* mutation carriers that have been identified through genome-wide association studies [19, 47–51]. [55, 117] (Fig. 2).

The genomic location of a patient's *BRCA1/2* mutation and the risk from modifier genes suggests that the *BRCA1* mutation carriers in the highest risk category may have an 81% or greater chance of breast cancer and a 63% or greater chance of ovarian cancer by age 80, whereas *BRCA2* mutation carriers at greatest risk may have more than an 83% chance of breast cancer by age 80 [19, 52]. In conjunction with other variables modifying risk in *BRCA1/2* mutation carriers, these emerging

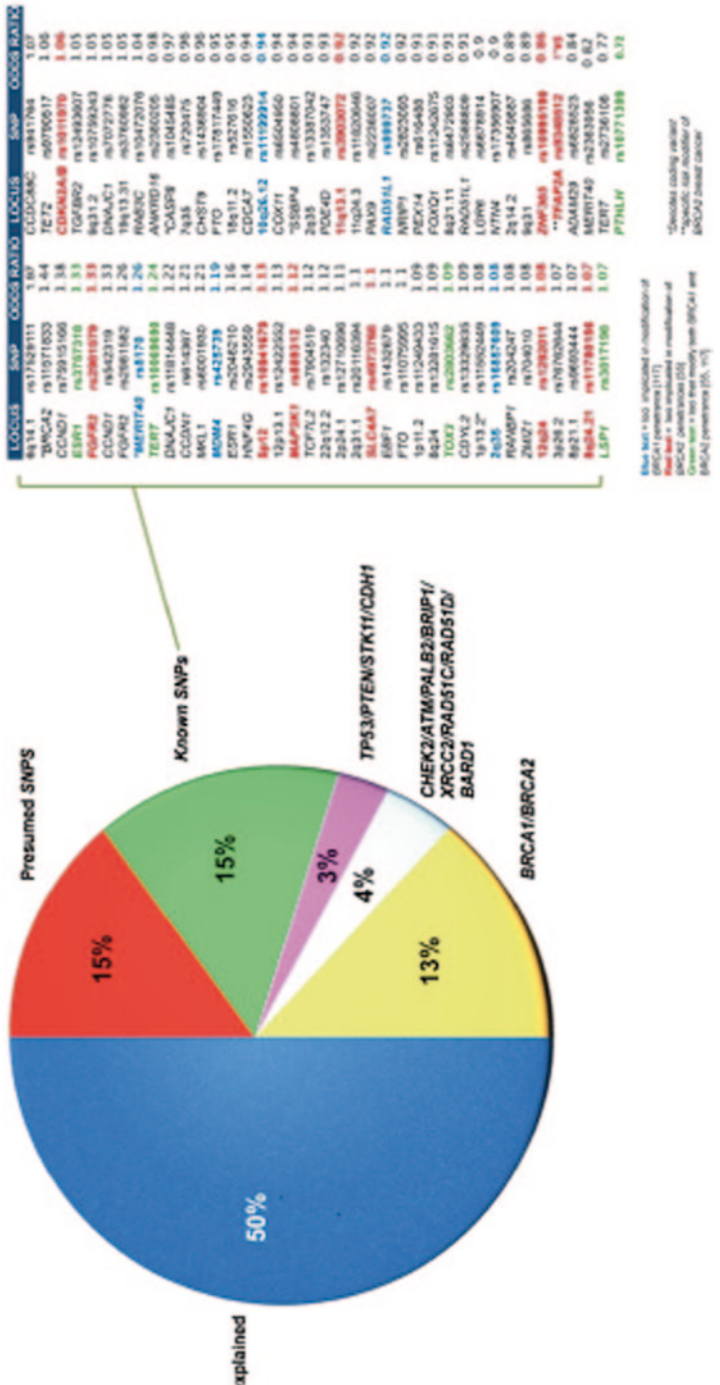

Fig. 2 Breast cancer biomarkers

biomarker data on mutation location and modifier genes offer the potential for more precise risk estimates. It is also possible that such biomarkers may correlate with disease behavior. As breast cancer patients with *BRCA1* mutations tend to have tumors that display features of more aggressive disease [53–56], genomic biomarkers of risk may also impact on the phenotype (e.g. estrogen receptor status) of hereditary disease.

As alluded to previously, VUS, including missense, intronic, and small in-frame insertion/deletion variants, continue to pose clinical challenges in terms of interpreting test results. Although one large testing company has classified many *BRCA1/2* variants as neutral or pathogenic using data collected over years, that data have thus far not been placed into public access. Thus, laboratories now entering the clinical sequencing space have had challenges classifying variants encountered during testing. In an effort to improve the classification process for variants in all genes now offered as part of clinic genetic testing, the Clinvar (www.ncbi.nlm.nih.gov/clinvar) database has been curating variants and attempting to capture clinical information, efforts pioneered for BRCA1/2 by the international Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) Consortium (see below). In 2014, the Global Alliance announced a demonstration project to create an international database of *BRCA1/2* variants. These steps are crucial to allow the most accurate interpretation of these genetic biomarkers for inherited risk. In the absence of these definitive databases, evaluation of VUS has often relied on in-silico models or animal models that predict the functional impact of variants on the basis of amino acid conservation and/or structure or try equate the human disease to a different species that is not a direct homologue to humans.

To provide algorithms to the interpretation of variants of uncertain significance, expert and evidence-based committees focused on the development of quantitative risk prediction methods. One such effort is ENIGMA, which has substantially improved assessment of the pathogenicity of VUS [57]. The following elements are assessed for each variant: conservation, family history, tumor pathology, and the effects of RNA splicing [39, 57–59]. This effort also estimates the probability of pathogenicity for each variant using combined evolutionary sequence conservation (Align-GVGD) [39, 59–61], and has resulted in classification of many *BRCA1/2* VUS as pathogenic or of neutral/low effect [59]. Due to the lack of statistical power for rare variants or individual VUS, high throughput quantitative cell-based *in vitro* assays have been developed to evaluate the effect of variants on established functions of the BRCA1 and BRCA2 proteins, with known controls of normal and pathogenic mutations as controls to asses sensitivity and specificity for VUS [39] or variant specific biomarker.

A special challenge in interpreting gene variants is the example of hypomorphic mutations, which retain some protein activity. Insights are being gained for some specific variants, i.e. the p. Arg1699Gln (R1699Q) missense mutation in the BRCT domain of *BRCA1* that abrogates the repression of microRNA-155 [62] and is associated with a cumulative risk of breast cancer of 24% by age 70 [57], and the well-known polymorphic stop codon in *BRCA2*, p.Lys3326X, which is associated with only a modest increase in breast cancer risk [odds ratio (*OR*) = 1.26] [63] and

appears to have little clinical relevance. As more moderate risk variants or biomarkers in breast cancer predisposition genes are detected and clinically validated, personalized surveillance and prophylaxis measures may be developed**.**

Impact on Clinical Management for *BRCA1 and BRCA2* Mutation Carriers

Genetic testing informs both medical decisions and family planning. While evidence-based medicine continues to evolve*, BRCA* mutation carriers should undergo a triple assessment for breast surveillance, including self-examination, clinician examination and mammography/Magnetic resonance imaging (MRI) [64–66].

Mammography is of limited sensitivity in *BRCA* mutation carriers; in one study 29% of new tumors were missed by mammography [16]. This limitation may be due to higher breast density in younger women and as hereditary breast cancers are often more rapidly growing "triple negative" tumors (negative for estrogen and progesterone receptors and lacking HER2/*neu* overexpression or amplification) [67]. It is strongly recommended that women at hereditary risk begin annual mammography/MRI screening at age 25 (http://www.nccn.org/professionals/physician_gls/pdf/f_guidlines.asp#breast_risk) [68]. MRI detects twice as many breast cancers in *BRCA1/2* mutation carriers as mammography or sonography [16], and is considered the standard of care. Alternatively, risk reducing mastectomy (RRM) decreases the risk of breast cancer by at least 90% in *BRCA1/2* mutation carriers [69, 70], but only 36% of women in the United States and 22% in Canada choose to undergo this surgery [71]. In contrast, risk-reducing salpingo-oophorectomy (RRSO) has become the standard of care for all women with *BRCA1/2* mutations because ovarian cancer screening methods using serum markers and imaging are ineffective [72, 73]. RRSO has been shown to reduce the risk of *BRCA*-associated gynecologic cancer by 80–96% [15, 69, 74] and to reduce the risk of breast cancer by ~50%, most likely through the induction of premature menopause [15, 69, 75]. Most significantly, RRSO reduces overall mortality of women with *BRCA1/2* mutations by 60% [76]. This reduction in mortality occurs despite the 0.2% annual risk of cancer of the peritoneal lining around the ovaries and fallopian tubes, which remains as these tissues cannot be surgically removed by RRSO [74]. Genetic testing for *BRCA1/2* mutations and RRSO provided an early example of the deployment of 'personalized' prevention through genetics [16, 77].

Data pertaining to chemoprevention based on inherited biomarkers such as *BRCA1/2* are limited. Efficacy of tamoxifen for *BRCA1/2* mutations carriers was conducted as a sub-analysis as part of the 13,388 women enrolled in the National Surgical Adjuvant Breast and Bowel Project Prevention Trial (NSABP-P1). In this study, 19 *BRCA1/2* mutation carriers were identified among 288 that developed breast cancer, with risk ratios for developing breast cancer with tamoxifen estimated to be 1.67 (95% confidence interval (CI): 0.32–10.7) for *BRCA1* mutation carriers and 0.38 (95% CI: 0.06–1.56) for *BRCA2* mutation carriers [78]. In a larger study of 2464 mutation carriers, tamoxifen use after a first breast cancer was associated with a reduced risk of contralateral breast cancer [79]. More refined chemopreven-

tion options for women with mutations in *BRCA1/2* may evolve. In patients with no mutations in *BRCA1/2*, other selective estrogen receptor modulators and aromatase inhibitors have been shown to prevent breast cancer,(http://www.nccn.org/professionals/physician_gls/pdf/f_guidlines.asp#breast_risk). Some data have also begun to emerge suggesting that modulators of RANKL signaling may be a target for chemoprevention [80].

Ovarian cancer chemoprevention studies have produced somewhat conflicting results bearing on benefits for *BRCA* mutation carriers [81–83], although most believe that oral contraception does decrease risk of hereditary as well as sporadic ovarian cancer. In that regard, treatment and standard of care for *BRCA1/2* mutation carriers must address ovarian cancer detection and prevention. Given the unproven methods of screening and the high mortality at time of diagnosis associated with ovarian cancer, definitive counseling and recommendations for prophylactic removal of ovaries after childbearing are standards of care for *BRCA1/2* mutation carriers or for women with two or more first degree relatives with ovarian cancers in the family (http://www.nccn.org/professionals/physician_gls/pdf/f_guidlines.asp#breast_risk). [15, 69, 84–88].

Finally, the identification of mutated genes as biomarkers has led to therapeutic applications. *In vitro* and *in vivo* experiments and clinical trials have shown that platinum chemotherapy is effective against *BRCA1* (and, by analogy, *BRCA2*) mutant tumors, in part because platinum generates interstrand cross-links that can only be adequately repaired by *BRCA1*- and *BRCA2*-dependent homologous recombination DNA repair [89]. A new class of drugs that inhibit poly(ADP-ribose) polymerase (PARP), an enzyme involved in base excision repair [90, 91] shows antitumor activity in the background *of BRCA*-associated defects in homologous recombination-mediated DNA repair [92]. Clinical trials have explored the efficacy of PARP inhibitors in the treatment of *BRCA1/2* mutant breast, ovarian, pancreatic, prostate, and other cancers, and one such compound was recently licensed for use in the U.S. for patients with previously treated *BRCA* mutant ovarian tumors [93]. Not all *BRCA* mutation carriers respond to these agents; mutations in the N-terminal BARD1 binding domain of *BRCA1,* such as the relatively common p.Cys61Gly (C61G), may not confer hypersensitivity to PARP ihibitors [94, 95]. Acquired resistance to PARP inhibitors has been associated with multiple mechanisms, including drug metabolism and efflux, post-transcriptional alterations of *BRCA1/2*, secondary mutations that restore the homologous recombination activity of *BRCA1/2*, and accumulation of somatic genetic alterations that counteract the sensitivity associated with *BRCA1/2* mutations [95–97]. Whether combination therapies can overcome these complications remains to be determined.

Other Highly Penetrant Breast Cancer Predisposing Genes

TP53 and CDH1

Compared to *BRCA1/2* mutations, *TP53* mutations are rare. However when testing for *BRCA1/2* is non-revealing or determined not causative, testing of *TP53* may be warranted in cases with a strong family history of cancer and negative *BRCA1/2* testing. Li-Fraumeni syndrome (LFS) is a multi-cancer predisposing syndrome driven by genomic alterations in the *TP53* gene. *TP53* encodes the tumor suppressor protein p.TP53. Patients with *TP53* mutations have an increased risk of breast cancer [98]. In determining the importance to variants detected by next generation sequencing similar steps taken by ENIGMA's efforts in assessing the *BRCA* genes are required. The International Association Cancer Research (IARC) hosts the *TP53* locus specific database. The database curates frequency of variants, if the variant has been detected in the germline, been found in the tumor, seen in a cell line, segregation information of the variants and functional prediction of the genomic variant on protein function. National guidelines for patients with Li-Fraumeni Syndrome support *TP53* testing concurrently for women ≤35 years of age or as a follow- up test after negative *BRCA1/2* testing (http://www.nccn.org/professionals/physician_gls/pdf/genetics_screening.pdf).

For carriers of *TP53* mutations, it seems reasonable to consider adding annual MRI starting at age 20–25 years of age or based on earliest age of onset in the family. When patients are found to harbor a *TP53* mutation, there is some laboratory based evidence that radiation exposure may be deleterious, although this remains incompletely documented. Ongoing trials are testing other approaches such as whole body MRI, PET, and other focused screening; patients should discuss approaches to novel screening and technology with their providers [99].

Reports of germline *CDH1* mutations emerged in patients with hereditary diffuse gastric cancer in the late 1990s [100–104] and it was soon observed that these families also included individuals with lobular breast cancer. In screening of over 400 cases of breast cancer, three patients were found to harbor germline mutations in *CDH1*. Families with multi-generations affected with gastric cancer have a 30% chance of harboring a mutation in E-Cadherin *(CDH1),* and 70% of carriers of mutations in this gene develop gastric cancer. In addition to the diffuse gastric cancer risk individuals with *CDH1* mutations also have approximately a 40–50% risk of lobular cancer of the breast (http://www.nccn.org/professionals/physician_gls/pdf/genetics_screening.pdf).

While no formal testing recommendations are established for patients with *CDH1* mutations and breast cancer, Petridis et al. recently proposed *CDH1* mutation screening should be considered in patients with bilateral lobular carcinoma *in situ* with or without invasive lobular breast cancer and with or without a family history. Gastrectomy for patients with *CDH1* mutations is routinely advised. However, the identification of families with *CDH1* mutations through multi-gene panel testing and no family history of gastric cancer are proving difficult to counsel, as

the risk of gastric cancer in those patients is unknown. Patients are also presented options regarding mastectomy given the frequency of breast cancer in these patients or cumulative risk for breast cancer for females by age 75 years is 52% [104].

PTEN/STK11

The majority of patients that undergo inherited genetic testing do not have overt physical manifestations of a syndrome. However, a few constitutional syndromes with overt phenotypes and genetic testing or "biomarkers" do have an increased risk of breast cancer such as Cowden syndrome/Bannayan-Riley-Ruvalcaba syndrome/*PTEN* hamartoma tumor syndrome (PHTS), and Peutz-Jeghers syndrome [105–107]. Major criteria to assess in diagnosing female patients suspected of having Cowden syndrome include breast cancer, endometrial cancer, follicular thyroid cancer, multiple gastrointestinal hamartomas or ganglioneuromas, macrocephaly (> 97%), and mucocutaneous lesions (trichilemmoma, palmoplantar keratosis, extensive mucosal papillomatosis or verrucous facial papules). Minor criteria include autism spectrum disorder, colon cancer, ≥ three esophageal glycogenic acanthosis, lipomas, intellectual disability, papillary or follicular variant of thyroid cancer, thyroid structural lesions, renal cell carcinoma, single gastrointestinal hamartoma or ganglioneuroma, testicular lipomatosis, and vascular anomalies. Individuals with a family member with a known mutation, patients with autism and macrocephaly, two or more biopsy proven trichilemmomas, two or more major criteria where one has to equal macrocephaly, three major criteria without macrocephaly or one major and three minor criteria and four minor criteria [108]. Screening for patients with Cowden is as per National Comprehensive Cancer Network (NCCN) guidelines; breast MRI is part of this strategy and preventive surgeries can also be considered (http://www.nccn.org/professionals/physician_gls/pdf/genetics_screening.pdf) (Table 1).

STK11

Peutz-Jeghers syndrome (PJS) is an autosomal dominant cancer predisposition syndrome with clinical characteristics of mucocutaneous pigmentation and gastrointestinal polyps. Patients with PJS are at increased risk of colon cancer, breast cancer, ovarian (mucinous tumors and sex cord tumors with annular tubules) [109–112]. Most mutations are small deletions/insertions or single base substitutions resulting in aberrant protein function with loss of kinase activity. In the analysis of greater than 400 patients, and close to 300 of these individuals with known *STK11* mutations, the cancer risk for the development of breast cancer was 50% by age 60 [113, 114]. However, in the largest study to date of PJS patients no differences in breast cancer risk have been found [113, 114] but the absolute numbers of kindreds with this syndrome collected for study is still small.

The major phenotype of PJS is gastrointestinal polyps. Patients require frequent endoscopic surveillance with polypectomy, which decreases the rate of intussuscep-

tion and potential bowel loss. Patients with PJS should be counseled required the high rate of breast cancer and the benefits of prophylactic mastectomy and bilateral salpingo-oophorectomy after the age of 35 to prevent malignancy. In addition to monitoring of the gastrointestinal tract, routine screening of the breast (e.g. mammography and possibly MRI) should be standard of care for individuals with PJS. In addition, patients should be offered investigational pancreatic cancer screening (e.g. magnetic resonance cholangiopancreatography (MRCP) or endoscopic ultrasound) starting at an early age, as well as small bowel visualization, and pelvic exam with consideration of transvaginal ultrasound (although unproven, to address ovarian cancer risk) and annual physical exam [113, 114] .

Moderate Penetrance Breast Cancer Genes or Biomarkers: CHEK2, ATM, PALB2, BRIP1, RAD51C, RAD51D, BARD1

There are no standardized guidelines for the management of other cancer risks or for the relatives of carriers with moderate penetrance gene mutations; screening recommendation should be established based on the patient's personal and family histories.

CHEK2

CHEK2 normally functions by preventing cellular entry into mitosis when DNA is damaged. In 2000, Lee et al. reported that *CHEK2* function in DNA damage by phosphorylating *BRCA1* [115]. Further experiments revealed *CHEK2* and *BRCA1* interaction is necessary for *BRCA1* to restore the survival after DNA damage. Heterozygous mutations were initially reported in a LFS-like family, suggesting CHEK2 serves as a tumor suppressor and mutations predispose individuals to cancer [116]. Subsequently mutations were shown to be associated with a moderate risk of breast cancer, rather having any association with LFS. Population studies have aimed to determine the role of *CHEK2* in patients without an identifiable mutation in *BRCA1/2* but a suggestive family history [117]. The truncating mutation *CHEK2**1100delC affecting kinase activity was revealed in 1.1 % of healthy individuals compared to 5.1 % coming from over 700 families with breast cancer (male and female breast cancers both included) and negative *BRCA1/2* testing. These data suggest a greater than a two-fold increase of breast cancer risk in females and 10-fold increase in men with the *CHEK2**1100delC. As a means to assess for additional mutations in *BRCA* negative families with breast cancer, Shutte et al. assessed 89 kindreds with three or more individuals with breast cancer and did not find other appreciable site specific variation in *CHEK2* [118]. Although studies are still in progress, it appears that the detection of a *CHEK2* deleterious mutation in the setting of a strong family history of breast cancer may warrant clinical use of this biomarker in the pre-symptomatic assessment for screening. Whether the absolute level of *CHEK2*-associated risk meets threshold for MRI screening can be determined on an individualized basis, taking into account population derived as well as family history data.

ATM

ATM is a gene encoding a protein that allows for the efficient repair of DNA. *ATM* when altered manifests phenotypes from bi-allelic and arguably mono-allelic genomic alterations. Individuals with two mutations or bi-allelic or homozygous mutations develop severe disease of the immune system and are predisposed to developing leukemia and lymphoma, called Ataxia-Telangiectasia (A-T). Various degrees of evidence support or refute individuals harboring a single mutation in the *ATM* gene as having an increased risk of developing breast cancer, stomach, ovarian, pancreatic, or lung cancer [119–122, 123]. Approximately 1% of the population is heterozygous for mutations in the *ATM* gene.

Mutant specific evidence for *ATM* p.S49C and p.F858L in association with increased breast cancer susceptibility show an odds ratio of 1.44 combining data from an American and Polish study [124]. When mutations that have been identified specifically in patients with *ATM* have been studied in mono-allelic carriers the estimated relative risk for familial breast cancer was = 2.37. The data are based on the evaluation of individuals from 443 familial breast cancer kindreds [120, 125–127]. Breast cancer-associated *ATM* mutations tend to be missense mutations whereas missense mutations are uncommon in individuals with A-T, even in the same host population [121].

Individuals who are carriers for *ATM* gene mutations should be aware that they might be sensitive to radiation, although the magnitude of this radiation sensitivity requires further study. There are no *ATM* mutation specific sets of recommendations for therapy, treatment, or tailored management options [128–131]. No definitive evidence has emerged regarding increased risk of mammograms in *ATM* mutation carriers, however, MRIs and ultrasound remain an important screening strategy. Annual breast MRI screening is recommended for women with a lifetime risk for breast cancer of 20–25% or greater and it is generally recommended that MRI be used in conjunction with mammogram.

Regarding prevention, prophylactic mastectomy has not been evaluated extensively in individuals who are carriers for *ATM* gene mutations. There is no evidence concerning the effectiveness of chemoprevention in the *ATM* gene carrier population, although there is also no evidence that it will not be as effective as in the general population.

PALB2

PALB2 is a gene encoding a necessary protein of the Fanconi complex and is also known as the partner and localizer of BRCA2 and FANCN. PALB2 interacts with the BRCA2 protein and work together to correct and fix DNA breaks. PALB2, as it helps control the rate of cell growth and division, is a tumor suppressor (http://ghr.nlm.nih.gov/gene/PALB2). Moreover, by limiting mistakes in DNA repair, *PALB2* aids in maintaining the stability of genetic information.

Literature is emerging in regards to the contribution of germline mutations of *PALB2* and hereditary breast cancer. Approximately a dozen mutations have been identified in *PALB2* and familial breast cancer. Mutations in *PALB2* are estimated to lead to a two-fold increase in breast cancer risk. In 2007, investigators sequenced the *PALB2* gene in close to 1000 individuals with breast cancer who were negative for *BRCA1/2* mutations [132]. Ten out of 923 harbored *PALB2* mutations conferring a 2.3-fold higher risk of breast cancer. The Q775X variant was identified in 1/50 high-risk women or 2/356 breast cancer cases and not present in any of > 6000 controls [133]. Assessing 559 women with contralateral disease and 565 women with unilateral disease as controls, fine truncating pathogenic mutations were identified. A study of Australian and New Zealand women who were negative for *BRCA1/2* mutations underwent *PALB2* testing and 26 out of 747 women were detected having *PALB2* genomic alterations. Two women harbored nonsense mutations and two frameshift mutations. Investigators concluded that ~1.5% of Australasian women in families with multiple members affected with breast cancer segregate *PALB2* mutations in their families.

Recent studies analyzing the risk of breast cancer in > 150 families assessing truncating, splicing or deletions in *PALB2* and family history estimated the risk of breast cancer for female carriers compared to the general population was eight to nine times as high among women younger than 40, six to eight times as high among those 40–60 years of age and five times as high for those females older than 60 years of age [134]. The estimated cumulative risk of breast cancer among female mutations carriers was 35% by age 70 and the absolute risk ranged from 33 to 58% depending on the extent of family history [134]. The investigators of this study concluded the breast cancer risk from *PALB2* potentially overlap with that for *BRCA2* mutation carriers and that loss of function mutations account for roughly 2.4% of familial aggregation of breast cancer [134]. These data would support the role for MRI breast screening in this genetically defined population.

BRIP1

BRIP1, or alternatively named *FANCJ,* similar to *PALB2* manifests disease in both the heterozygous and homozygous state. *BRIP1* is also known as the *BRCA* interacting helicase. Patients with constitutional bi-allelic mutations in these two genes are notable for a Fanconi anemia phenotype. One study suggests that constitutional heterozygous carriers have a relative risk of breast cancer of 2.0 [135], however further validation studies need to be done.

RAD51C and RAD51D

Nonsense, frameshift, splice and non-functional missense mutations have been described in *RAD51C*, however the evidence that they are a driver of familial breast cancer is limited [136, 137]. Evidence of *RAD51C* mutations in familial ovarian

cancer is greater than familial breast cancer [137, 138]. In a cohort of familial breast and ovarian cancer cases a distinct difference was noted between ovarian and breast cancer i.e., data revealed a relative risk of 5.88 in mutation carriers for ovarian cancer and 0.91 for breast cancer [139].

Loveday et al. also demonstrated a similar risk ratio for patients harboring mutations in *RAD51D*. Regarding therapeutics, the group showed that cells deficient in RAD51D are sensitive to treatment with a PARP inhibitor, suggesting a possible therapeutic approach for *RAD51D* mutant patients with a family history of breast and predominantly ovarian cancer.

BARD1

In Finnish families with breast and/or ovarian cancer, 5.6% of individuals were detected to have a cys557-to-ser substitution (C557S) in the *BARD1* gene compared to healthy controls (5.6 vs. 1.4%, $p=0.005$) [140]. The highest prevalence of C557S was detected in a subgroup of 94 patients with breast cancer whose family history did not include ovarian cancer (7.4 vs 1.4%, $p=0.001$). The C557S mutation is located in a region of *BARD1* needed for induction of apoptosis and possibly also transcriptional regulation. The investigators concluded that C557S may be a breast cancer-predisposing allele.

Low Penetrant Polygenes

Other single nucleotide variations or single nucleotide polymorphisms have been detected conferring a moderate to low penetrant breast cancer genes or biomarkers [23] (Fig. 2). Genome-wide association studies (GWAS) have identified common genetic variants in 76 loci associated with small increases in the risk of breast cancer (Fig. 1) [63, 141]. However, most of these variants have weak effects on risk (OR < 1.10) [63]. Little is known about the relevance of these risk factors to the different molecular subtypes of breast cancer, although three of these loci (*MDM4*, 19p13.1, and *TERT-CLPTM1L* rs10069690) are exclusive to triple-negative breast cancer [142–145] and *BRCA1*-associated breast cancer [19]. Although the identification of causal variants and mechanism of action for most remain unclear, some variants are near known genes such as *BRCA2, TGFBR2, MYC*, and *TET2* [63]. One mechanism of action of common variants is on gene transcription, as evidenced by the 11q31.1 locus and Cyclin D1 expression via a transcriptional enhancer and a silencer of the *CCND1* gene [146], and FGFR2 expression via induction of FOXA1, ER*a*, and E2F1 binding to enhancers [142].

The clinical utility of these common variants as a paradigm of polygenic risk assessment for human cancer remains a work in progress [144–146]; breast cancer–associated common variants combined with traditional breast cancer risk markers had minimal impact on risk prediction models [147] or discriminatory accuracy [148]. A polygenic risk score calculated as the sum of the ORs for each allele, correlated with

risk of early onset breast cancer ($OR = 3.37$, $P = 0.03$) [148] and other such studies are now under way [145], with the goal of leading to better identification of women who will benefit from enhanced screening and intervention [22].

New Paradigms for Genomic Biomarkers of Risk

Two decades of molecular biologic and genetic epidemiologic research have resulted in tests for inherited genomic variants as useful biomarkers for breast cancer risk. Tests for highly penetrant (high-risk) genetic mutations have been incorporated into clinical practice. Currently, "panel" tests for large numbers of genes, including some of unclear clinical utility, are commercially available. A pressing challenge posed by these developments is the interpretation and actionability of the large number of variants and "low penetrance" mutations discovered. To address this challenge, in 2014, the Prospective Registry of Multiplex Testing (PROMPT) began as an academic-commercial-and patient-centered initiative, and readers are encouraged to access it at https://connect.patientcrossroads.org. Such longitudinal studies would also add to the evidence base for targeted screening and prevention in genetically defined high-risk cohorts. Other federal initiatives are underway to catalogue and interpret the emerging array of genomic biomarkers of inherited cancer risk, which will only increase as screening of entire exomes and genomes becomes more feasible.

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Epigenetic Biomarkers of Breast Cancer Risk: Across the Breast Cancer Prevention Continuum

Mary Beth Terry, Jasmine A. McDonald, Hui Chen Wu, Sybil Eng and Regina M. Santella

Abstract Epigenetic biomarkers, such as DNA methylation, can increase cancer risk through altering gene expression. The Cancer Genome Atlas (TCGA) Network has demonstrated breast cancer-specific DNA methylation signatures. DNA methylation signatures measured at the time of diagnosis may prove important for treatment options and in predicting disease-free and overall survival (tertiary prevention). DNA methylation measurement in cell free DNA may also be useful in improving early detection by measuring tumor DNA released into the blood (secondary prevention). Most evidence evaluating the use of DNA methylation markers in tertiary and secondary prevention efforts for breast cancer comes from studies that are cross-sectional or retrospective with limited corresponding epidemiologic data, raising concerns about temporality. Few prospective studies exist that are large enough to address whether DNA methylation markers add to the prediction

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of tertiary and secondary outcomes over and beyond standard clinical measures. Determining the role of epigenetic biomarkers in primary prevention can help in identifying modifiable pathways for targeting interventions and reducing disease incidence. The potential is great for DNA methylation markers to improve cancer outcomes across the prevention continuum. Large, prospective epidemiological studies will provide essential evidence of the overall utility of adding these markers to primary prevention efforts, screening, and clinical care.

Keywords Biomarker · Breast cancer · DNA methylation · Plasma · Prevention · Prognosis · Recurrence · Serum · Survival · Breast tissues

Introduction

Breast cancer mortality rates have steadily decreased since 1990; however, breast cancer remains the second leading cause of cancer deaths in women in the United States [1]. Breast cancer is the most common cancer in women in the United States, and the incidence is increasing dramatically in very young women under age 40 years [2]. Women at higher risk of breast cancer due to family history and/or specific genetic alternations have an earlier age of onset than women at average risk and screening mammography is less sensitive in younger women [3]. Early detection of breast cancer increases treatment options, including surgical resection and therapeutic interventions [4]. Thus, finding markers that can help detect cancer early, particularly in younger women, that complement and/or improve existing methods will help in reducing incidence and mortality from breast cancer.

Biomarkers can be a useful tool for monitoring disease risk and prognosis. For example, in cardiovascular disease, blood pressure and blood markers such as lipid levels are measured routinely throughout adulthood. These markers prove particularly useful when combined with other cardiovascular disease risk factors in predicting risk through models that can readily be employed in the community and clinic. Breast cancer risk assessment models provide estimates of the absolute risk of breast cancer within a fixed time horizon (e.g., 5 or 10 years) or for the remaining lifetime of a woman. For example, women with a 5-year risk of 1.67% or higher are classified as "high-risk" and are eligible for taking tamoxifen or raloxifene to reduce breast cancer risk based on the FDA guidelines. The Gail model is the most frequently used risk prediction tool in United States clinics; however, the model is not recommended for high-risk women such as those with a strong family history of breast cancer [5, 6]. Breast cancer risk assessment methods, just like cardiovascular disease models, may benefit from the addition of biomarker and intermediate marker information. However, at present, there are no existing validated plasma/serum biomarkers for breast cancer. Only a few biomarkers (such as estrogen receptor) have utility for diagnosis and prognosis (reviewed in [7]). Thus, there is a great need for sensitive biomarkers to detect early neoplastic changes and to facilitate the detection of breast cancer at an early treatable stage.

Epigenetic modifications (e.g., DNA methylation) refer to heritable and modifiable markers that regulate gene expression without changing the underlying DNA sequence. DNA methylation may play an important role in tumorigenesis by silencing tumor suppressor genes [8–12]. Emerging evidence suggests that aberrant DNA methylation can begin very early in breast tumor progression [13] and can be detected in body fluids [14]. Similarities between methylation patterns found in primary tumor specimens and in blood plasma indicate the potential utility of blood-based molecular detection of breast cancer [15–18]. Emerging evidence has shown that DNA methylation of select genes measured in plasma results in sensitivities >90% for detecting breast cancer [15, 19]. These results suggest that DNA methylation has promise for screening. As we review, however, the evidence base is far from complete with many small studies and of a cross-sectional design that limit any inferences about temporality. Where there are gaps, we suggest study designs and the types of evidence that may prove useful in addressing these gaps.

Breast cancer is a heterogeneous disease with very different therapeutic responses and outcomes. Gene expression profiles have been used for breast cancer classification and have served as prognostic and therapeutic predictors. However, there are still major challenges in accurate early prediction of breast cancer incidence, detection and prognosis. Given that DNA methylation changes are plausibly critical components of the molecular mechanisms involved in breast cancer, distinct DNA methylation profiles may help improve the accuracy of prediction of incidence, detection and prognosis. The number of genes identified as being aberrantly methylated in breast cancer is rapidly growing (reviewed in [20]). These genes encompass multiple pathways leading to malignancy, including the six alterations proposed by Hanahan and Weinberg required to transform a healthy cell into a cancer cell: unlimited replication potential, self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, sustained angiogenesis, and tissue invasion and metastasis [21].

In this chapter, we review the methods used to assay DNA methylation in human studies and the evidence to date from clinical and epidemiological studies on DNA methylation and breast cancer. We focus our review on describing the most common measurement techniques used to ascertain DNA methylation in human studies and then evaluate the evidence base for DNA methylation to enhance tertiary prevention (reduction of morbidity after diagnosis and improving overall survival), secondary prevention through early detection of disease, and primary prevention as a risk marker to reduce overall breast cancer incidence.

DNA Methylation, Definitions and Measurement Methods

Epigenetics is defined as changes in gene expression in the absence of changes in DNA sequence. Levels of DNA methylation, histone modifications and microRNA expression are the three main epigenetic drivers of altered gene expression. As the evidence base is largest for DNA methylation biomarkers, here, we concentrate

on studies of DNA methylation, specifically 5-methylcytosine (5mC), which results from the addition of a methyl group to the 5′ position of cytosine primarily in CpG sequences. DNA methylation is essential in development and cell differentiation, silencing of transposable elements, genomic imprinting and X-chromosome inactivation. In cancer, it is well established that tumors have lower levels of total 5mC than adjacent tissues (reviewed in [22]). This hypomethylation is primarily in repetitive elements which make up the majority of our DNA and leads to their reactivation, increased illegitimate recombination, and genomic instability. This loss of methylation is an early event in carcinogenesis. Gene-specific hypomethylation can also occur and results in the re-expression of affected genes. Gene-specific hypermethylation, particularly in CpG island promoters, is the more common and well-studied event and is associated with gene inactivation. Thus, we now know that inactivation of tumor suppressor genes is not only the result of mutation but also of DNA methylation. In breast cancer, as discussed below, a large number of genes have been identified as having hypermethylated CpG island promoters and include those involved in DNA repair, cell-cycle regulation, apoptosis, chromatin remodeling, cell signaling, transcription and tumor cell invasion.

In addition to 5mC, which is present at levels of about 4 % of the cytosines, 5-hydroxymethylcytosine (5hmC) is present but at much lower levels. This is the result of Tet enzyme oxidation of 5mC [23]. This family of enzymes can further oxidize 5hmC to 5-formylcytosine and 5-carboxylcytosine, both of which are substrates for thymidine–DNA glycosylase, a DNA repair enzyme. This pathway of oxidation and base removal and repair is believed to be a mechanism for removal of the methyl group from cytosine.

A large number of methods have been developed for analysis of DNA methylation including evaluation of total 5mC; levels of methylation in repetitive elements that are a large fraction of the human genome as an indirect measure of global methylation; and levels in specific genes, primarily in CpG-rich promoter regions, but also in gene bodies and regions more distant from genes. While a large number of methods have been developed for the analysis of DNA methylation (reviewed in [24–28]), a much more limited range of assays has been applied to human health studies. These methods as well as their strengths and limitations are given in Table 1. Early studies digested DNA to nucleosides and analyzed levels of 5-methyldeoxycytidine (5mdC) by high performance liquid chromatography (HPLC) or used antibodies to bind to 5mC to obtain qualitative data. More recently, liquid chromatography-mass spectrometry (LC/MS) that allows the use of an internal standard for highly accurate and sensitive quantitation has been used [29]. This has also facilitated the quantitation of 5hmdC; however, this method as well as HPLC generally requires 1μg of DNA [30]. Another method takes advantage of the ability of the *Sss*I prokaryotic methylase enzyme to indiscriminately methylate all unmethylated CpG sequences using [^{3}H]*S*-adenosylmethionine as the methyl group donor [31]. Therefore, the ability of DNA to incorporate [^{3}H] methyl groups *in vitro* is inversely related to endogenous DNA methylation. Another method that looks at general levels of DNA methylation is the luminometric methylation assay (LUMA) which specifically analyzes 5mC in C^{m}CGG regions. It takes advantage of a pair of

Table 1 Methods commonly used for analysis of DNA methylation in epidemiologic studies

Assay	Method	Advantages	Disadvantages
5methylC by HPLC or LC/MS	Enzymatic digestion of DNA followed by analysis	Absolute, specific quantification of total levels of 5mdC; can also measure 5hmdC	Expensive equipment especially for LC/MS; MS need internal standard
[^{3}H] Methyl acceptance assay	Enzymatic radioactive labeling of non-methylated CpG sites	Global measure of methylation	Uses radioactivity, variable with batch of enzyme and *S*-adenosylmethionine; requires highly accurate DNA quantitation
Luminometric methylation assay (LUMA)	Methylation sensitive restriction digestion followed by pyrosequences	Global measure of methylation at CCGG sites	Limited CpG sites evaluated; requires high quality DNA
Bisulfite treatment-based assays			
Combined bisulfite restriction analysis (COBRA)	Restriction digestion of PCR amplified regions	Provides semi-quantitiative data at specific regions	Analysis limited to specific restriction target sites; gel analysis limits high throughput
Methylation- specific PCR (MSP)	Separate primers for methylated and non-methylated DNA followed by gel analysis	Requires limited equipment; sensitive to 0.1% methylated alleles	Gel analysis limits high throughput; not quantitative; only one region analyzed per assay
Fluorescence-based real time methylation-specific PCR	MSP but with fluorescence detection	Suitable for high throughput; highly quantitative; sensitive to $1/10^5$	Requires more expensive instrumentation than PCR; only measure DNA fully methylated on sites covered by primers; only one region analyzed per assay
MethyLight	Adds Taqman probe to real time PCR	Suitable for high throughput; highly quantitative	Requires more expensive instrumentation than PCR; only measure DNA fully methylated on sites covered by primers; sites covered by probe must also be fully methylated; only one region analyzed per assay

Table 1 (continued)

Assay	Method	Advantages	Disadvantages
Pyrosequencing	Sequencing by synthesis after amplification with non-methylation-specific primers; sequencing probe also does not contain CpG sites	Relative level of methylation at each CpG analyzed; control for efficiency of bisulfite conversion	Requires expensive instrumentation; only one region analyzed per assay; sometimes impossible to design appropriate primers and probe for specific region
Illumina Infinium 27k and 450k CpG HumanMethylation BeadChip arrays	Two types of assays; type 1 uses two probes per CpG (methylated and unmethylated); type 2 uses degenerate probes and two colors	Information from across the genome; easily interpretable beta values for methylation level	Expensive instrumentation and arrays; only interrogates sites on the array; two chemistries, specific probes and SNPs require careful data analysis
Next generation sequencing	Varies by platform	High resolution analysis of each cytosine in the genome; also obtain genetic information	Expensive; large fraction of C converted to T (reduced sequence complexity) complicates sequence alignment

isoschizomer restriction enzymes that cut DNA differentially based on methylation status. Sequencing of the product allows determination of methylation but only in CCGG sequences [32].

A major advance in analysis of DNA methylation resulted from the demonstration that treatment of DNA with sodium bisulfite resulted in deamination (the removal of an amine group) of unmethylated cytosines converting them to uracil while leaving 5mC intact. Since uracil pairs with adenine, polymerase chain reaction (PCR) primers can be designed with either an A or a G opposite the position of the C in CpG sequences. Cs in non CpG sequences, since generally not methylated, will be converted to U and an A will be used in the PCR primer. Upon PCR, the U is amplified as a T. Thus, Cs in unmethylated CpG sites are converted to Ts while methylated CpG sites remain as Cs. In methylation specific PCR (MSP), two sets of primers are designed specifically for the modified DNA strand encompassing several CpG sites, one assumes a C and the other a T in Cs in CpG sites. PCR is then followed by gel analysis for qualitative determination of whether methylated and/or unmethylated DNA is present [33].

This basic bisulfite treatment methodology has also been applied to real time fluorescence PCR eliminating the need to run gels, as well as to microarray analysis, sequencing and other types of assays. There are a number of variations of the real time assays, some using a combination of methylated and unmethylated primers with cyber green for quantification of amplified DNA and others using a control gene [21–23]. A specific variation of real time PCR, the MethyLight assay uses TaqMan probes for quantification [34]. The fluorescence-based PCR assays are much more sensitive than MSP, but also allow high throughput since they can be run on 96- or 384-well plates. All the PCR methods that use methylation specific primers/probes detect only those DNA strands that are fully methylated for the CpG sites that are interrogated by the primers or probe; they cannot discriminate between 5mdC and 5hmdC. While small quantities of DNA are required for each PCR reaction, bisulfite modification is generally carried out on a minimum of 250 ng of DNA. All bisulfite-based assays also are dependent on the complete conversion of C to T for accurate data. In addition, differential PCR efficiency with methylated and non-methylated primers can impact results.

Bisulfite sequencing has been used extensively in epidemiologic studies for analysis of methylation. For both analysis of specific genes as well as repetitive elements (e.g., LINE-1, Alu), pyrosequencing has been the method of choice due to its relatively low cost [35]. In contrast to real time PCR, primers do not contain CpGs so that both methylated and unmethylated DNA will be amplified. The sequencing probe that sits adjacent to the region of interest also does not contain CpG sites. Synthesis of the DNA strand from the 5′ to 3′ direction is carried out one base at a time by incubation with the appropriate triphosphate (dNTP) based on known DNA sequence. Each incorporation event is accompanied by the release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide. ATP sulfurylase coverts the PPi to ATP in the presence of adenosine 5′phosphosulfate and this ATP drives an enzymatic reaction that generates light. When sequencing through positions that might contain either a C or a T, both G and A dNTPs are sequen-

tially added, which allows calculation of average level of methylation of each CpG site in the region sequenced, which is generally <300 base pairs in length. Allele-specific methylation data, or methylation along a single strand of DNA, can only be obtained if PCR products are cloned prior to sequencing, but this is not feasible in epidemiologic studies. Pyrosequencing is also not accurate at very low or high levels of methylation. The sensitivity limitation for pyrosequencing is ~5%. Next generation bisulfite sequencing is the most comprehensive method of analysis as it allows determination of methylation of multiple regions at the same time or even across the genome. Different platforms utilize different technologies, but all provide large amounts of data even with relatively small amounts of DNA. However, there are cost limits in the utilization of these platforms in epidemiological studies.

Bisulfite treated DNA has also been analyzed using Illumina Infinium HumanMethylation BeadChips that evaluate methylation of >27,000 or >450,000 CpGs. Two types of chemistries are used on the 450 K chips that lead to some differences in data, but both provide beta values or percent of methylation at each site. The low cost per data point and ease of data interpretation have made these arrays commonly used in epidemiologic studies. While results are frequently referred to as genome-wide analysis data, they are limited to the specific CpG sites on the chip. However, the 450 K array covers 99% of RefSeq genes, with an average of 17 CpG sites per gene region distributed across the promoter, 5′UTR, first exon, gene body, and the 3′UTR [36]. The 450 K array covers 96% of CpG islands, with additional coverage in island shores and the regions flanking them. One challenge with methylation studies is knowing which region of the DNA to analyze [37]. Most gene-specific methylation studies evaluate promoter regions upstream and downstream of transcription start sites. While these regions are clearly important, we now know that that intra-genic CpG sites as well as CpG shores may also be important (reviewed in [38, 39]). While levels of gene expression are often of primary interest, the relation between methylation levels and gene expression is rarely evaluated.

DNA methylation is dynamically changing over the lifecourse, but most studies only have samples from one time point. Here, we describe how DNA methylation markers may be useful in improving prognosis and overall survival (tertiary prevention), early detection (secondary prevention) and primary prevention. The importance of DNA methylation markers across all stages of the prevention continuum is strengthened by the recent data from The Cancer Genome Atlas (TCGA) on DNA methylation of over 800 breast tumors using Illumina Infinium HumanMethylation BeadChips. The data have dramatically expanded the number of genes identified as aberrantly methylated in breast cancer [40]. Knowing whether these aberrantly methylated genes in the tumor tissue are influenced by modifiable factors across the lifecourse, and/or affect early detection and tumor growth, and/or response to treatment and overall survival will have major implications for primary, secondary, and tertiary prevention efforts. In TCGA, unsupervised clustering analysis of the methylation array data identified five distinct DNA tumor groups. Group 3 showed a hypermethylation phenotype, was significantly enriched in the *luminal-B* mRNA subtype, and was under-represented for *PIK3CA, MAP3K1* and *MAP2K4* mutations. Group 5 showed the lowest levels of DNA methylation, overlapped with the

basal-like mRNA subtype, and had a high frequency of *TP53* mutations. Other studies examining the associations between whole-genome DNA methylation and breast cancer classification found that there were distinct methylation patterns by hormone receptor status [41, 42] and by *BRCA* mutation state [43]. Methylation profiling was also shown to reflect the cell type composition of the tumor microenvironment, specifically T lymphocyte infiltration [44]. In addition, methylation patterns in selected genes were significantly associated with disease progression [41, 42] and survival [45]. Thus, DNA methylation markers by enhancing molecular characterization of breast tumors show potential utility in population health prevention and screening and clinical care. Here we review the evidence to evaluate its potential across the cancer prevention continuum starting with improving outcomes after diagnosis and ending with primary prevention.

DNA Methylation Markers and Tertiary Prevention and Role in Prognosis

Extensive data examining DNA methylation in tissue samples at the time of diagnosis exist, however, there are far fewer studies that have prospectively followed breast cancer cases to examine how DNA methylation patterns at the time of diagnosis relate to overall survival and prognosis after breast cancer diagnosis. For example, although there have been several thousand studies that report DNA methylation and breast cancer, when we used the following search strategy in MEDLINE from the earliest available publication to September 2014 (the following search terms included forms of methylation + breast cancer + prognosis or recurrence or survival + serum or plasma in varied combinations) using two separate and independent reviewers, we only found 82 studies of DNA methylation in tissue or plasma at the time of diagnosis that examine DNA methylation and prognosis. Of these 82 studies, we reviewed the subset that specifically followed up patients longitudinally to evaluate whether DNA methylation markers are related to overall prognosis and mortality and that met the following additional criteria: (1) reported on either disease-free survival (DFS) or overall survival (OS) using survival regression methods and (2) had at least 30 events of either relapse or death (Table 2). We used these criteria because we specifically wanted to focus on whether DNA methylation markers predicted DFS or OS, over and beyond the standard clinical prognostic markers. As evidenced by TCGA, many DNA methylation markers map to subtypes of tumors [40]. For clinical utility, it is necessary to know whether new markers add to the prediction of DFS and OS after considering standard clinical metrics like stage, grade, tumor size, and nodal status. To do so, multivariable regression models are needed; such models require large sample sizes to yield precise estimates. For example, in one study that we did not include in Table 2 because it did not meet the criterion for the number of events, the overall unadjusted association of methylation in the *NEUROD1* gene with relapse free survival was 0.8 (relative risk (RR) = 0.8, 95% confidence interval (CI) = 0.3–1.8) but the adjusted association was over six-fold (relative

Table 2 Summary of studies evaluating the tertiary prevention potential of DNA methylation markers using breast tissue or plasma

	Sample size (N)	Follow-up time	Genes	Outcomes (HR (95% CI))		
				Disease free survival	Recurrence	Overall survival/mortality
Tissue studies						
Huang 2013 [52]	175 primary breast cancer samples	Mean 45.81 months (15–124 months)	*PTPRO*	–	–	Reference group: Unmethylated 3.27 (0.75–14.21)
Xu 2013 [56]	167 triple-negative breast cancer treated with adjuvant chemotherapy	Median 9 years (0.41-15.1 years)	*BRCA1*	Reference group unmethylated	–	Reference group unmethylated
	675 non-triple-negative breast cancer treated with adjuvant chemotherapy			0.45 (0.24–0.84) for triple-negative		0.43 (0.19–0.95) for triple-negative
				1.56 (1.16–2.12) for non-triple negative		1.53 (1.05–2.21) for non-triple negative
Hsu 2013 [57]	139 early stage breast cancer samples including 21 triple-negative	Up to 120 months[a]	*BRCA1*	Reference group unmethylated: 12.19 (2.29-64.75)	–	Reference group unmethylated: 16.38 (1.37–195.45)
Lu 2012 [53]	348 primary breast cancer samples	Median 86 months (8-108 months)	*HOTAIR*	–	Reference group low methylated: 0.95 (0.52–1.73)	Reference group low methylated: 1.15 (0.58–2.31)

Table 2 (continued)

	Sample size (N)	Follow-up time	Genes	Outcomes (HR (95% CI))		
				Disease free survival	Recurrence	Overall survival/mortality
Lo Nigro 2012 [62]	157 primary breast cancer samples including 119 ER-positive, including 11 HER2+ only, and including 26 triple negative	Up to 12.5 years[a]	*NT5E*	Reference group methylated: HR 2.7, $p=0.001$ (95% CI not listed)	–	Reference group methylated: HR 3.0, $p=0.001$ (95% CI not listed)
Van Hoesel 2012 [55]	222 early stage breast cancer samples	Median 19.9 years (0.25–23.0 years)	*MINT17, MINT31, RARβ2*	Reference group meth-Neutral: meth-High: 1.51 (0.99–2.32)	Reference group meth-Neutral: meth-High: 1.70 (0.99–2.92)	Reference group meth-Neutral: meth-High: 1.36 (0.89–2.08)
				meth-Low: 1.35 (0.91–2.02)	meth-Low 1.44 (0.85–2.92)	Meth-Low: 1.14 (0.75–1.72)
Li 2014 [59]	98	Median 60 months (43–70 months)	*PTPRO*	–	–	Reference group unmethylated: HR 2.7 (1.1–6.2)
Zeng 2012 [156]	302	Median 86.3 months (8.2–107.8 months)	*L3MBTL1*	Reference group Low Meth: Med Meth: 1.26 (0.73–2.18) High Meth: 0.97 (0.55–1.71) $P_{trend}=0.97$	–	Reference group Low meth Med Meth: 1.3 (0.66–2.56) High Meth: 1.45 (0.75–2.77) $P_{trend}=0.27$

Table 2 (continued)

	Sample size (N)	Follow-up time	Genes	Outcomes (HR (95% CI))		
				Disease free survival	Recurrence	Overall survival/mortality
Krasteva 2012 [157]	135 sporadic invasive primary breast cancer samples	5 years	*BRCA1*	–	–	Reference group unmethylated: 0.91 (0.24-3.41)
Cho 2012 [60]	670 invasive breast cancer samples	Mean 8 years (0.3-9.4 years)	*HIN1*, *RASSF1A*, *DAPK1*, *GSTP1*, *CyclinD2*, *TWIST*, *CDH1* and *RARβ*	–	–	Breast cancer-specific mortality: Reference group unmethylated: *HIN1* 1.17 (0.75–1.83) *RASSF1A* 1.77 (0.86–3.67) *DAPK1* 1.27 (0.72–2.22) *GSTP1* 1.71 (1.10–2.65) *CyclinD2* 1.18 (0.71–1.99) *TWIST* 1.67 (1.01–2.79) *RARβ* 1.78 (1.15–2.76) All-cause mortality: Reference group unmethylated: *HIN1* 1.07 (0.77–1.47) *RASSF1A* 1.21 (0.76–1.93) *DAPK1* 1.10 (0.72–1.66) *GSTP1* 1.49 (1.08–2.07) *CyclinD2* 1.27 (0.89–1.81) *TWIST* 1.37 (0.93–2.01) *RARβ* 1.45 (1.05–2.02)

Table 2 (continued)

	Sample size (N)	Follow-up time	Genes	Outcomes (HR (95% CI))		
				Disease free survival	Recurrence	Overall survival/mortality
Noetzel 2010 [58]	195 primary, unilateral, invasive breast cancer Patients did not undergo neoadjuvant chemotherapy	Up to 140 months based on longest median recurrence free survival reported	*SYNM*	Reference group unmethylated: 2.94 (1.12, 7.71)	–	–
Akhoondi 2010 [48]	68 (C1)b and 93 (C2)b breast cancer samples	Up to 5 years following diagnosis[a]	*FBXW7/ hCDC4-β*	–	–	Reference group unmethylated: C1: 0.53 (0.23–1.23) C2: 0.50 (0.23–1.08)
Chen 2009 [158]	536 breast cancer samples	Median 8 years (0.4–11.6 years)	*BRCA1*	Reference group unmethylated: Disease-free survival: 1.23 (0.84–1.80) Disease-specific survival: 1.27 (0.81–1.99)	–	–
Xu 2009 [159]	851 breast cancer samples	Mean 5.6 years (0.2–7.4 years)	*BRCA1*	–	–	Reference group unmethylated: Breast cancer-specific mortality: 1.67 (0.99–2.81) All-cause mortality: 1.40 (0.94–2.08)

Table 2 (continued)

	Sample size (N)	Follow-up time	Genes	Outcomes (HR (95% CI))		
				Disease free survival	Recurrence	Overall survival/mortality
Hartmann 2009 [50]	241 breast tumor samples All patients treated with adjuvant anthracycline-based chemotherapy	Median 81.5 months	*PITX2* and 60 additional candidate genes	–	Reference group Q1 methylated: *PITX2P2* Q4 methylated 1.28 (1.03–3.83)	–
Nimmrich 2008 [61]	412 (all LNN/HR+)c	Median 98 months (1–233 months)	*PITX2*	–	–	Reference group low methylation: 1.53 (1.21–1.92)
Van Hoesel 2012 [54]	379 primary ductal breast cancer patients	Median 19 years (0–23 years)	*LINE-1*	Reference group methylated: 2.05 (1.14–3.67) for younger cases 0.83 (0.57–1.20) for older cases	Reference group methylated: 2.83 (1.53–5.21) for younger cases 0.67 (0.40–1.10) for older cases	Reference group methylated: 2.19 (1.17–4.09) for younger cases 0.73 (0.50–1.07) for older cases
Plasma						
Gobel 2011 [70]	428 primary breast cancer samples	Median 51 months (IQR[e] 35–68)	*PITX2* *RASSF1A*	Reference group unmethylated: *RASSF1A* 3.4 (1.6–7.3)	–	Reference group unmethylated: *PITX2* 3.4 (1.2–9.8) *RASSF1A* 5.6 (2.1–14.5)

[a] Follow-up time not provided and estimated from Kaplan Meier curve
[b] Cohort 1 (C1) and Cohort 2 (C2)
[c] Lymph node-negative, steroid hormone receptor positive (LNN/HR+)
[d] Odds ratios (OR) were provided
[e] Interquartile range (IQR)

risk (RR) = 6.2, 95 %CI = 1.6–24) after adjusting for tumor size, grade, lymph node metastases, and menopausal status [46]. There were only 10 events in the group with low methylation in *NEUROD1* and 11 events in the group with high methylation [46]. Thus, with so few events, large associations in multivariable models may result from model over-fitting.

As reviewed in Table 2, only 17 studies of 82 studies on DNA methylation met the criteria that were large enough to adequately address the relation between tissue-based DNA methylation markers and DFS or OS have identified a number of markers that are independently related to outcomes after diagnosis. For example, the methylation patterns in selected genes including *RASSF1A* have been associated with disease progression [42, 47] and relapse-free survival [13, 42, 47–49]. Paired-like homeodomain transcription factor 2 (*PITX2*) DNA methylation has also been validated using a robust assay for paraffin-embedded tissue for clinically relevant outcome prediction in early breast cancer patients treated by chemotherapy [50], suggesting that DNA methylation signatures have important therapeutic implications in guiding the use of epigenetic drugs in anticancer therapy [51]. The Long Island Breast Cancer Study Project, a population-based case-control study that followed cases for prognosis and survival, evaluated ten genes in breast tumors of 670 invasive cancers and found that methylation of the tumor suppressor genes *GSTP1, Twist* and *RARβ* was significantly associated with higher breast cancer-specific ($n = 86$) mortality with a mean follow-up time of 8 years [47, 49]. Compared to cases with an unmethylated promoter in tumor tissues, those with a methylated promoter had a 71, 67 and 78 % increased risk of dying from breast cancer at the end of follow up for methylated *GSTP1* (hazard ratio (HR) = 1.71, 95 % 1.10–2.65), *Twist* (HR = 1.67, 95 %CI 1.01–2.79), and *RARβ* (HR = 1.78, 95 %CI 1.15–2.76), respectively. Similar associations between methylation status and all-cause mortality ($n = 161$) were observed [47].

In Table 2, with one exception [52], all studies have a follow-up of at least 5 years. Among the studies that do not report mean or median follow-up time, based on the Kaplan Meier curves, we assume follow-up time spanned at least 5 years. Few studies examined recurrence [50, 53–55] with only two observing significant associations with methylation [50, 54]. Among chemotherapy-treated patients, higher methylation of *PITX2*, a gene associated with tumor aggressiveness and tamoxifen resistance, was associated with time to distant metastasis [50]. Gene-specific methylation, the number of methylated genes, as well as global gene methylation were all significantly associated with poor DFS (HR 2–3 fold) [54, 56–58] and poor OS (HRs in the range of 1.2–3.0) [54, 57, 59–61]. One study exceeded these observed effect sizes. Among women treated with adjuvant chemotherapy, *BRCA1* methylation was associated with poor survival with effect sizes between 12–16 fold; however, measures were imprecise given the large confidence intervals [57]. While the majority of studies found methylation associated with poor prognostic outcomes, methylated *NT5E*, another gene linked to tumor aggressiveness, was associated with improved survival [62]. Table 2 also demonstrates that when examining methylation and tertiary prevention, the association between methylation and prognosis can vary in direction and magnitude across subpopulations which can be

based on tumor (e.g. triple-negative) and/or sociodemographic characteristics (e.g. age). In addition to these studies, three large studies that do not report event rates, with median follow-up time exceeding 5 years, found higher methylation associated with worse OS in single gene analyses [63] or gene panels [64, 65]. As is evidenced in Table 2, a number of important genes for breast cancer are also methylated and the methylation status affects outcomes. In summary, although limited prospective evidence exists, studies reported to date suggest that promoter methylation, particularly for a panel of tumor suppressor genes, has the potential to be used as a biomarker for predicting breast cancer prognosis; however, the data so far are very limited and the predictive value of the small number of DNA methylation signatures that have been identified is unclear.

Although the bulk of the epidemiologic evidence is with breast-tissue specific methylation, associations of plasma and serum DNA methylation and prognosis have also been observed. For example, patients with methylated *RASSF1A* and *APC* had worse prognosis than those without [66]. Several studies have examined recurrence [67–69] and survival [52, 64, 66, 67, 70–73]; with some focusing on the prognostic value of serum/plasma DNA methylation post therapeutics [64,69,66]. The source of the blood sample for the sera or plasma can also vary and may lead to different prognostic results. Peripheral blood plasma and bone marrow plasma samples were collected from 428 breast cancer patients during primary surgery with a median observation time of 51 months (interquartile range 35–68 months). In Kaplan-Meier analyses, methylated *PITX2* and *RASSF1A* in peripheral blood plasma were significantly associated with DFS and OS while associations were weaker in bone marrow plasma. Moreover, there was stronger prognostic value for DFS and OS when combining methylated *PITX2* and *RASSF1A* [70] indicating that plasma source, as well as gene panels, are important factors when testing prognostic biomarkers. Studies have also shown that methylation patterns change over time [74–76]. In one study, using cell-free plasma DNA, methylation patterns changed after surgery and tamoxifen treatment suggesting that methylation may also be used to monitor treatment [77]. Serum markers may also have prognostic utility. Studies have shown that tumor methylation patterns are highly correlated with serum methylation [15–17]. For example, the correlation coefficient of *GSTM*1 methylation in breast tumor tissues and serum was 0.365 [15]. Therefore, DNA methylation in plasma or serum is an attractive prognostic tool as it can be measured repeatedly and may help monitor response to therapeutics, DFS, and OS over time. In addition, plasma or serum DNA is an easier sample to procure in comparison to tissue samples.

In summary, the evidence that markers of DNA methylation, both in breast tissue, plasma, and serum collected at diagnosis may be important prognostic markers is intriguing and growing. As the evidence to date has primarily been relatively weak with breast cancer specific outcomes (e.g., breast cancer specific mortality), and also with only a select sample of markers, larger prospective studies that address a panel of markers are needed. It will be critical to identify those studies that collect extensive data on other clinical markers so that the contribution these methylation biomarkers make over standard clinical markers such as stage, grade,

tumor size and molecular subtype in predicting DFS and OS can be determined. In addition to general prospective observational cohorts, clinical trial data using stored breast tissue and plasma samples from diagnosis, where available, have the advantage of examining the impact of gene-methylation over and beyond detailed therapeutic information among a cohort of individuals who may be more homogenous with respect to stage and overall treatment than participants in an observational epidemiologic study. In addition to the markers measured at baseline, it will be very useful to evaluate if repeated plasma samples can be useful to complement screening protocols after diagnosis.

DNA Methylation Markers for Secondary Prevention and Early Detection

Regular mammographic screening has greatly improved breast cancer mortality among women ages 40–74 [78, 79]; however, mammography has limited sensitivity and specificity particularly in women with dense breasts [80, 81] and in younger women [82, 83]. Moreover, breast cancer is a complex disease that is difficult to detect in early stages by a single-marker approach. A variety of different markers and risk factors combined and weighted using robust and validated statistical models are needed to improve disease screening sensitivity [84]. Thus, identification of other markers for improved early detection is critical. In addition, more accurate risk assessment and risk stratification will improve the population effectiveness of these screening modalities.

While plasma and serum biomarkers have been used as a prognostic tool to determine treatment and diagnosis, there has been limited use of blood biomarkers as a reliable secondary prevention screening tool. For a plasma/serum biomarker to have adequate screening ability, the marker must be able to identify as positive those with the disease (high sensitivity) and be able to identify as negative those without the disease (high specificity). The most well-known examples of plasma/serum cancer screening biomarkers are the prostate-specific antigen (PSA) test for the detection of prostate cancer and CA-125 for ovarian cancer. While both screening tools are widely used, the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial has demonstrated that neither PSA nor CA-125 screening meet the criteria of a good sensitive and specific screening tool [85, 86]. Cancer research continues to identify serum/plasma biomarkers as independent and synergistic cancer screening tools for secondary prevention as the biological processes involving cancer detection in blood is well established.

That cell-free DNA is released from tumors and can be found circulating in the plasma was first discovered by analysis of mutations in *KRAS* and *P53* [87, 88]. Although they can vary widely, in general, levels of circulating DNA are higher in cancer cases than controls, ranging from 0 to >1 μg/ml of plasma; healthy individuals generally have <25 ng/ml (reviewed in [89, 90]). In individuals without cancer, an increase in DNA in the blood can be caused by exercise, inflammation, and

tissue injury [91]. In cancer cases, tumor circulating DNA is thought to originate from necrotic and apoptotic tumor cells. While initial studies analyzed mutations in circulating DNA, it was also found to contain methylation patterns similar to those found in primary tumors, suggesting the potential utility of blood-based molecular detection of cancer including breast cancer (reviewed in [89, 92–94]).

DNA hypermethylation of selected biomarkers, such as *RASSF1A* and *RARβ2*, was found to occur early in breast cancer development, suggesting that plasma DNA methylation might be useful as an early marker of disease [13]. Multiple genes that are now more frequently evaluated include *CDH1, RASSF1A, APC, BRCA1, GSTP1, RARβ*, and others (reviewed in [19, 93, 94]), with many studies showing reasonably consistent results. However, methylation of a single gene often results in low sensitivity; using a panel of epigenetic markers seems to achieve a more reasonable sensitivity with high specificity in breast cancer detection [93]. Most studies of methylation in serum/plasma DNA used samples collected at or just after breast cancer diagnosis. This retrospective design can result in bias if methylation levels are affected by disease progression or treatment. In order to evaluate the usefulness of DNA methylation markers in plasma as potential screening tools, it is important to understand whether methylation markers can be detected in plasma years prior to diagnosis. In a pilot study, we measured methylated *RASSF1A* in plasma DNA collected before diagnosis from 28 women with breast cancer and 10 of their unaffected siblings as well as from 33 women with breast cancer and 29 age- and ethnicity-matched population-based controls [95]. We found 18 % of cases were positive for methylation of *RASSF1A* in their plasma DNA collected before diagnosis, while only 5 % of controls were positive. Tumor tissue was available for 12 cases and all were positive for *RASSF1A* methylation. Our results suggest that aberrant promoter hypermethylation in serum/plasma DNA may be common among high-risk women and may be present years before cancer diagnosis. However, another study measured methylation in *RASSF1A, GSTP1, APC* and *RARβ2* using a nested case-control ($n = 50$ cases and 100 controls) study design within the prospective New York University Women's Health Study cohort [96]. While the frequency of methylation in each gene was lower than expected among cases and higher than expected among controls, the frequencies did not differ between cases and controls.

While many studies have used plasma for early detection [14, 59, 70, 97–99], studies of plasma DNA methylation must take into account the technical aspects of plasma collection. For example, the time between blood collection and processing may impact the amount of DNA obtained due to the potential for lysis of white blood cells (WBC). The ratio of tumor to normal DNA in plasma may also be low given that plasma DNA can come from all tissues. There are conflicting data on the percentage of circulating DNA that comes from the tumor, with a range of 10–90 % reported (see review in [89]). Another limitation is that circulating DNA is highly fragmented, typically 160–180 bp in length [100]. So care must be taken in the design of PCR primers to ensure that most samples will give a PCR product. In our own studies of plasma DNA in liver cancer, we found that the success of PCR decreased from 80 to 100 % for reactions with products < 200 bp to 63 % for one reaction with a 248 bp product [101]. Finally, it is clear that plasma DNA is lost rapidly

when the source is removed. This was first observed in studies of pregnancy where it was found that fetal DNA disappeared from the mother's blood within hours of delivery [102]. Much optimization remains to be done, both in terms of increasing the sensitivity of both assays and guarding against false positives.

The great potential of plasma markers for screening enhancement is that it could complement the existing protocol of Magnetic Resonance Imaging (MRI) and mammography in very young women if the plasma markers are sensitive and specific. Currently, according to National Cancer Institute (NCI) Guidelines [103] as well as the National Comprehensive Cancer Network (NCCN) [104], women who are high-risk should be screened with MRI and also mammography starting as early as 25. Criteria to define high-risk vary by guidelines but include (*BRCA1* or *BRCA2* carrier, lifetime risk of >20% as defined by models that are largely dependent on family history, or 5-year risk of >1.7%. This means that a woman with lower DNA repair capacity because of mutations in *BRCA1* or *BRCA2*, among other genes, typically may have 15–20 mammograms by their early 40s. Instead, if MRI could be coupled with sensitive plasma markers for monitoring, this would translate into a substantially lower radiation dose. In order to evaluate this, prospective observational cohorts, ideally utilizing a range of sample collection and/or repeated blood collection so that a panel of genes can be evaluated in the plasma would be essential for uncovering whether or not plasma markers can enhance the potential of screening with MRI. For average risk women who are already postmenopausal, mammography has already been shown to be highly effective at reducing mortality [1, 105] so the main question for subsequent research would be whether plasma markers can help make the findings from mammography more useful in terms of informing the screening interval (number of years between screens) as well as improve the overall specificity of mammography to reduce the false positives.

In addition to plasma markers, an intraductal approach to early breast cancer detection, which includes nipple aspiration, ductal endoscopy, and ductal lavage (DL), has also been explored within the context of DNA methylation (as reviewed in [94, 106, 107]). Epigenetic analysis of DNA methylation in DL fluid for early breast cancer detection has been evaluated [108–111] including in women at high genetic risk of breast cancer [112, 113]. Analysis of methylation of *Cyclin D2, RAR-β*, and *Twist* using cells from DL fluid found cancer-specific methylation in patients with ductal carcinoma *in situ* (DCIS); abnormal methylation in cells from some of the healthy women, who later developed breast cancer was also observed. These results provided the first direct evidence that DNA methylation can be used to detect cancer in asymptomatic individuals with non-suspicious mammograms and normal breast examinations [108]. However, the Breakthrough Breast Cancer Research group has provided evidence that, while DNA methylation detected in DL fluid may be a strong prognostic marker for cancer patients, such methylation lacks specificity. DNA methylation was assessed in six tumor suppressor genes from tumor tissue, adjacent tissue and bilateral DL fluid of cases and from normal tissue and DL fluid of healthy controls. Between bilateral DL fluid and tumor tissue, the highest sensitivity for methylation markers was observed for *SCGB3A1* (90%), *CDH13* (91%), and *RAR-β* (83%). There was poor discriminatory ability of DL biomarkers. The

area under the curve (AUC) for the receiver operator characteristic curve for cancer DL DNA methylation ($n=54$ samples) compared to healthy control DL DNA methylation ($n=46$ samples) was 0.76 with a specificity, or error, of 22% [109]. Biomarkers present promising utility for high-risk populations and limited studies have examined DL fluid and epigenetic analysis for women at high genetic risk [112, 113]. A prospective study of 34 *BRCA* mutation carriers (16 *BRCA1* and 18 *BRCA2)* measured hypermethylation of *CDKN2A, RASSF1A, Twist*, and *RAR-β* in DL fluid collected prior to breast cancer development in seven women. There was a significant association between *RASSF1A* methylation and the development of breast cancer and hypermethylation of *CDKN2A* was associated with *BRCA1* mutation status [112]. Larger prospective epidemiological studies are needed with larger gene panels to determine if DL fluid is a promising, non-invasive, screening tool for early breast cancer detection or diagnosis.

Studies examining the correlation of DNA methylation in breast tumor tissues and plasma show similarities between methylation patterns found in primary tumor specimens and those in plasma, indicating the potential utility of blood-based molecular detection of breast cancer. Overall, DNA methylation is a candidate biomarker because of numerous characteristics: (1) in the process of carcinogenesis, promoter hypermethylation is a more frequent event than mutations [114], with estimates varying from 600 to 1000 aberrantly methylated genes per tumor [115], (2) methylation has been shown to be an early event in breast tumorigenesis [116–118], (3) not only the malignant cells but also the surrounding tissue shows methylation defects [15–18, 119], (4) DNA methylation is stable and can be amplified by PCR, which means that aberrations can be relatively easily analyzed in very small amounts of DNA [120] as opposed to other approaches such as gene expression profiling, (5) a hypermethylated sequence forms a positive signal against an unmethylated background, which makes it more easily detectable than genetic alterations such as loss of heterozygosity [121]. Emerging evidence has shown that DNA methylation of select genes measured in plasma results in sensitivities >90% for detecting breast cancer. These results suggest that DNA methylation has promise for screening. Yet, these small clinical studies were cross-sectional with no or limited corresponding epidemiologic data. Further, unlike more easily collected blood, tissue is not suitable for use as a screening method. Of those studies that used blood, samples were collected at diagnosis, raising concerns about temporality.

DNA Methylation Markers and Primary Prevention

In addition to studies of tertiary and secondary prevention, DNA methylation markers have been evaluated to see if they are useful biomarkers for ascertaining risk. In these studies of individuals with and without breast cancer, the primary source for the DNA methylation markers are peripheral blood cells, as it is often difficult to get breast tissue in women without breast cancer and plasma markers measure circulating tumor cells may not be useful to examine in samples collected many years prior to cancer diagnosis.

Studies examining the relationship between methylation of DNA from peripheral blood cells and breast cancer risk have largely been case-control investigations in which blood samples are collected from cases after the diagnosis of breast cancer. This study design feature makes it challenging to determine whether any observed differences in case vs. control DNA methylation levels are a consequence of the disease (or treatment), as opposed to a causative factor for breast cancer development. These studies have evaluated both gene specific and global methylation (reviewed in [122, 123]).

One early case-control study evaluated global methylation levels in WBC DNA using both the 5mdC and LINE-1 methodologies, initially in a subset of 19 breast cancer cases and 18 controls [124]. Blood from all cases in the study were collected prior to surgery or any chemotherapy. Levels of 5mdC were significantly lower in cases than controls, but there was no difference observed in LINE-1 methylation, nor did level of 5mdC correlate with LINE-1 methylation within this subset. The 5mdC assay was subsequently used to ascertain global methylation levels in a total of 179 cases and 173 controls, and lower methylation was significantly associated with breast cancer (odds ratio (OR) and 95 % CI comparing lowest tertile of methylation to highest tertile of methylation = 2.86 (1.65–4.94)). Xu and colleagues made use of data from the Long Island Breast Cancer Study Project, a population-based case-control study, utilizing both the LUMA assay and analysis of LINE-1 methylation to assess global DNA methylation in WBC DNA isolated from 1055 cases and 1101 controls [125]. Blood was collected from cases following diagnosis and it was possible to stratify the case population into those from whom blood samples were pre- vs. post-chemotherapy and also pre- vs. post-radiation therapy. No relationship with breast cancer was found for LINE-1, but for LUMA, higher levels of global methylation were associated with increased risk (OR 95 %CI) comparing quintile 5 of methylation to quintile 1 of methylation (OR = 2.41, 95 % CI 1.83–3.16). This is likely due to the fact that LUMA, which evaluates CCGG sites, is primarily measuring methylation in promoter regions. This over two-fold increase in breast cancer risk persisted when comparing prechemotherapy cases to controls and preradiation cases to controls, suggesting that the association was likely not a treatment effect. In our case-control studies, global methylation levels in Sat2 were correlated between 40 breast tumor tissues and matched WBC DNA isolated from blood samples collected from cases at the time of surgery and prior to chemotherapy [126]. Although intriguing, the literature is far from consistent and we did not observe an association with LUMA and breast cancer risk in a family registry of women at high-risk of breast cancer [127], and another case-control study found lower levels of methylation by the LUMA assay to be associated with breast cancer risk [128]. In addition to LUMA, other markers of global methylation have been examined, and we compared methylation of repetitive elements (Sat2, LINE-1, and Alu) in WBC and granulocyte DNA isolated from blood samples donated by sisters discordant for breast cancer development (282 cases and 347 sister controls; cases donated blood following breast cancer diagnosis) [129]. WBC DNA Sat2 hypomethylation was again associated with breast cancer risk, but no association was observed with

granulocyte DNA Sat2 methylation, suggesting that differential global DNA methylation of some repetitive elements may be associated with blood cell type counts.

In contrast to retrospective case-control studies, the nested-case control design has the same efficiency as in case-control studies but also ensures temporality, an important attribute for biomarker studies. A nested case-control study conducted within the prospective Breakthrough Generations Study and European Prospective Investigation into Cancer and Nutrition (EPIC) examined global DNA methylation in the LINE-1 repetitive element among the WBC DNA from 640 cases and 741 controls, but did not find any differences [130]. The Sister Study is another of the few prospective studies to examine global DNA methylation in WBC and breast cancer risk [131] and is methodologically strong because of its case-cohort design, with blood collected from cases prior to diagnosis. A total of 294 incident breast cancer cases and a sample of 646 non-cases in the study were selected for examination of global DNA methylation of LINE-1. Hypomethylation of WBC DNA LINE-1 was associated with subsequent development of breast cancer comparing quartile 1 of methylation to quartile 4 (HR = 1.75, 95% CI 1.19–2.59).

With respect to gene-specific WBC DNA methylation, one of the earliest epigenotyping case-control studies made use of data on cases and age-matched controls from the ESTHER study [132]. The investigation utilized a multistep method consisting of selection of a broad array of 49 genes of interest based on those known to be methylated in breast cancer; methylation analysis of these genes in a small group ($n = 83$) of healthy, postmenopausal women to narrow down the genes investigated to 25, based on observed methylation patterns as related to *a priori* hypotheses; and examination of methylation of this smaller group of 25 genes among peripheral blood cell DNA isolated from individuals in the larger ($n = 353$ cases and 730 controls) case-control study. Adjusting for age and family history of breast cancer, the authors found differences in methylation of five out of 25 genes between cases and controls, with cases in each of these situations exhibiting lower levels of methylation. The genes with observed methylation differences were estrogen receptor-2 (ER-2) target genes (*NUP155, ZNF217*) and polycomb group target genes (PCTG) that play a role in stem cell biology (*TITF1, NEUROD1, SFRP1*). Lack of DNA methylation at these gene loci conferred a statistically significant 1.4- to 1.5-fold increased risk of breast cancer. In addition, invasive ductal and invasive lobular breast cancer was characterized by methylation of different sets of genes and methylation of ER-α target genes predicted estrogen receptor-positive breast cancer. Elevated *BRCA1* methylation has been observed in cases compared to controls in several case-control studies [17, 133]. In one study that enrolled 255 cases diagnosed with breast cancer prior to age 40 years and compared them to 169 controls, the prevalence of detectable WBC *BRCA1* methylation tended to increase as the tumors from cases contained more *BRCA1* mutation-associated morphologic features and methylation of *BRCA1* in WBC DNA was associated with a 3.5-fold (95% CI 1.4–10.5) increased risk of breast cancer [17]. However, the largest case-control study carried out to date of *BRCA1* methylation did not observe a significant difference between cases and controls, although there was a trend of *BRCA1* promoter hypermethylation in cases vs. controls [134]. Other studies have evaluated the relationship be-

tween methylation of *ATM* in WBC DNA and breast cancer [130, 135], with the single prospective nested case-control examination reporting an increased risk of breast cancer associated with higher levels of methylation at the *ATMmvp2a* locus comparing quintile 5 of methylation to quintile 1 of methylation (OR = 1.89, 95% CI 1.36–2.64) [130].

The prospective Sister Study also used an efficient approach of a case-cohort design to study WBC methylation and breast cancer risk [136]. Specifically, they applied the Illumina Infinium 27k CpG HumanMethylation BeadChips arrays and identified 250 differentially methylated CpG sites between cases and controls in WBC DNA [136]. The AUC for the receiver operator characteristic curve estimated for five of these methylation markers (66%) was larger than for the Gail model (56%) or nine highly ranked single nucleotide polymorphisms from genome-wide association studies of breast cancer (56%). The mean time from baseline blood draw to diagnosis among the breast cancer cases was only 1.3 years in the Sister Study [136]. Thus, the data cannot tell us whether epigenetic changes can predict risk years into the future or are, instead, a response to underlying disease. In addition, the differences in mean percent methylation for the identified sites, while statistically significant, were very small and almost entirely within two percentage points. Cohort studies with longer follow-up time and serial blood collections are needed to estimate lead times, clarify biology, and apply appropriate methods for evaluating predictive value of DNA methylation for diagnosis [137]. It is also necessary to validate data from Illumina arrays, which can be problematic at very high or low methylation levels. For example, in trying to validate some of the top candidates in the Sister Study in our subjects, we found that several sites with low methylation (~10%) in the original Illumina data showed nondetectable methylation by pyrosequencing. In addition, there are also a number of potential problems with Infinium data such as cross-reactive probes, probes containing single nucleotide polymorphism (SNP) sites and probes giving high intensity data, which further underscores the importance of array data validation by other methodologies such as pyrosequencing [138].

A limitation of WBC DNA analysis is the concern that results may be related to differences in cell populations. This is particularly problematic for bloods collected at the time of diagnosis, since it is known that cancer patients have altered proportions of specific cell types compared to healthy controls [139]. We have demonstrated that global methylation profiles vary by different blood cell types [140]. A method has been reported that allows the use of DNA methylation array data to estimate WBC populations [141, 142]. A second limitation is that DNA methylation is known to be impacted by age, genetics, and environmental as well as lifestyle factors (reviewed in [122, 143]). Smoking, air pollution, heavy metals, micronutrients, and even stress have all been shown to alter DNA methylation in WBC. As mentioned above, differences in DNA methylation between cases and controls are small. The biological significance of these small differences is terms of gene expression and function is not clear. Finally, there have been objections to the use of WBC for DNA methylation analysis, given the lack of information on relationship to target tissue, in this case breast tissue. One paper has evaluated the use of WBC

DNA as a surrogate for evaluating imprinted loci methylation in mammary tissue [144]. Of the six loci studied, after correction for multiple comparisons, for only one was there a correlation between tissue and blood and only for invasive tumor tissues not benign breast disease.

In addition to primary prevention studies of WBC DNA methylation, limited studies examined DNA methylation in breast tissue from mammoplasty patients. For example, one study of DNA methylation enrolled 141 healthy European and African American women (mean age (standard deviation (SD))=35 years (11) and mean body mass index (BMI) (SD=32 kg/m^2 (7)) undergoing non-surgical breast reduction [145]. The focus of the study was to detect the likelihood of promoter methylation of *CDKN2A/p16INK4, BRCA1, ER-α, and RAR-β* across a spectrum of breast cancer risk factors. In age-adjusted models, family history of cancer was associated with a two to seven fold greater odds of *p16INK4, BRCA1* or *ER-α* hypermethylation compared to those without a family history of cancer. In race-stratified analyses of European-American women, hypermethylated *p16INK4* was independently associated with having a family history of any cancer and ever consuming alcohol. In African-American women, a family history of cancer was associated with *BRCA1* hypermethylation. While the large number of mammoplasty samples is a strength in this study, there was limited power, especially in the race-stratified analyses. Some of the cell counts had as few as two events, thus the results need to be interpreted with caution. In addition, though the women represent a high breast cancer risk population given their elevated average BMI, results of this study may not be generalizable. Confirmation in a larger population is required, but, mammoplasty samples are difficult to procure, as underscored by the recruitment timeline for this study [145].

In summary, the evidence base for DNA methylation markers and breast cancer incidence is accumulating but major gaps remain. First, most data come from retrospective studies where disease itself or cancer treatment may affect blood, saliva, and tissue methylation, limiting a determination of temporality and with methylation changes being a consequence rather than a cause. Second, the prospective studies that do exist, although growing, generally lack repeated measures making it difficult to establish whether environmental exposures change the levels of DNA methylation markers and, in turn, whether these changes alter breast cancer risk. Third, the evidence base until recently has focused on selected gene targets and markers of global methylation. As techniques improve to scan for a larger set of genes, using prospective studies with extensive environmental data, the evidence base for using DNA methylation markers as biomarkers will build.

Summary and Next Steps

Although the scientific literature on DNA methylation and breast cancer is extensive, for specific questions about primary, secondary and tertiary prevention, as described above, the literature is in its infancy. In particular, methodological consider-

Table 3 Methodological considerations when conducting DNA methylation study across the breast cancer prevention continuum

	Primary prevention	Secondary prevention	Tertiary prevention
Types of questions to address	Are DNA methylation markers related to breast cancer incidence?	Can DNA methylation markers augment standard screening?	Can DNA methylation markers predict prognosis (disease-free and overall survival)?
Study design considerations	Prospective studies needed to assure temporality	Repeated measures over time, prospective	Prospective studies with enough events to adjust for standard clinical markers
Source of DNA	WBC DNA	DNA methylation markers measured in plasma	DNA methylation markers in breast tissue at diagnosis
	Some markers in saliva have very different levels than blood		DNA methylation markers in plasma repeated over time for recurrence
	Breast tissue will mean sample may be less generalizable		

ations for the type of prevention study need to be carefully considered. Table 3 summarizes some of the key methodological considerations that we have observed to be lacking from many of the existing studies. A significant consideration is whether or not the study design is prospective or retrospective, with the former needed to determine temporality. Another important consideration is whether the study design is large enough to have sufficient number of events for multivariable modeling to allow for assessment of the independence of the DNA marker over and beyond standard clinical markers.

Common to all types of studies is a consideration of the source of DNA. Using data from multiple DNA sources from the same individuals, we have observed variation in genomic DNA methylation within specific WBC types [140]. Given the variation in function and gene expression levels of specific WBC types, it is not surprising the both gene-specific and global methylation levels vary by type. This complicates investigation of the association of DNA methylation with disease. It is well known that there are differences in total WBC counts in healthy individuals, with a range of 5000–10,000/μl, but there are also differences in cell populations. Cancer patients also demonstrate alterations in specific cell types. For example, neutrophil and lymphocyte counts were elevated and reduced, respectively, in advanced stage uterine cervical cancer [139]. It is also clear that in metastatic cancer, there is a dynamic range of circulating tumor DNA [146]. Martin [147] suggested that definitive analyses require specific methodologies to account for shifts in cellular population heterogeneity. Moreover, a significant proportion of DNA variation might be due to the disparity in protocols for sample processing [148], and in time of storage of blood samples [149]. In addition, as future studies will need to address repeated measures of DNA methylation markers, issues surrounding the temporal stability of DNA methylation need to be understood. For example, comparing DNA

methylation levels in blood collected at two visits, we have observed that changes in DNA methylation over time are highly associated with baseline values of the assay and vary by assay type [76]. These findings suggest that assays that change more over time may warrant consideration for studies that use DNA methylation as biomarkers. An additional challenge is that different assays measure varying aspects of DNA methylation. For example, MSP measures the relative amounts of fully methylated regions, while pyrosequencing measures average methylation levels at several CpG sites in a pool of DNA. It is important to verify and validate differences in DNA methylation using various assays.

These methodological considerations can be overcome by more research utilizing large, prospective studies that have careful biospecimen collection. The great potential of DNA methylation markers across the prevention continuum and in improving risk assessment may soon be realized. Absolute risk prediction models provide useful information for health care providers and patients and aid in the design and recruitment phase of studies of preventive interventions [150–152]. Clinical prediction modes such as Gail [153], and Breast and Ovarian Analysis of Disease Incidence and Carrier Estimation Algorithm (BOADICEA) [154] have been developed to estimate absolute age-specific breast cancer risk. The most frequently used risk prediction tool in United States clinics is the Gail model, which takes only first-degree family history into account and focuses on nongenetic risk factors [153]. Although the Gail model has been found to be well calibrated for women at average risk, its discriminatory ability is moderate and limits its clinical applicability, particularly for screening [155]. Moreover, the Gail model has not been recommended for high-risk women such as those with a strong family history of breast cancer [4,6]. Using information from the New York site of breast cancer family registry (BCFR), we previously reported that models developed using extended family and genetic data, such as the IBIS model, showed better discrimination (AUC = 69.5 %) than did the Gail model [6]. As mentioned above, Xu et al. [136], using prospectively collected blood samples, identified five methylation markers (AUC = 66 %). Extending such models to include additional genetic or epigenetic information may improve performance in women across the breast cancer risk continuum.

In summary, DNA methylation markers are compelling candidate biomarkers because of numerous characteristics: (1) in the process of carcinogenesis, promoter hypermethylation is a more frequent event than mutations [114], with estimates varying from 600 to 1000 aberrantly methylated genes per tumor [115], (2) methylation has been shown to be an early event in breast tumorigenesis [116–118], (3) not only the malignant cells but also the surrounding tissue shows methylation defects [15–18,119], (4) DNA methylation is stable and can be detected by PCR methods, which means that aberrations can be relatively easily analyzed within very little material [120] as opposed to other approaches such as gene expression profiling, and (5) a hypermethylated sequence forms a positive signal against an unmethylated background, which makes it more easily detectable than genetic alterations such as loss of heterozygosity [121]. It will be essential as we move forward to fully evaluate and quantify the potential gains that can be made in primary, secondary, and tertiary prevention efforts by measuring through large, prospective studies the impact DNA methylation markers have in predicting incidence and OS after diagnosis.

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Incorporating Biomarkers in Studies of Chemoprevention

Carol J. Fabian and Bruce F. Kimler

Abstract Despite Food and Drug Administration approval of tamoxifen and raloxifene for breast cancer risk reduction and endorsement by multiple agencies, uptake of these drugs for primary prevention in the United States is only 4% for risk eligible women likely to benefit from their use. Side effects coupled with incomplete efficacy and lack of a survival advantage are the likely reasons. This disappointing uptake, after the considerable effort and expense of large Phase III cancer incidence trials required for approval, suggests that a new paradigm is required. Current prevention research is focused on (1) refining risk prediction, (2) exploring behavioral and natural product interventions, and (3) utilizing novel translational trial designs for efficacy.

Risk biomarkers will play a central role in refining risk estimates from traditional models and selecting cohorts for prevention trials. Modifiable risk markers called surrogate endpoint or response biomarkers will continue to be used in Phase I and II prevention trials to determine optimal dose or exposure and likely effectiveness from an intervention. The majority of Phase II trials will continue to assess benign breast tissue for response and mechanism of action biomarkers. Co-trials are those in which human and animal cohorts receive the same effective dose and the same tissue biomarkers are assessed for modulation due to the intervention, but then additional animals are allowed to progress to cancer development. These collaborations linking biomarker modulation and cancer prevention may obviate the need for cancer incidence trials for non-prescription interventions.

Keywords Risk biomarker · Surrogate endpoint biomarker · Proliferation · Mammographic density · Clinical trials

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Need for New Approaches to Breast Cancer Prevention

Tamoxifen and Raloxifene Effective for Primary Prevention

Positive results of the landmark National Surgical Adjuvant Breast and Bowel Project (NSABP) P-1 trial comparing 5 years of tamoxifen to placebo in high-risk pre- and postmenopausal women over the age of 35 was announced in 1998 with Food and Drug Administration (FDA) approval for risk eligible women the same year [1]. Longer term follow-up of the NSABP P-1 trial, and similar trials conducted in Europe, continue to underscore the benefit of prevention with tamoxifen not only while taking the drug but for more than 5 years after completion [2–7]. The magnitude of effect according to a recent meta-analysis is substantial with a 44% reduction in risk of invasive cancer and 38% reduction in risk for carcinoma *in situ* [8]. Raloxifene, another selective estrogen receptor modulator (SERM) received FDA approval for breast cancer risk reduction in 2007 following demonstration of reduced breast cancer risk in postmenopausal women [9] and a head to head comparison of raloxifene and tamoxifen showing fewer side effects for raloxifene although a slightly greater efficacy for tamoxifen [10].

Poor Uptake of Tamoxifen and Raloxifene for Primary Prevention

Despite effectiveness, uptake of tamoxifen and raloxifene in the United States for primary prevention by risk eligible women is reported as only 4% [11]. In 2010, it was estimated that ~21,000 women were taking tamoxifen and ~97,000 were taking raloxifene for prevention of breast cancer [12], a small fraction of the 2.5 million women whose risk is high enough to derive benefit [13]. There has been essentially no change in uptake of a SERM for primary breast cancer prevention since 2005 despite endorsement for use in risk appropriate women by the American Society of Clinical Oncology (ASCO), National Comprehensive Cancer Network (NCCN) National Clinical Practice Guidelines, and the US Preventive Task Force [14–18]. The primary reasons for poor uptake appears to be fear of side effects combined with incomplete efficacy and lack of a survival advantage [7] as education about risks and benefits from chemoprevention with these drugs is negatively associated with uptake [19]. Once tamoxifen is started, almost half discontinue prior to the prescribed period of 5 years with an average drop rate of 10% per year [20].

Tissue Biomarkers Identify Women Likely to Receive the Greatest Benefit from Endocrine Therapies and May Enhance Uptake

Uptake of prevention interventions is positively correlated with perceived risk [11, 20] in that biomarkers such as atypical hyperplasia which are associated with both

increased risk and benefit from an intervention are likely to enhance uptake of chemoprevention. Women with a prior diagnosis of atypical hyperplasia or lobular carcinoma *in situ* (LCIS) frequently have an absolute risk of 1 % per year or higher, five times that of the average risk of a 55 year old. The higher relative risk reduction with tamoxifen in women with precancerous lesions (75–86 % with atypical hyperplasia) is probably due to the higher proportion of estrogen receptor-positive cells and proliferative rate than is seen in normal tissue [1, 2, 21–25]. Thus, it is not surprising that in a recently reported Boston series the uptake of chemoprevention after a diagnosis of atypical hyperplasia was ~20 % [26]. Although the majority of high-risk women have never had a biopsy, benign breast tissue can be sampled inexpensively in a minimally invasive fashion by random periareolar fine needle aspiration (RPFNA). With this non-lesion directed technique, hyperplasia with atypia appears to provide similar short term risk stratification [27], and is associated with increased uptake of prevention therapies [28].

Prevention Trials with Later Generation SERMs and Aromatase Inhibitors

Biomarkers can also be used to objectively assess toxicity. Attempts to decrease uterine side effects and improve both bone mineral density and risk of breast cancer gave rise to trials with the later generation SERMs arzoxifene and lasofoxifene in postmenopausal women [7, 29, 30]. Both showed significant reduction in breast cancer risk, bone mineral density preservation, and lack of uterine agonist effects [7, 29, 30]. Despite the lack of uterine side effects, neither arzoxifene nor lasofoxifene are likely to be pursued for an FDA indication for breast cancer risk reduction because, like tamoxifen and raloxifene, both increase the risk of thromboembolism.

Women randomized to receive aromatase inhibitors in adjuvant therapy trials had fewer recurrences than those randomized to receive tamoxifen [31]. Although there are no direct comparative prevention trials, aromatase inhibitors appear to be associated with a higher degree of relative risk reduction (50–65 %) than tamoxifen or raloxifene, without the excess thromboembolic risk [8, 32, 33]. However, the increased incidence of arthralgias, bone mineral loss, fatigue, and estrogen deprivation symptoms [8, 33, 34] make poor uptake and adherence as likely for aromatase inhibitors in the primary prevention setting as tamoxifen and raloxifene [35].

Roles That Biomarkers Will Play in Development of New Prevention Therapies

Biomarkers will play a major role in improving accuracy of risk prediction and development of new more acceptable risk reduction approaches with fewer side effects. In this chapter we will discuss biomarkers used in breast cancer prevention research and how they are incorporated into chemoprevention trials.

Risk Biomarkers

The most important biomarker for prevention trials is the risk biomarker as if it is modifiable it also may serve as an indicator of response as a surrogate for cancer incidence [36].

Ideal Characteristics of Risk Biomarkers

An ideal risk biomarker has all of the following properties:

- biologically plausible
- associated with cancer in prospective cohort studies
- high discriminatory accuracy (can separate individuals into high vs low probability of developing cancer)
- is present in a reasonable proportion of a high-risk population
- obtainable by minimally invasive techniques
- minimally influenced by normal physiologic processes
- assessment method is readily reproduced

Although many biomarkers have been associated with risk for breast cancer, none truly meet all the above ideal criteria. Table 1 indicates various classes of risk biomarkers (discussed in detail below) and in general how well they meet risk criteria.

Germline Mutations

High penetrance germline mutations such as *BRCA1, BRCA2, P53, PTEN*, and *PALB2* satisfy all the above criteria for an ideal risk biomarker except that they are present in a small minority (~5%) of women with breast cancer and in less than 1% of the general population [37–39]. Despite their rarity, high penetrance mutations dramatically influence type of screening, age at screening initiation, and prevention modalities and clinical trial interventions selected. Currently women with deleterious mutations in *BRCA2, P53, PTEN*, and *PALB2* have a 40% or higher lifetime chance of breast cancer and often a 10–20 fold relative risk of breast cancer in their 30s and 40s compared to average risk women. Approximately 70% of cancers in women with *BRCA1* mutations do not express estrogen receptor (ER) which raises concerns about the use of a SERM or an aromatase inhibitor for primary prevention. A recent large study in *BRCA1* or *BRCA2* mutation carriers suggests that tamoxifen reduces risk of contralateral breast cancer whether the woman has a mutation in *BRCA1* or *BRCA2* (cancers developing in *BRCA2* mutation carriers unlike those in *BRCA1* mutation carriers are most often hormone receptor-positive) [40]. Most women with *BRCA1* mutations are likely to be most interested in prevention in-

Table 1 Risk biomarkers commonly used for cohort selection or as response indicators in prevention trials

Risk factors	Associated relative risk	Reference
High penetrance breast cancer susceptibility genes (*BRCA1*/*BRCA2*)	10–20 (age dependent)	[39]
Mammographic breast density		
<5 vs 75%	4–5	[82]
<5 vs 50%	3	
Breast histology		
Hyperplasia	1.4–2	[55]
Atypical hyperplasia	4–5	[21]
In situ cancer	10–20	
Total estradiol		
Postmenopausal <5 vs 9 pg/ml	2	[89]
Free estradiol		
Postmenopausal <0.064 vs >1.48 pg/ml	2	[89]
Follicular total estradiol		
Premenopausal <29 vs > 66 pg/ml	2	[93]
Follicular free estradiol		
Premenopausal <0.4 vs >0.56 pg/ml	2	[93]
Total testosterone		
Postmenopausal <15 vs >26 ng/dl	1.6	[89]
Premenopausal < 20 vs > 32 ng/dl	2	[93]
Free testosterone		
Postmenopausal <0.016 vs >0.32 ng/ml	1.8	[89]
Premenopausal <0.15 vs >0.21 ng/ml	2	[93]
Body mass index		
Premenopausal >age 35		
< 25 vs 25–30 kg/m^2	1.6	[100]
< 25 vs >30 kg/m^2	1.7	[100]
Postmenopausal 28–30 kg/m^2	1.2–1.5	[101]

terventions which target prevention of both ER-negative and ER-positive cancers. Since screening starts at age 25, young women with *BRCA1*/*2* mutations are good candidates for natural product or behavioral intervention trials that will not interfere with fertility.

Women age 35 and older are likely to have undergone prophylactic salpingo-oophorectomy, but given the information that the combination of salpingo-oophorectomy and yearly magnetic resonance imaging (MRI) produces similar survival as salpingo-oophorectomy and bilateral mastectomy [41], carriers often opt to forgo or delay a commonly recommended option, prophylactic mastectomy, for these women [21], until or unless they actually develop breast cancer. *BRCA1*/*2* mutation carriers who wish to preserve their breasts after salpingo-oophorectomy are also ideal candidates for prevention trials.

Single Nucleotide Polymorphisms

Single nucleotide polymorphisms (SNPs) are more common than the high penetrance breast cancer susceptibility gene mutations described above; risk increase is modest when used independently [42]. SNPs are being explored to help stratify risk based on common assessment models such as the Gail model which has a concordance little better than chance [43, 44]. A case control study from the Women's Health Initiative suggests that addition of a SNP panel to the Gail model increases the c-statistic from 0.55 to 0.59 but that SNP associated risk was nearly independent of Gail model risk [45]. Other studies combining SNP panels with breast density or SNP panels with body mass index (BMI) +/− breast density suggest the concordance statistic can be raised from 0.55 with the Gail model to over 0.60 with SNPs plus mammographic density [46, 47].

Whether SNP panels will come into common clinical use as adjuncts to risk assessment with commonly used models such as Gail and Tyrer–Cuzick [48, 49] and/or give women more confidence in their model risk estimates remains to be seen. However, select SNPs are likely to be utilized as part of prevention trials to select or exclude individuals from participation in a study of a particular intervention or explain aberrant results. Although it no longer seems that *CYP2D6* alleles are of major critical importance in tamoxifen response, a recent report suggests that variant alleles in *ZNF423* and *CTSO* genes involved with *BRCA1* regulation can predict up to five fold difference in response to SERMs [50]. Polymorphic alleles of other genes may impact new agents being evaluated in ongoing prevention trials such as *CYP17* polymorphisms important in lignan metabolism and arachidonate 5-lipoxygenase (*ALOX5*) gene variants important in metabolism of the marine omega-3 fatty acids, eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) [51–53].

Breast Hyperplasia and Atypical Hyperplasia

After high penetrance mutations for hereditary breast cancer, chest radiation before age 30, and carcinoma *in situ*, atypical hyperplasia found in 3.5–10% of diagnostic biopsies is associated with the highest term risk of any of the biomarkers (Table 1). Recent long-term follow-up data from the Mayo Clinic suggest that the absolute risk for either invasive cancer or ductal carcinoma *in situ* (DCIS) after a biopsy showing atypical ductal hyperplasia or atypical lobular hyperplasia is close to 1% per year and was predicted to be 30% at 25 years from diagnosis [54]. The Vanderbilt series [55] originally suggested that women with a biopsy showing atypical hyperplasia and a family history of breast cancer had twice the relative risk as those with atypical hyperplasia alone. However, if other unfavorable histologic variables such as multiple atypical foci and limited lobular involution are considered, then family history was not significant [54]. This is not surprising as women with a strong family history of breast cancer may be more likely to have multifocal precancerous changes and thus multiple atypical foci.

Women with atypical hyperplasia are often referred to specialty clinics for risk counseling and risk reduction management. For women with atypical hyperplasia, the Gail model generally underestimates risk and the Tyrer–Cuzick (IBIS) model generally overestimates risk with a concordance statistic for both close to 0.5 [56, 57]. A new model, the BBD-BC based on findings from the Mayo Benign Breast Disease cohort which requires knowledge of number of lobules with atypical foci and extent of lobular involution [58], has a concordance statistic of 0.62 in women with atypical hyperplasia [59]. The BBD-BC model is not likely to be widely used unless the number of atypical foci and the extent of lobular involution becomes a matter of routine reporting by pathologists.

Surgical re-excision after a core biopsy showing atypical ductal hyperplasia is considered standard of care because of a 10–30% possibility of upgrading, usually to DCIS [21]. Although controversy remains, there is again movement away from automatic surgical re-excision of atypical lobular hyperplasia if pathology and radiology findings are concordant, as upgrading in this circumstance is only 0–6% [21]. Despite a lifetime risk of 25% or more, neither yearly surveillance breast MRI nor prophylactic mastectomy is recommended by NCCN guidelines and is not likely to be covered by insurance. The observation in the Mayo Clinic Benign Breast Disease cohort that women with atypical hyperplasia and three or more identified atypical foci have a breast cancer incidence approaching 50% at 25 years [21] may result in greater third party coverage for surveillance MRI and prophylactic mastectomy for women with high-risk atypical hyperplasia.

At present, anti-hormonal therapy with a SERM or aromatase inhibitor is considered the standard prevention option after local excision for women with a diagnosis of atypical hyperplasia. Those who do not wish to do so (currently the majority) are an ideal cohort for prevention trials.

Because women with atypical hyperplasia, particularly atypical ductal hyperplasia, are likely to have had the index lesion excised, use of histologic evidence of atypical hyperplasia as a response biomarker in Phase II chemoprevention trials is not practical as the lesion cannot be resampled. However, presence of cytologic atypia in RPFNA in high-risk women has been associated with a five-fold increase of DCIS and invasive breast cancer at a median follow-up time of 4 years similar to its histologic counterpart [27]. Evidence of hyperplasia with atypia in RPFNA may then be used as a risk biomarker for cohort selection for chemoprevention trials and as a response biomarker as well.

Proliferation (Ki-67), ER, Methylation, and Other Molecular Markers in Benign Breast Tissue

The proportion of breast epithelial cells in terminal lobular duct units expressing ER and Ki-67 varies with age, menstrual cycle phase, menopausal status, and type of benign breast disease (Fig. 1). In normal premenopausal breast epithelium, proliferation (Ki-67) correlates with systemic concentrations of progesterone, not estradiol,

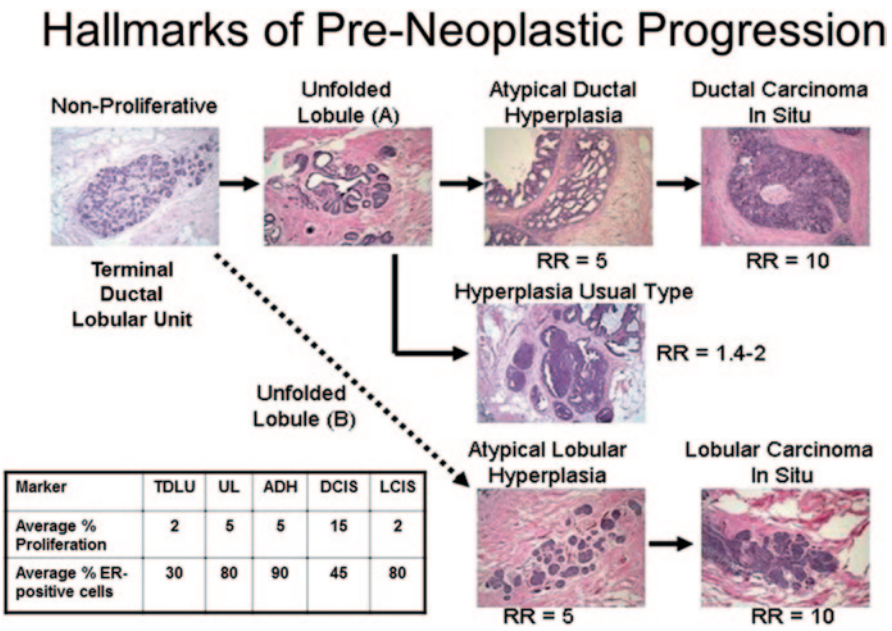

Fig. 1 The association between histologic abnormality and relative risk (*RR*) for development of invasive breast cancer. (Modified from Fig. 1 in Fabian et al. [66], with added information from Allred et al. [22]. *ADH* atypical ductal hyperplasia, *DCIS* ductal carcinoma *in situ, LCIS* lobular carcinoma *in situ, TDLU* terminal ductal lobular units, *UL* unfolded lobule)

and is lowest in the follicular phase (~1%) and highest in the luteal phase (2–3%) [60, 61]. Ki-67 labeling averages 0–1% in normal postmenopausal epithelium [61]. A Ki-67 labeling index of 2% or higher in usual ductal hyperplasia or atypical hyperplasia is associated with increased risk of breast cancer compared with women with lower levels of proliferation in hyperplastic foci [62, 63].

ER-alpha expression in normal breast epithelial cells displays an opposite pattern from Ki-67 and is lowest in the luteal phase (0–5%) and highest in the follicular phase (10%) of premenopausal women. With declining systemic hormone levels, ER-alpha expression increases to ~30% of cells in the terminal lobule duct unit of postmenopausal women [22, 61, 64, 65]. ER-alpha also shows progressive increases to 45% or more of epithelial cells in usual duct hyperplasia and 90% or higher for atypical ductal hyperplasia [22, 54, 61] (Fig. 1). The proportion of cells positive for ER-beta (over 90% in normal lobules) relative to ER-alpha declines in hyperplasia (~75%) and invasive breast cancer (60%) [23].

Few cells in the normal terminal lobule duct unit co-express ER-alpha and Ki-67. In premenopausal women, this is 0.01% of normal epithelial cells with higher levels in postmenopausal women despite a lower overall labeling with Ki-67 [61]. The proportion of dual labeled cells increases in hyperplasia and in atypical hyperplasia the negative association between ER-alpha and Ki-67 is lost. High cyclooxygenase-2 (COX-2) immunocytochemical expression in women with atypical

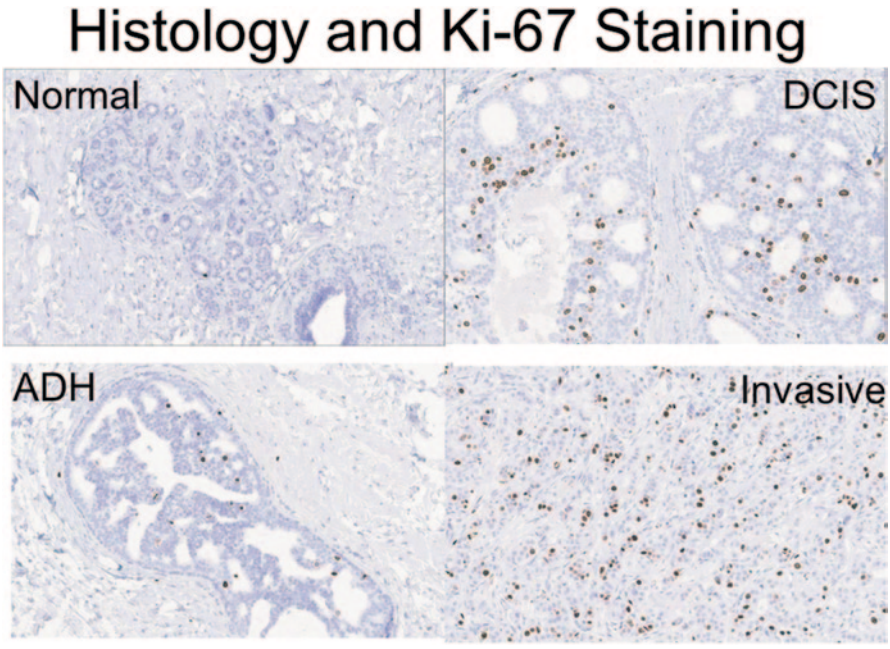

Fig. 2 Assessment of proliferation by MIB-1 immunohistochemical staining in normal breast tissue to invasive breast cancer. (Images provided by Ossama W. Tawfik, M.D., Ph.D., Department of Pathology, University of Kansas Medical Center. *ADH* atypical ductal hyperplasia, *DCIS* ductal carcinoma *in situ*)

hyperplasia, has also been associated with risk of subsequent risk of cancer in the Mayo Clinic Benign Breast Disease cohort [67].

Ki-67 in hyperplastic or atypical foci and/or dually staining Ki-67/ER-alpha-positive cells is likely to be a good risk biomarker in premenopausal women or postmenopausal women taking hormone replacement therapy. Terminal duct lobule involution is negatively associated with higher serum estradiol and testosterone in postmenopausal women [68]. Assays can be readily performed in tissue sections from core needle biopsies or surgical resections (Fig. 2) or on breast epithelial cells acquired by RPFNA (Fig. 3) [69–72].

However, Ki-67 is not a practical risk or response biomarker for most postmenopausal women who often have near complete lobular involution, few epithelial cells, and little detectable Ki-67.

Methylation of the promoter regions of multiple tumor suppressor genes that code for *p16(INK4), BRCA1, ER-alpha, RASSF1A, TWIST1, HIN1*, and *RAR-beta* in benign breast tissue, ductoscopy washings, ductal fluid, and blood has also been associated with breast cancer risk [73–76]. Promotor methylation is probably an early event in carcinogenesis, particularly in the evolution of the Luminal B subtype. Relative risks have not been well-defined and may vary depending on the methylated gene and/or the number of methylated tumor suppressor genes identified [77].

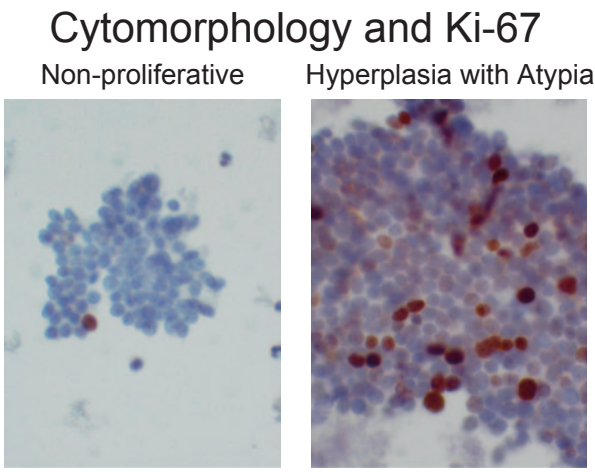

Fig. 3 Assessment of proliferation by Ki-67 immunocytochemical staining in benign breast epithelial cells acquired by random periareolar fine needle aspiration (*RPFNA*)

Benign breast tissue expression of estrogen response genes that code for ER-alpha and ER-beta, pS2, PR, GREB-1 and GREB-2; and genes associated with cell cycle or proliferation regulators such as Cyclin D1, Cyclin B1, or BRCA1 may eventually be identified as important risk modifiers or response indicators in a prevention trial and can be assessed in very small quantities of formalin fixed or frozen tissue by reverse transcription polymerase chain reaction (RT-qPCR) [78, 79].

Alterations in protein and phosphoprotein expression have been identified in multiple pathways important in carcinogenesis. Assessment of a large number of proteins and phosphoproteins can be performed on very small amounts of tissue using reverse phase protein arrays and similar assays. These assessments are highly dependent on type of tissue specimen and pre-analytic processing. Protein arrays are currently not used in risk assessment but are used in clinical trials as mechanism of action biomarkers [80].

Mammographic Breast Density

As opposed to germline genetic testing and breast biopsies, most women 50 years of age and older have a mammogram every 1–2 years as part of screening for breast cancer. Thus, mammographic density expressed as either absolute dense area or percent dense area relative to the total area of the breast can be readily and inexpensively obtained for use as a risk or surrogate response endpoint [81]. Women with >75% breast density have a risk of breast cancer four to five times that of an individual with no density (about 10% in each of the extremes). A meta-analysis suggests that percent dense area versus absolute dense area is the strongest predictor of risk [81–83]. It is clear that not all conditions associated with increased cancer risk are also associated with increased breast density. Obesity is associated with increased breast cancer risk but reduced percent dense area on a mammogram.

Likewise 10-year risk of breast cancer is generally lower in young premenopausal women which often have the highest breast density. In the International Breast Cancer Intervention Study (IBIS)-1 trial of tamoxifen vs placebo, breast density adjusted for age and BMI was a stronger measure of breast cancer risk than unadjusted percent dense area [81]. For studies in which a single measurement of density is meant to classify risk, correction of the density measurement for BMI and age is recommended but is probably not needed in prevention studies using change in mammographic density as an endpoint. Recently, there has been considerable effort devoted to using automated volumetric approaches to assess breast density directly from digital mammography [84] and to correlate these with MRI assessment of fibroglandular tissue [85, 86] for use in risk prediction.

High density has long been recognized as having a strong hereditary component [87]. A recent genome-wide association study (GWAS) involving over 18,000 women with cancer identified a number of genes as correlated both with density and breast cancer of which *AREG* associated with amphiregulin, *ESR1* associated with the ER, a Zinc finger gene, and *IGF1* were some of the most important [88].

Blood Hormones and Growth Factors

Endogenous total and free estradiol and testosterone concentrations in postmenopausal women have been consistently associated with increased risk for breast cancer in prospective cohort, case-cohort, and case control studies [89, 90]. The increase in relative risk between highest and lowest groups is generally in the two-fold range [89] (Table 1). Estradiol and testosterone appear to stratify risk based on the Gail model [91]. No association was noted between serum estradiol, testosterone and sex hormone binding globulin (SHBG) and the risk biomarker mammographic breast density after adjustment for BMI in the Women's Health Initiative [92].

Results from serum hormone studies in premenopausal women are not entirely in agreement probably due to different sampling times in the cycle and different assays. In the large nested case-control study from the Nurses' Health Study II in which blood was obtained in both the mid-follicular and luteal phases, there was a two-fold increase in risk in the highest compared with the lowest quartiles of total and free follicular phase estradiol. No association was observed with luteal phase estradiol concentrations [93] (Table 1). A two-fold higher risk was also noted for highest vs lowest quartile of luteal phase total and free testosterone with no association for SHBG, estrone, or estrone sulfate [93]. A nested case-control study within the EPIC cohort noted no difference in risk between quartiles of serum estradiol obtained from a single blood sample obtained during a non-specified portion of the cycle. An increase in relative risk (1.7) for the highest compared with the lowest quartiles of testosterone was reported as well as a decrease in risk between the highest and lowest quartiles of serum progesterone (0.60) [94]. A nested case-control study in the New York University Women's Health Study also found a 1.8 relative increase in risk with the highest compared to the lowest quartiles of free and total

testosterone [95]. These data would appear to indicate that in both pre- and postmenopausal women, endogenous free or bioavailable testosterone may be used as a risk and a response biomarker. The same is true of free and total estradiol in postmenopausal women and probably follicular phase free and total estradiol in premenopausal women. The finding in the Nurses' Health Study that higher endogenous progesterone in premenopausal women is protective deserves follow-up but higher endogenous progesterone was associated with greater terminal lobular duct involution in a recently published study utilizing normal breast tissue and blood [68].

Blood Lipid Metabolism and Inflammatory Markers

Although breast cancer has traditionally been thought of as a hormonally promoted cancer, it is increasingly acknowledged that inflammation may play a role in the development of more aggressive ER-negative and ER-positive cancers [96]. Breast inflammation may be part of a systemic disorder such as that observed in obesity related insulin resistance, with reduced adiponectin:leptin ratio, elevated pro-inflammatory cytokines and eicosanoids; and increases in aromatase activity and estrogen production in adipose [97–99]. BMI >30 kg/m^2 has long been associated with a modest increase in risk of postmenopausal breast cancer but increasing evidence suggests a high BMI in premenopausal women >35 years of age is also associated with increased risk [100, 101] (Table 1). Evidence is increasingly suggesting that it is the presence or lack of metabolic health (defined by normal serum insulin levels, lack of insulin resistance, and normal levels of pro-inflammatory cytokines) which is truly the operative risk factor—not weight or BMI [102]. Serum concentrations of C-reactive protein (CRP), insulin-like growth factor -1 (IGF-1), and homeostasis model of insulin resistance (HOMA IR) are positively associated and the adipokine adiponectin and adiponectin:leptin ratio negatively associated with increased risk of benign breast disease and cancer [97, 103–107].

However, proliferative breast disease, even in the absence of obesity and systemic elevation of cytokines, is also an inflammatory process, although perhaps a more localized one. A progressive increase in activated macrophages and T cells between normal breast tissue, proliferative breast disease, and breast cancer has been observed which might be due to abnormal antigen presentation or tissue breakdown products [108]. We do know that activated macrophages are an important determinant of cytokine production in the microenvironment [109], and the pro-inflammatory cascade results in mTOR activation which is acknowledged as important in breast carcinogenesis [110]. There is some evidence that genetic variants in interleukin 6 (IL-6), tumor necrosis factor (TNF) alpha, and their receptors may be associated with altered breast cancer risk [111].

More work is needed in determining the relevance of elevated systemic and local levels of a large number of inflammatory markers including IL-6, macrophage chemoattractant protein (MCP-1), plasminogen activator inhibitor 1 (PAI-1), and TNF-alpha.

Use of Biomarkers in Prevention Intervention Trials

Risk Biomarkers to Select the Cohort

A certain level of risk is usually mandated to warrant the risk or inconvenience of clinical trial participation. Often biomarkers are used to supplement estimated risk based on family and personal history models. For example, eligibility criteria for a trial might specify any of the following as risk criteria for entry: a relative risk of 2X that of the average risk woman in her age group based on the Gail or Tyrer–Cuzick model, mammographic breast density > 50 %, RPFNA evidence of atypia, or mutation in a moderate to high penetrance hereditary breast cancer gene.

Matching the Participant and Intervention

Biomarkers may also be used to enhance the probability of clinical trial success, i.e., precision medicine for prevention. Examples might be a germline *BRCA1* mutation for a PARP inhibitor trial, high serum insulin or HOMA IR index for a metformin or weight loss trial [112], and a low ratio of EPA + DHA to arachidonic acid in erythrocytes in women considering a marine omega-3 fatty acid supplementation trial.

Pharmacodynamic Measures

A biomarker that may have little to do with breast cancer risk but that can document that an agent is having a physiologic effect can be used for preliminary dose finding as well as compliance. Increases in blood concentrations of enterolactone and enterodiol are examples for lignans, and decrease in triglycerides are examples for omega-3 fatty acids.

Surrogate Endpoint (Response) Biomarkers

Potential response biomarkers should satisfy a number of criteria, as delineated previously.

How some of the commonly used classes of risk/response biomarkers compare as far as satisfying the criteria is described in Table 2.

Optimally the risk biomarker would be validated as a response indicator or surrogate endpoint biomarker by demonstrating that modulation is associated with reduction in breast cancer incidence in a Phase III prevention trial. None of the biomarkers we currently use as response indicators are truly validated but perhaps

Table 2 Utility of commonly used risk and response biomarkers. (Modified from Fabian et al. [66])

	Morphology	Proliferation	Mammographic density	Serum hormones/ growth factors	Serum inflammatory markers	Tissue molecular markers[a]
Biologically plausible	Y	Y	Y	Y	Y	Y
Strong statistical association w/ cancer	Y	Y	Y	+/−	N	+/−
Not affected by normal physiologic processes	Y	N	+/−	N	Y	N
Can be measured in majority of at-risk population	Y	Y	Y	Y	Y	Y
Easily sampled	N	N	Y	Y	Y	N
Quantified	+/−	Y	+/−	Y	Y	?
Modulated by known prevention drugs	Y	Y	Y	Y	+/−	?

[a] Includes genomics (mRNA and mRNA methylation by PCR), proteomics, and metabolomics

proliferative breast disease with and without atypia, Ki-67 in proliferative lesions, and mammographic density come the closest. Tamoxifen was associated with reduction in benign breast biopsies and a finding of atypical hyperplasia in NSABP P-1; but it is unclear if tamoxifen eradicates atypical hyperplasia and if so how long this would take [113]. Reduction in breast density appeared to be associated with reduction in risk of breast cancer with tamoxifen in IBIS-1 with little evidence of risk reduction in those with no change in density [114]; but density is not modulated with all anti-estrogenic therapy. Low Ki-67 after short term tamoxifen treatment or reduction in Ki-67 from baseline has been shown to be associated with reduced risk of recurrence in early cancer treatment trials [115] but we have little information as to whether decrease in Ki-67 in benign breast tissue with prevention treatment leads to decreased cancer incidence.

Proliferative breast disease and atypical hyperplasia are in the direct pathway of breast carcinogenesis, theoretically modifiable, minimally affected by normal physiologic processes such as phase of menstrual cycle, and would thus be the preferred surrogate response indicator. On the negative side it may not be attractive to participants to access tissue and this is likely to be a major study expense plus it is difficult to quantify morphologic change and the time course for reversing abnormalities is unclear.

Given these considerations and the 6–12 month treatment interval for most Phase II trials, change in Ki-67 in an area of hyperplasia or atypical hyperplasia may be a more practical surrogate response indicator than morphology alone and thus is one of the most common primary endpoints used for Phase II prevention trials. Since the majority of postmenopausal women not on hormone replacement, as well as many parous premenopausal women, have partial or complete lobular involution, if Ki-67 is chosen as the primary endpoint it is imperative to demonstrate measureable Ki-67 prior to participant entry onto a study. Due to low epithelial cell number and proliferation rates in postmenopausal women not on hormone replacement, Ki-67 is not a practical primary endpoint for this group of women.

Choice of tissue sampling method is largely principal investigator dependent. Ductal lavage is no longer used for studies requiring cells as ductal fluid is often poorly cellular [66, 116]. RPFNA is used as the sampling method for Phase II trials by multiple investigators in the United States and works well in multi-site trials [117]. Multiple random biopsies or biopsies directed towards sonographically dense areas are generally the most expensive sampling procedures and potentially the most complicated to arrange, particularly if the trial is in premenopausal women and sampling must be in a particular phase of the menstrual cycle. We use RPFNA and aspirate in the early follicular phase (day 1 to 10) as Ki-67 is thought to be most stable during this phase.

Tissue sampling also provides the opportunity to preserve fresh frozen tissue for gene expression or proteomics analysis for exploratory studies if the underlying mechanism is not known or for hypothesis driven marker assessment [71, 79, 118–120].

Mammographic density is quantifiable with computer assisted programs, is not invasive, and may be minimally expensive for the trial as yearly mammography is part of standard of care for most high-risk women 40 and older. Unfortunately, change in breast density is not an appropriate surrogate endpoint for all interventions [121]. Although it is likely to be good surrogate for most SERMs [114], it is not modulated by aromatase inhibitors known to reduce breast cancer risk [122]. Weight reduction may actually increase percent dense area [119, 123]. Breast density, even with computer assisted calculations, is subjective and pre and post intervention assessments should be performed by the same individual at the same setting with the rater blinded as to which image was baseline [124]. In addition there are a number of technical factors that can introduce error when change is being evaluated including variation in type of imaging, positioning and degree of compression. Mammographic density is validated for trials involving tamoxifen [114]. However, despite the findings from IBIS-1, in which women with $<10\%$ reduction in breast density appeared not to have a reduced risk of breast cancer [114], it is premature to stop tamoxifen or other SERMs in women who do not have a measurable change in density after a year or more of treatment.

Selection of a biomarker as a surrogate response endpoint is often dependent on many variables including the intervention to be tested, how much is known about the molecular mechanisms responsible for effects, cohort characteristics, anticipated duration of intervention, and budget. A variety of biomarkers are available

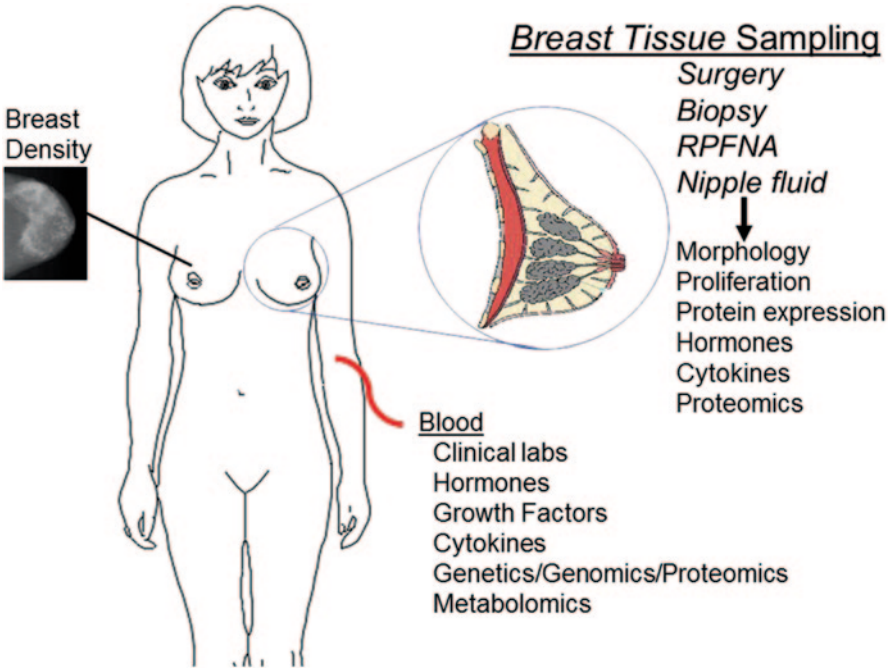

Fig. 4 Sources for biomarker assessments used in prevention trials, including several approaches for the sampling of breast tissue

from several sources (Fig. 4); however, particularly in Phase II trials, use of only serum markers as a primary endpoint is discouraged and in general for Phase II trials at least one biomarker selected should be directly reflective of breast tissue or physiology.

Trial Models for Clinical Prevention Trials

Dose Finding

When the effective dose of an intervention is not known, a dose finding or Phase I trial is performed with the aim of establishing the lowest effective dose at which the desired biologic effect is likely to be reliably obtained. Phase IA trials usually assess multiple markers and multiple dose levels whereas Phase IB is placebo or no treatment controlled and confirms modulation of both a pharmacodynamic and a risk biomarker at a given dose level [66]. One of the most popular dose finding models is the so called pre-surgical or window of opportunity trial (Fig. 5) in which women with DCIS or a small invasive cancer are randomized between one of several doses in the interval (usually 4 weeks) between biopsy and the definitive surgical procedure. Change in Ki-67 in the cancer is generally used as the primary endpoint and

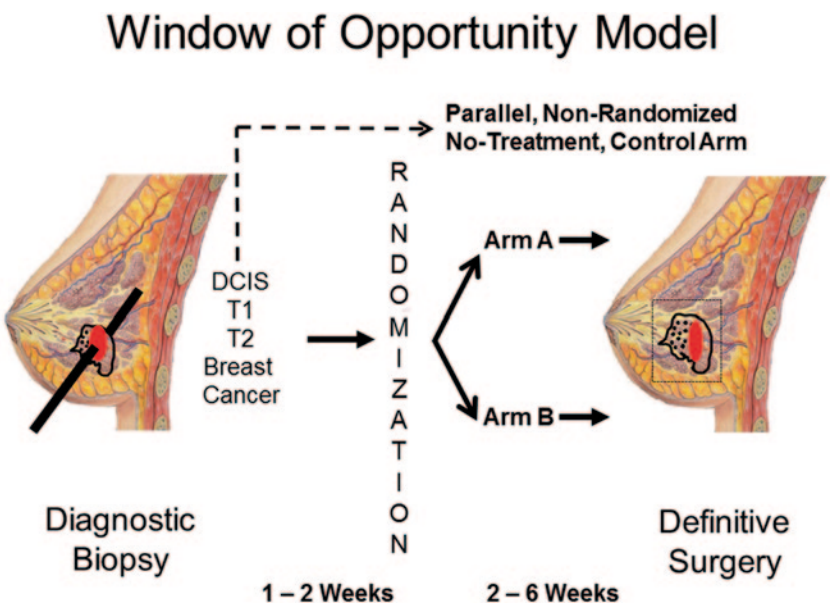

Fig. 5 Schematic of the "window of opportunity" trial model. The diagnostic biopsy and the surgical specimen acquired clinically as standard of care provide the pre- and post-intervention sources for biomarker assessment. While there is no assurance that a specific biomarker will be expressed in both specimens, assays are amenable to batch processing to reduce variability. The treatment time is restricted to the clinically mandated interval between diagnosis and surgery. *DCIS* ductal carcinoma *in situ*

8–10 women are enrolled per dose level. In Phase IIB the dose that is associated with change in Ki-67 is compared against a no treatment control arm or placebo [125, 126]. Generally speaking, due to a wound healing response after core needle biopsy, Ki-67 exhibits an increase in the placebo or no-treatment control group from baseline, usually minimal for ER-positive tumors and much larger (~5 %) for hormone receptor-negative tumors [127]. The statistically significant differences between control and intervention observed in these trials is due not just to Ki-67 reduction in the intervention arm but also the increase in Ki-67 in the control arm.

Phase IIA pilots are generally performed when feasibility of the intervention needs to be demonstrated, it is uncertain which risk biomarkers may be modulated, and/or an effect size for the primary endpoint biomarker needs to be estimated for the purpose of determining sample size for a Phase IIB trial. Phase IIA pilots and full Phase IIB randomized trials typically involve healthy volunteers who are at high-risk for development of breast cancer, rather than patients who have already been diagnosed with *in situ* or invasive neoplasia. Phase II pilots may be single arm or placebo-controlled but rarely exceed 30–40 biomarker evaluable participants. The most frequent primary endpoint is change in Ki-67 in hyperplastic benign breast tissue acquired by RPFNA (Fig. 6) with a trial length of 6–12 months. If a change in Ki-67 or another strong risk biomarker is demonstrated, the intervention progresses to a Phase IIB trial.

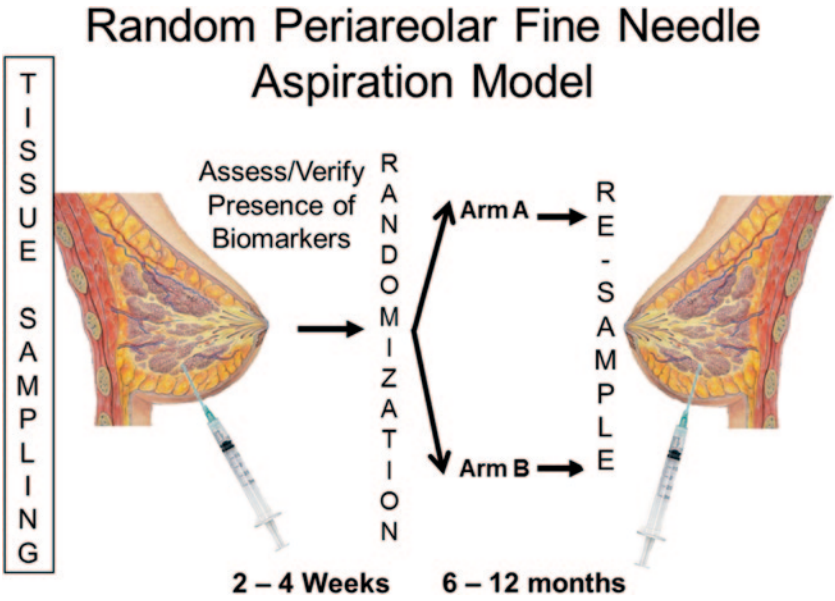

Fig. 6 Schematic of the random periareolar fine needle aspiration (*RPFNA*) trial model. A baseline specimen is screened to confirm biomarker eligibility criteria are met. Eligible participants receive study agent for 6–12 months, followed by repeat RPFNA

A Phase IIB trial is always placebo-controlled and generally involves over 200 biomarker evaluable participants. Benign breast tissue is generally sampled unless there was prior clear evidence of modulation of another strong risk biomarker such as mammographic density in the Phase II pilot. The primary endpoint is change in Ki-67 in women with hyperplasia or hyperplasia with atypia. Selecting a robust and meaningful primary endpoint biomarker for prevention trials in benign breast tissue of postmenopausal women not on hormone replacement is often problematic in that this cohort has a high proportion of women with lobular involution, a low rate of proliferation and low mammographic density. Depending on the intervention, assessing blood risk biomarkers as a primary endpoint with breast tissue sampled for select gene expression, cytokines, and proteins and phosphoproteins in pathways of interest may be the best alternative.

A Phase IIB trial generally explores mechanism of action biomarkers through assessment of gene expression, proteomic, and/or cytokine change in breast tissue; and hormonal, growth factor and cytokine changes in blood. A Phase IIB trial showing modulation of one or more response biomarkers combined with minimal side effects would generally signal the need to move to a Phase III trial of cancer incidence. While this approach is likely needed for FDA approval of a pharmaceutical agent, other clinical benefit endpoints are possible such as reduction in the incidence of biopsies. For behavioral interventions and natural products not requiring a prescription, Phase II-III trials in which biomarker modulation is the primary endpoint may be reasonable (see below).

Future: Integrated Phase II Human and Phase II–III Animal Co-Trials

The cost of Phase III prevention trials combined with lack of uptake of effective interventions for primary prevention has dramatically reduced if not halted the launch of large Phase III prevention trials of pharmaceutical agents in which cancer incidence is the primary endpoint. The current focus is on development and testing of behavioral prevention interventions and natural products and their derivatives available without prescription and the need for FDA approval. Phase II trials with risk biomarkers as an endpoint are performed in conjunction with a similarly designed animal model studies which utilize a similar level of exposure and biomarker assessment. In the animal model studies, however, designated cohorts are allowed to go on to cancer development such that biomarker modulation can be correlated with reduction of cancer incidence (Fig. 7). Several of these co-trials are ongoing.

Summary

A variety of biomarkers have been recognized/developed over the past two decades which can help stratify risk estimates based on traditional variables of personal reproductive and family history. Those associated with the highest relative and absolute risks are useful in identifying women likely to be interested in standard risk reduction interventions or clinical trials. Further biomarkers which are modifiable and readily quantitated are useful as surrogate response indicators as a substitute for cancer incidence in Phase II trials. Currently the most frequently used surrogate response indicator is Ki-67 in DCIS or small invasive cancers in short term window of opportunity trials as well as Ki-67 in hyperplastic benign breast epithelium longer term Phase II trials. Mammographic density is also used as primary or secondary endpoint particularly in Phase II trials of SERMs. Tissue and serum adipokines, cytokines and growth factors, methylation of oncogenes, and serum hormones are additionally used as risk and response indicators and may assume particular importance for clinical trials depending on the intervention and menopausal status of the cohort. Newer types of assessments such as RT-qPCR assessing gene expression in benign breast tissue and protein and phosphoprotein arrays targeting specific pathways are important in defining the underlying mechanism of action. The current emphasis on behavioral interventions and natural products combined with the lack of enthusiasm for large Phase III cancer incidence trials has resulted in development of novel Phase II-III translational trial design models for interventions not requiring FDA approval. Human and animal co-trials are where both human and animal participants receive similar intervention exposure and have the same biomarkers assessed before and after the intervention. Whereas the human trial ends with reassessment of biomarkers in blood and benign breast tissue, cohorts of animals are allowed to continue on to cancer development allowing investigators to associate biomarker modulation with cancer development at least in the animal trial.

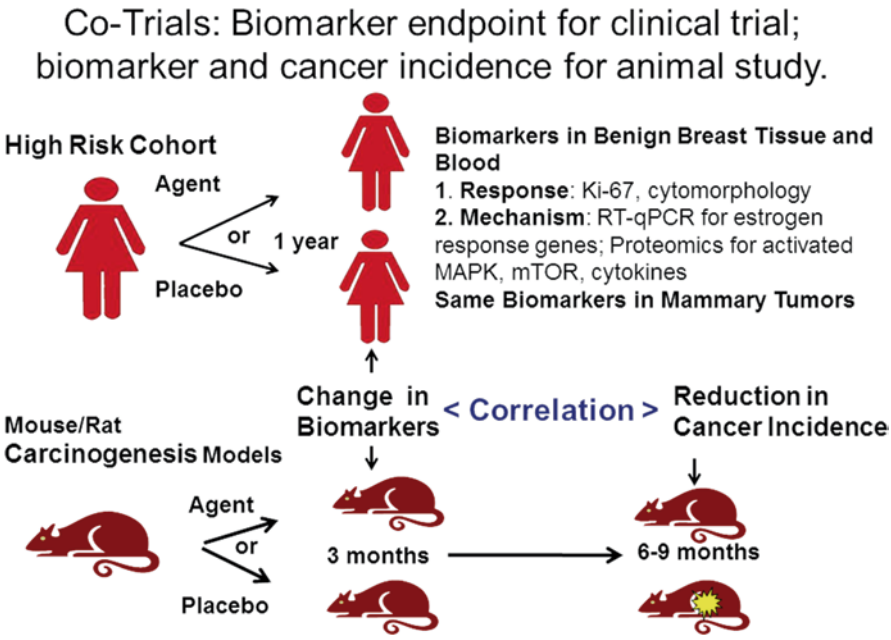

Fig. 7 Schematic of the co-trial model, where a human clinical trial is conducted in parallel with animal studies using rodent carcinogenesis models. Humans and animals receive the same effective dose of study agent (adjusted if necessary for differences in pharmacology) and the same biomarkers are assessed pre-and post-intervention for both. However, additional animals are allowed to progress to actual cancer development so as to allow correlation between biomarker expression and cancer development; and between modulation of biomarkers and reduction of cancer incidence

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Breast Molecular Profiling and Radiotherapy Considerations

Omar Mahmoud and Bruce G. Haffty

Abstract The last decade has seen major changes in the management of breast cancer. Heterogeneity regarding histology, therapeutic response, dissemination patterns, and patient outcome is evident. Molecular profiling provides an accurate tool to predict treatment outcome compared with classical clinicopathologic features. The genomic profiling unveiled the heterogeneity of breast cancer and identified distinct biologic subtypes. These advanced techniques were integrated into the clinical management; predicting systemic therapy benefit and overall survival. Utilizing genotyping to guide locoregional management decisions needs further characterization. In this chapter we will review available data on molecular classification of breast cancer, their association with locoregional outcome, their radiobiological properties and radiotherapy considerations.

Keywords Breast cancer · Molecular signature · Subtyping · Radiotherapy

Introduction

Radiotherapy (RT) plays an integral role in improving locoregional control and survival in patients with breast cancer following lumpectomy or mastectomy [1, 2]. The magnitude of benefit varies based on tumor size, extent of disease, lymph node involvement, and a patient's age [3, 4]. Yet, the clinical outcome differs significantly among patients sharing the same clinicopathologic features.

Ever since the discovery of estrogen receptor (ER) and progesterone receptor (PgR) expression in breast cancer cells [5], treatment strategies changed significantly to adapt these markers in the treatment decision making. High throughput technology interrogating thousands of genes regarding their relative expression identi-

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fied four distinct breast cancer molecular subtypes [6]. The molecular subtypes, Luminal A (LumA), Luminal B (LumB), Basal-like and Human epidermal growth factor receptor (Her-2/*neu*) enriched subtypes (HER2), illustrated the heterogeneity of breast cancer disease spectrum and the limitations of classic clinicopathologic factors in predicting outcomes.

This classification was incorporated into the clinic to direct systemic therapy decisions [7]. In contrast, inclusion of breast cancer subtyping in guiding local therapy needs further characterization [7] . The evidence supporting biologic marker use in RT treatment decisions has not been strong due to many reasons: conflicting findings reported by different study designs, various end points, non-systematic RT use and multiple management paradigms spanning across time [8].

Breast cancer subtyping may refine the treatment decision in controversial situations such as:

1. Identifying patients without tangible RT benefits after wide excision of small early stage disease
2. Deferring treatment in patients at high-risk of radiation induced side effects
3. Recommending RT for patients with one to three positive axillary lymph nodes at high-risk of locoregional failure (LRF)
4. Excluding the patient subgroup not suitable for partial breast irradiation
5. Discovering new agents that target pathways enhancing radiation response and/or reducing radioresistance.

Hints Based on Immunophenotypic Classification

In spite of immunohistochemistry (IHC) limitations [9] in reconstructing molecular subtypes, the use of ER, PgR, HER2, Ki-67 and Cytokeratin 5/6 (CK) as markers has been widely adopted as a surrogate to genotyping. The less prohibitive tissue requirement, cost, complexity and technical availability made IHC a practical approach instead of the logistically demanding molecular profiling [10]. Table 1 dem-

Table 1 Immunohistochemical criteria for constructing molecular subtypes

Subtypes	ER	PgR	HER2	CK5/6	EGFR	Ki-67
LumA	Positive	±Positive	Negative	Any	Any	Low
LumB	Positive	±Positive	Negative	Any	Any	High
	ER-positive	±Positive	Positive	Any	Any	Any
HER2	Negative	Negative	Positive	Any	Any	Any
TN Basal	Negative	Negative	Negative	±Positive	±Positive	Any
TN non-Basal	Negative	Negative	Negative	Negative	Negative	Any

ER estrogen receptor, *PgR* progesterone receptor, *HER 2* human epidermal growth factor receptor type 2, *TN* triple-negative breast cancer, *EGFR* Epidermal growth factor receptor, *CK5/6* Cytokeratin 5/6, *LumA* Luminal A

onstrates the IHC based approximation including two hormone receptor-positive and two hormone receptor-negative subtypes. The hormone receptor-positive group (LumA and LumB) is characterized by prominent hormone receptor gene expression; with LumB displaying a comparatively higher expression of proliferative genes. The opposite expression pattern is seen in receptor-negative Basal and HER2 subtypes. The Basal subtype, exhibiting strong myoepthilial expression pattern, is deficient in hormone receptors and HER2 markers; explaining the triple-negative breast cancer (TNBC) nomenclature. Conversely, HER2 subtype displays high level of HER2 receptor protein or amplifications of the gene (Table 1) [11].

These four subtypes are characterized by distinct biology, evident in their presentation [9], chemotherapy response [12, 13], and failure pattern [14–16]. In general the TNBC subtype is more aggressive and is associated with poor prognosis in comparison to other subtypes, in particular LumA, which carries the best prognosis [17].

Triple Negative Breast Cancer: Clinical Characteristics and Outcome

Distribution and Characteristics

As shown in Table 2, TNBC subtype constitutes 7–24% of all breast tumors [18]. In contrast to other tumor types, TNBC is more often associated with Black race [9], younger age [9, 19, 20], large tumor size [19, 20], high tumor grade [19], and the presence of *BRCA1* mutations [15, 21]. Interestingly, lymphatic involvement is independent of this aggressive presentation and its rate is comparable with other subtypes [22].

TNBC immunophenotypic pattern was used as a proxy to Basal breast cancer, whereas, the later represents a gene expression profile [23] described initially by Sørlie et al. [24]. The Basal TNBC is distinguished from the non-Basal subtype by overexpression of epidermal growth factor receptor (EGFR), Basal CK 5/6/17, and c-Kit [24]. Therefore, sole reliance on the low hormone receptor and HER2 expression results in up to 30% misclassification rate [25, 26]. Apart from Millar et al. [27] and Voduc et al. [14], most investigators grouped Basal and non-Basal subtypes TNBC. Out of the 498 patients with breast cancer, in the Millar study, TNBC was observed in 68 patients that included 52 patients satisfying the Basal subtype five markers requirement [27]. In the Voduc study, the entire patient cohort (2985) included 9.8 and 8.7% Basal-like and non-Basal TNBC, respectively [14]. However, this distinction did not reveal a significant difference in 10-year local or regional free survival. In this review we will use TNBC as a proxy to a Basal subtype.

Table 2 Type of surgeries and the distribution of patients by constructed breast subtype or by molecular signature

Author	Year	N	Stage	N0	Age (%)	BCT (%)	LumA (%)	LumB (%)	LumB HER (%)	HER (%)	TN-Basal (%)	TN non-Basal (%)
Nguyen [55]	2008	793	I–II	62	<55(50)	100	75	9.7		4	11.2	
Kyndi [16]	2008	1000	II–III	0	Pre(50)	0	63	10		12	15	
Millar [27]	2009	482	I–II	70	61[b]	100	79.1	4.6		2.6	10.4	13.6
Freedman [131]	2009	753	I–III	70	57[b]	100	79.6			7.3	13	
Gabos [37]	2010	618	I–II	54	NR	38	44		29	15	12	
Voduc [14]	2010	2985	I–II	50	<55(38.8)	48.9[d]	43.6	23.8	6.1	7.6	9.8	8.7
Arvold [20]	2011	1434	I–II	67	<55(53)	100	63.1	13.8	7.3	3.8	11.9	
Meyers [39]	2011	149	II–III	40	48[b]	33	37	17		13	33	
Dominici [46]	2012	819	I–III	71	<50(34%)	0	70	7		6.2	11.5	
Caudle [51]	2012	595	I–III	50	50[b]	100	51.9	8.5		7	32.4	
Park [30]	2012	1006	I–III	53	47[b]	27.7	53.1	21.7		9	16.2	
Kneubil [54]	2013	1742	I–II[a]	38	<50(66)	0	20.3	45.4	15.3	12.4	7	
Gangi [19]	2014	1851	I–III	73.8	<50(25.6)	100	72.4	11.5		12.4	14	
Haffty [15]	2006	482	I–II	75	<50(50)	100	75.7				24.2	
Dent [29]	2007	1601	I–II	50	53–57[c]	47	88.8				11.2	
Solin [38]	2009	519	I–II	78	<50(16)	100	83				17	

BCT Breast conservation therapy with lumpectomy and radiotherapy, *HER* Her-2-neu positive subtype, *LumA* Luminal A, *LumB* Luminal B, *N* Number of patients, *NR* Not reported, *Pre* premenopausal, *TN* triple negative subtype

[a] Mastectomy with breast reconstruction 61.4% did not receive any radiation, 13% received standard chest wall radiotherapy and 25% received inta-operative RT to the nipple

[b] Median age

[c] Mean age

[d] 51.1% patients had total mastectomy of whom 25% received post-mastectomy RT

Interdependent Association of Subtype and Covariates on Locoregional Failure and its Implications on Radiotherapy Decision Making

Independent of the more aggressive clinicopathologic features or treatment received [28], TNBC subtype is characterized by inferior progression-free survival (PFS) and overall survival (OS) [9, 29]. In the era prior to the availability of trastuzumab, both hormone receptor-negative subtypes (HER2-positive and TNBC), were associated with early relapse, and propensity to lung and brain metastasis [29–31]. The prognosis following local recurrence [32, 33] or distant metastasis was poor with short median survival [34]. Furthermore, two meta-analyses confirmed the high LRF observed in patients with TNBC [18, 35] in several studies [14, 16, 20, 27], but the results were not shared by others [15, 36–39].

The patient population, surgical intervention and distribution of subtypes in these studies were variable (Table 2). Many factors have been conventionally linked to a higher LRF probability, such as multiple positive lymph nodes [3, 40], large tumor size [3, 41], presence of lymphovascular invasion [41], younger age [42], positive surgical margins [41], high tumor grade [42], pectoral fascia involvement [41], skin invasion [41], lymph node ratio >0.2 [40] and extra-capsular extension [40]. The conflicting reports on TNBC LRF may be resolved by analyzing pattern of failure by the breast cancer subtypes while adjusting for the previously mentioned classic risk factors.

Based on the adopted surgical approach (mastectomy or lumpectomy), the studies are grouped into two broad categories to minimize the variability within the patient population. Although the majority of patients with TNBC included in the studies (Table 3) had early stage breast cancer managed with breast conserving therapy (BCT), the 5-year LRF in these select studies varies widely (4.7–17%). A similar wide range is demonstrated in the post mastectomy setting where the 5-year LRF rate ranges from 7.4 to 14% (Table 4).

Follow Up Duration

The variable follow up time may be a potential confounder due to possible higher event rate in one subtype versus the others. For instance, shorter follow up could only accentuate the LRF difference between TNBC and other subtypes, with the formal having a notorious early relapse pattern [29, 30].

Age

Most studies report an association between young age and high LRF risk [43, 44]. Moreover, a larger proportion of patients with TNBC present at a young age [17, 19, 45]. To clarify this confounding variable, Arvold et al. analyzed the LRF pattern by age groups and breast cancer subtype [20]. In this study, 11.9% of the patients ($n=1434$) undergoing BCT had TNBC. Young age and TNBC status were the most

Table 3 Studies of locoregional recurrence by constructed breast cancer subtype after breast conservation therapy

Author	N	MFU	Systemic therapy (%)	RT	5 Y-LRF by subtype (%)				5 Y-Isolated regional failure (%)			
					LumA	LumB	HER	TN	LumA	LumB	HER	TN
Freedman [131]	753	3.6	100	Comp[b]	2.6	NI	4.6	5.3	NR	NR	NR	NR
Millar [27]	482	7	23 CT; 45 HT	Comp[b]	3.6[a]	8.7[a]	7.7[a]	9.6[a]	1.2	0	7.6	6.7
Arvold [20]	1434	7	90	Comp[b]	0.8	1.1	10.8	6.7	NR	NR	NR	NR
Caudle [51]	595	5.3	100[c]	Comp[b]	1.2	3.9	9.5	7.2	1.2	1.9	2.3	2.5
Nguyen [55]	793	5.8	90	Comp[b]	0.8	1.5	8.4	7.1	NR	NR	NR	NR
Gangi [19]	1851	5	CT 43	Non-comp	1.7	1.9	12.5	4.7	0.7	2.4	6.3	1.3
Haffty [15]	482	7.9	69	Comp[b]	17			17	1			6[d]
Solin [38]	519	3.9	20 CT; 27 HT; 7 Both	Comp[b]	2			8[d]	0			1

Comp Comprehensive including regional lymph nodes RT, *CT* chemotherapy, *HER* Her-2-neu positive subtype, *HER* Human Epidermal Growth Receptor, *HT* Hormonal therapy, *LumA* Luminal A, *LumB* Luminal B, *LRF* locoregional recurrence or isolated local recurrence, *MFU* Median Follow up, *N* Number of patients, *NI* Not included, *NR* Not reported, *TN* triple negative subtype, *RT* radiotherapy, *Y* year

[a] 10 y end point

[b] Per discretion of the treating physician mostly for T3N+ or >3 positive nodes

[c] Neoadjuvant chemotherapy was given to all patient

[d] P-value is significant for difference between the subgroups

Table 4 Studies of locoregional recurrence by constructed breast cancer subtype where the majority had mastectomy

Author	N	MFU	Systemic therapy (%)	RT	5 Y-LRF by subtype (%)				5 Y-Isolated Regional failure (%)			
					LumA	LumB	HER	TN	Lum A	LumB	HER	TN
Voduc [14]	2985	12	57(20 % C; 31 % HT)	Comp[d]	8[b,c]	10[b,c]	21[b,c]	14[b,c]	3[b,c]	8[b,c]	16[b,c]	14[b,c]
					8[b,h]	14[b,h]	17[b,h]	19[b,h]	4[b,h]	12[b,h]	12[b,h]	20[b,h]
Kyndi [16]	1000	17	100 (CMF 82b, Tamoxifen 83b)	Comp	2[a]	2[a]	10[a]	9[a]	NR	NR	NR	NR
				None	23[a]	28[a]	21[a]	25[a]				
Gabos [37]	618	4.8	57 CT; 63 HT	Comp[d]	3.4	8.5	14.7[+]	11[+]	NR	NR	NR	NR
Meyers [39]	149	4.5	100[f,e]	Comp[d]	4	4	5	14	0	2	0	6
Kneubil [54]	1742	6.1	18 CT[e]; 33HT; 45 both	Non-comp	2.5	9.8	3.8	10.9	1.8	4.4	1.8	5
Dominici [46]	819	4.8	47 CT; 67 HT[e]	Comp[d]	1[i]	6.5[i]	2[i]	10.9[i]	0.5[i]	1.8[i]	2[i]	8.3[i]
Park [30]	1006	5.8	82 CT;56 HT	Comp[d]	2.4[i]	9.6[i]	12.1[i]	7.4[i]	1.3[i]	7.3[i]	7.7[i]	4.9[i]
Dent [29]	1601	8.1	27CT; 51HT	NR	12			13	NR			NR

Comp Comprehensive including regional lymph nodes RT, *CT* chemotherapy, *HER* Her-2-neu positive subtype, *HER* Human Epidermal Growth Receptor, *HT* Hormonal therapy, *LumA* Luminal A, *LumB*, Luminal B, *LRF* locoregional recurrence or isolated local recurrence, *MFU* Median Follow up, *N* Number of patients, *NI* Not included, *NR* Not reported, *TN* triple negative subtype, *RT* radiotherapy, *Y* year

[a] 15 year end point

[b] 10 y end point

[c] Lumpectomy and adjuvant radiotherapy as a part of breast conserving therapy

[d] Per discretion of the treating physician mostly for T3N+ or > 3 positive nodes

[e] Trastuzumab delivered in Her-2-Neu positive subtypes

[f] Neoadjuvant chemotherapy was given to all patient

[h] Total mastectomy

[i] P-value is significant for difference between the subgroups

important prognostic factors independently predicting poor LRF on multivariable analysis (MVA). For example, the LRF in patients with TNBC was 10.2, 8.9, 8.3 and 6.5% in ages 23–46, 47–54, 55–63 and >63 year old, respectively. Notably, both HER2-positive breast cancer and TNBC were associated with higher LRF in each age quintile. A similar finding was reported by Voduc et al.; age less than 40 and hormone receptor-negative subtypes were the most significant independent regional recurrence predictors in patients who undergo BCT. The Hazard ratio (HR) for regional failure was 2.2 ($p=0.035$) and 2.7 ($p=0.009$) for younger age and Basal subtype, respectively [14]. In the post mastectomy setting, age did not retain significance; while tumor size, high grade, positive lymph nodes and non-Luminal subtypes were independent predictor of high LRF. Conversely, another study demonstrated the significant association between young age and LRF risk in the post-mastectomy setting (HR 3.32, $p=0.02$) [46].

Tumor Size and Lymph Node Involvement

With the exception of few studies that did not reveal the effect of TNBC subtype on LRF rate, either alone or in relation with other covariates [15, 47], the data regarding the effect of tumor size and lymph node positivity on LRF risk- in the BCT and Total Mastectomy ™ setting- are more consistent. Gabos et al. for example, reported that tumor size larger than 2 cm, TNBC status and positive lymph nodes were associated with increased LRF on MVA. However, when analyzing the LRF pattern by surgery type (approximately one third of patients underwent BCT), TNBC status and lymph node positivity were significant only in the TM group (HR 4.72; $p=0.0069$ and HR 3.23; $p=0.0047$, respectively) [37]. Contrary to the previous results, more than three positive lymph nodes and hormone receptor-negative tumor subtypes were associated with high LRF in both BCT- (HR 3.2 and 2.7, respectively) and TM-treated patients (HR 2.28 and 4.22, respectively) [14]. Adjusting for lymph node positivity, an inferior disease-free survival (DFS) and OS was observed in patients with TNBC presenting with node-positive disease, specifically DFS HR was 2.1 ($p=0.001$) in women undergoing BCT and 2.6 ($p<0.001$) in those undergoing TM. In contrast, patients with node-negative TNBC did not display statistically worse DFS or OS when compared with other node-negative subtypes [30]. In addition to a non-Luminal subtype, the lymph node positivity, was consistently reported as being the most significant predictor of LRF in multiple studies, either alone or in association with other risk factors such as lymphovascular invasion [46], extra-capsular extension [39], number of lymph nodes [27, 39, 46], extensive intraductal carcinoma [27] or tumor grade [14, 27].

Type of Surgical Approach

Voduc et al. reported on the LRF in 2985 breast cancer patients treated with different approaches (BCT: 1461; TM: 1492; TM and RT: 508). On MVA, local failure

HR in TNBC were 1.2 ($p=0.048$) and 1.9 ($p=0.018$) in BCT and TM, respectively; and regional recurrence HR were 2.7 ($p=0.009$) and 4.22 ($p<0.001$), respectively [14].

At first glance, this high LRF rate may justify a more aggressive surgical approach; however, the LRF rate in this study was comparable between the BCT and TM groups. The meta-analysis, conducted by Lowery et al., displayed a similar conclusion (Fig. 1 and 2); Compared to TNBC, the LRF HR in hormone receptor-positive tumors were 0.49 (0.33–0.73) and 0.66 (0.53–0.83) for BCT and TM, respectively [18]. Congruent with this philosophy, Ho et al. reported an excellent 5-year local control (95%) in patients with TNBC treated with BCT [36]; implying that an aggressive surgery is not warranted in this subtype and that a limited surgery and RT in the BCT setting achieves equivalent outcome.

Response to Neoadjuvant Chemotherapy

Neoadjuvant chemotherapy provides an attractive treatment option in patients with large operable breast cancer desiring BCT while maintaining equivalent DFS and OS to the administration of adjuvant chemotherapy [48]. Moreover, achieving pathological complete response (pCR) following neoadjuvant chemotherapy has been constantly associated with an improved disease outcome [49, 50].

Regarding breast cancer subtypes and the impact of achieving pCR on locoregional control, Caudle et al. reported a 38% pCR rate to neoadjuvant chemotherapy in a TNBC group (193 out of 595 patients) compared with 9% pCR rate in LumA

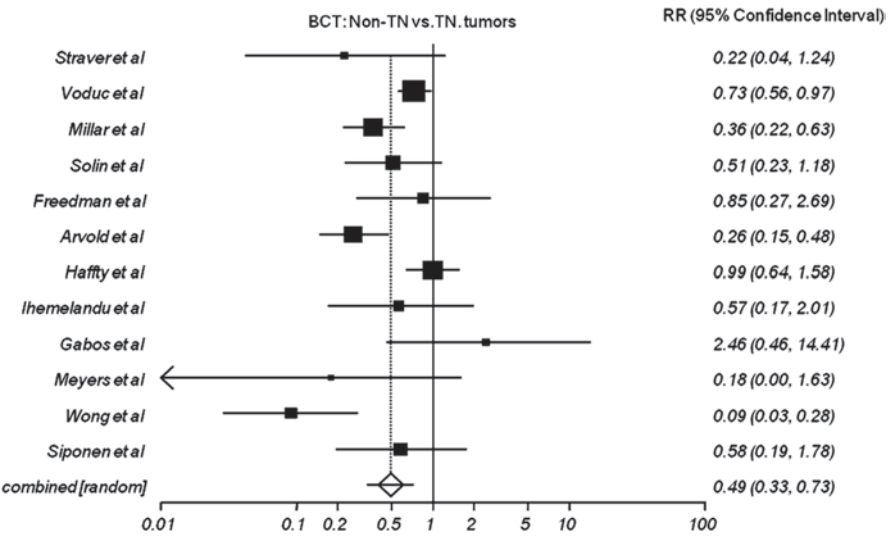

Fig. 1 Locoregional recurrence relative risk (RR) in patients with triple negative (TN) breast cancer compared with other subtypes after breast conserving therapy (BCT) [18]

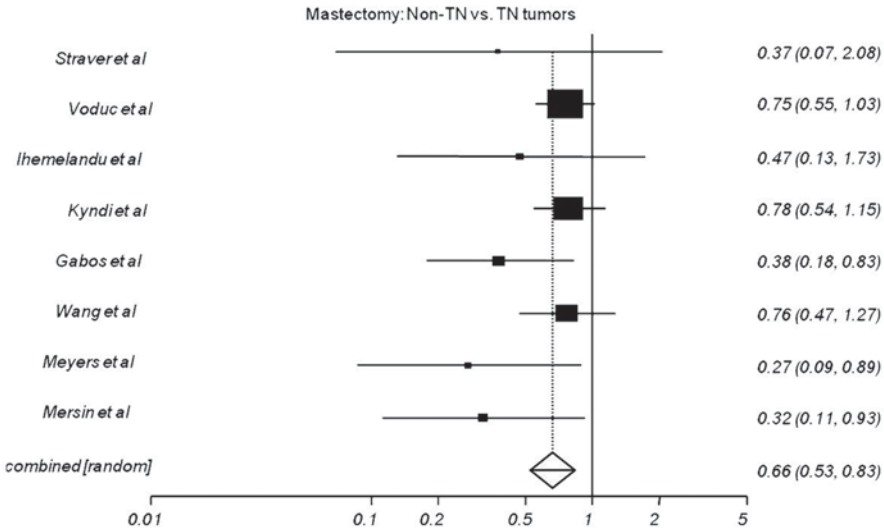

Fig. 2 Locoregional recurrence relative risk (RR) in patients with triple negative (TN) breast cancer compared with other subtypes after mastectomy [18]

subtype. On MVA, TNBC subtype and more than four positive lymph nodes were significant for increased LRF risk (HR 5.7 and 2.9, respectively), while, achieving pCR was associated with lower LRF risk (HR 0.22, $p=0.01$) [51]. In addition to the traditional covariates (number of positive lymph nodes, extra-capsular extension), the univariable (UVA) model identified that post treatment tumor size and stage were significantly associated with LRF risk in 149 patients with breast cancer receiving neoadjuvant chemotherapy for their locally advanced disease. In this analysis the relatively low number of events did not allow for MVA to examine the relation between subtypes and pCR [39].

Many reports confirmed that intrinsic breast cancer subtypes varies in their response to neoadjuvant chemotherapy with a pCR rate reaching as high as 30% in hormone receptor-negative tumors compared to < 10% in hormone receptor-positive tumors [12]; potentially representing a promising treatment strategy for these aggressive subgroups associated with advanced presentation and inferior prognosis [51, 52].

Adjuvant Systemic Therapy

The chemosensitive breast cancer subtypes are expected to derive a locoregional protective effect from adjuvant systemic therapy. As highlighted in Table 4, the LRF rate was lower when chemotherapy was used more systematically. Both TNBC and adjuvant systemic therapy administration were independent prognostic factors of LRF on MVA with the latter being protective [14, 53]. This conclusion is not uni-

versal though; as the majority of patients with TNBC received chemotherapy, some studies either did not adjust for chemotherapy receipt [38, 54], or chemotherapy was not a significant covariate when adjusting for other variables [27, 30, 46, 55].

Adjuvant Radiotherapy

Reducing the two components of LRF, local and regional, may result in reduction of distant metastasis [56] and potentially improve survival in patients with TNBC [57].

Reduction of the high LRF with the use of RT, resulted in 14% OS benefit in a prospective study in which 681 patients with stage I-II TNBC were randomized to chemotherapy and post-mastectomy radiotherapy (PMRT) or chemotherapy alone [58]. Contrary to these results, Kyndi et al. noted that PMRT did not improve survival in patients with TNBC despite their high LRF; suggesting possible radioresistance [16]. Consistent with these data, radiation use improved survival (HR 0.3; $p=0.0004$) in the patients with TNBC undergoing BCT but not in the PMRT setting (HR 0.79; $p=0.38$) [59]. Even in patients with T1-2N0 TNBC, RT reduced the LRF by 6% in those treated with BCT (including RT) compared with those treated with modified radical mastectomy (MRM) without RT [53], suggesting that this subgroup does not benefit from an extensive surgery, and that LRF reduction with RT use is a viable approach.

Kyndi and collegues suggested that the high LRF rate, observed in TNBC in a Danish study, is due to a predominantly locally advanced breast cancer population [16], unlike the Haffty study where 75% of patients presented with node-negative early stage disease [15]. Interestingly, the isolated LRF in patients with TNBC was significantly higher in the Haffty study (6% vs. 1%; $p=0.05$) [15]. Similarly Abdulkarim et al. exclusively included patients with early stage, node-negative disease and reported that, almost half of the LRF were regional [53]. By inspecting the rates of isolated regional failure separately (from the combined local and regional failure rates), a higher pattern is observed in TNBC denoting the need to revisit regional nodal irradiation (RNI) role in this patient population. Indeed, Wo et al. analyzed regional LRF in a cohort of 1000 women, attempting to establish a subset of patients that would benefit from RNI. On UVA, higher regional failure was associated with hormone receptor-negative subtypes, positive lymph nodes, lymphovascular invasion and high grade. RNI, delivered in approximately one third of the patients, was not a significant factor on UVA. The authors concluded that, although non-HR subtypes are associated with higher risk of regional failure, the rate is not sufficiently high to consider lymphatic irradiation in patients with low regional disease burden such as those with fewer than four positive nodes [60].

Collectively, the intrinsic biology of TNBC suggests a high propensity to LRF that may require modification of RT fields and dose. Thus, breast cancer subtyping may refine the RT decision when considered in concert with other risk factors. The information derived from subtyping may reflect intrinsic radioresistance while other risk factors may point to the disease burden and other competing failure patterns; both should be considered to refine the treatment decision.

For instance, true recurrence (within 3 cm from tumor bed) is relatively more common in hormone receptor-negative tumors. TNBC (HR 4.8; $p=0.01$) and tumor size (HR 2.1; $p=0.04$) were independent predictors of true recurrence as opposed to younger age, which was a predictor of elsewhere in-breast recurrence [61]. This notion may have implications on selecting patients for partial breast irradiation (PBI). In a recent dose escalation study treating patients with 32 Gy PBI, the 5-year actuarial local recurrence rate in TNBC was 33% compared with 2% in patients with non-TNBC [62]. A similar conclusion was found in the Vernonesi et al. PBI trial that included 1822 patients; along with age younger than 50 and tumor size larger than 2 cm, molecular subtyping was an important predictor of subsequent in-breast failure on MVA. All non-LumA subtypes were associated with high rates of breast recurrence (both, true and elsewhere) with a HR reaching 5.26 ($p=0.002$) for TNBC [63]. Conversely, two retrospective PBI studies demonstrated low rates of local in-breast failure in patients with TNBC comparable with those seen in other tumor types [64, 65]. Variable techniques and different patient characteristics can explain the conflicting failure rate reported in patients with TNBC treated with this technique. Alternatively, hormone receptor-positive tumors may display more elsewhere (more than 3 cm from tumor bed) in-breast recurrences due to higher incidence of a new primary [66], suggesting that PBI is not suitable for certain breast cancer subtypes. However, such recommendation cannot be made without considering other factors such as age or margin status, nor without allowing for sufficient follow up time (accounting for other competing risks such as death or metastasis) to separate true recurrence (in the lumpectomy region) from elsewhere in-breast recurrence (versus new breast primary). Certainly, a prospective PBI trial analyzing failure pattern by subtype could help answer that question.

Regarding dose fractions and the eagerness to adopt a shorter, more convenient radiation schedule, hypofractionated whole breast irradiation (HWBI) in early stage breast cancer disease, was widely accepted as a viable alternative to standard fractionation due to equivalent tumor control rate and comparable toxicity profile [67, 68]. Standardization of radiotherapy in breast cancer (START) trials A and B did not assess the receptor status; however, using tamoxifen as a surrogate for ER positivity, the percent of patients with hormone receptor-negative tumors were 12.2% and 11.7% in START-A and START-B, respectively. In these two trials, the HWBI treatment effect was equivalent regardless of age, tumor size, nodal status, grade or systemic therapy used [67]. Moreover, the mature results of the Ontario randomized trial confirmed HWBI (42.5 in 16 fractions) non-inferiority to standard fractionation (50 Gy in 25 fractions) in patients with early stage breast cancer [68]. Likewise, the treatment effect of HWBI was equivalent regardless of age, tumor size, systemic therapy use or ER status (ER-negative disease constituted 26.1% of the study population). However, HWBI appeared to be less effective in high-grade disease [68].

As a great proportion of TNBC and HER2-positive tumors present with high-grade histology, evaluation of the effect of hypofractionated scheme on different biologic subtype requires further clarification. Bane et al. reconstructed the molecular subtype in 989 out of the 1234 patients included in Ontario hypofractionated study. Interestingly, the 10-year local recurrence rate was equivalent for LumA

and TNBC (4.5%), and significantly lower than LumB (7.9%) and HER2-positive (16.9%) subtypes ($p<0.01$). Yet, neither tumor grade nor molecular subtype were significant predictors of response to hypofractionation [69]. Besides the heterogeneity of patients and treatment characteristics in these studies, it should be noted that the majority of patients in the trials presented with hormone receptor-positive tumors leading to underpowered results regarding hypofractionation effect in different breast cancer subtypes. Currently although whole breast hypofractionation data in TNBC and HER2-positive subtypes are not as prevalent, there does not appear to be a higher risk of local relapse comparing standard and hypofractionation.

Biologic Causes of Radioresistance, BRCA1 and Triple Negative Subtype

Breast cancer susceptibility protein 1 (BRCA1) is an integral part of homologous recombination, repairing double strand DNA breaks. *BRCA1* mutation is associated with defective DNA repair, accumulation of genetic damage, and ultimately, increased cancer susceptibility [70]. Carriers of *BRCA1* deleterious mutation are likely more susceptible to DNA damaging agents such as RT increasing their cancer risk. Notably, the tumor associated with this mutation is expected to be more radiosensitive.

BRCA1 carriers and those with the TNBC subtype share many molecular and phenotypic features [71]. TNBC occurred in 57% of *BRCA1* mutation carriers [72], and approximately 20–25% of patients with TNBC carry *BRCA* mutations [15, 73]. Even in the absence of *BRCA* mutation in patients with sporadic TNBC, the gene function can be hindered by *BRCA* promoter methylation [74]. Influencing RT management decisions, TNBC tumors with defective BRCA are expected to be more radiosensitive with a potential of increased new primary breast cancer risk and/or toxicity. Yet, there is no clinical evidence supporting these hypotheses; there was no increased radiation induced toxicities in *BRCA* mutation carriers treated with BCT compared with controls [75]. However, the 15-year local in-breast (not accounting for regional failure) events were 23.5% vs. 5.5% ($p=.0001$) without survival difference between 302 BCT treated versus 353 TM treated patients [76]. In addition to refuting increased radiosensitivity claims, these results suggest that BCT is a viable option; while acknowledging the increased life time risk of ipsilateral and contralateral breast cancer events in this patient population.

Compared to the expected radiosenstivity (due to close association with BRCA defective DNA repair), mechanisms of TNBC radioresistance are complex and under active research. For instance, 53BP1 loss was postulated to rescue cells from triggering imperfect DNA repair induced by BRCA deficiency [77]. In association with TNBC, absence of 53BP1- a DNA damage response gene- will reverse BRCA1 defect by preventing error prone repair of accumulated DNA breaks induced by radiation; thus, rendering cells radioresistant [78]. As with radiation, BRCA1-deficient cells are suspected to be hypersensitive to poly-ADP-ribose polymerase (PARP) inhibitors due to blocking a second DNA damage repair machinery (necessitating homologous recombination); potentially highlighting agents that can be used to enhance radiosensitization of TNBC [79]. Yet again, 53BP1 absence

leads to suppression of both spontaneous and agents induced radial chromosome formation; granting BRCA1-deficient cells immunity against DNA damaging agents (radiation, Platinum compound and PARP inhibitors) [78]. A recent study displayed a significant association between 53BP1 expression and TNBC in 514 patients treated with BCT. Remarkably, low 53BP1 was related to inferior outcome in all end points, including ipsilateral breast recurrence (76.8% vs. 90.5%; $p=0.01$) and this low expression level retained significance as an independent predictor on MVA. To ensure that TNBC is not a potential confounder, a separate analysis was conducted on the TNBC population demonstrating a significant lower recurrence-free survival (37.8% vs. 83.7%; $p=0.001$), distant metastasis-free survival (48.2% vs. 86.8%; $p=0.004$) and ipsilateral breast recurrence-free survival (72.3% vs. 93.9%; $p=0.036$) [80]. Additionally, mutation of *p53* tumor suppressor gene which, observed in as high as 50% in TNBC [15], is an independent risk factor of inferior response to RT [81] and OS [82].

Taming of the HER2-Positive Subtype

Her-2-*neu* (HER2) is one of four members of the human epidermal growth factor receptor (EGFR) family that has tyrosine kinase activity [83]. HER2 gene amplification or overexpression is observed in 30% of breast cancers [84], including LumB and HER2 subtypes. Limiting the scope on HER2-positive subtype, characterized by hormone receptor negativity, the incidence varies from 4 to 15% as shown in Table 2. This HER2 protein mediates a cascade of signaling molecules such as phosphatidylinositol-3-kinase (PI3-K) and mitogen-activated protein kinase (MAPK) which, in turn, result in activating mitogenic and survival signal transduction pathways that play a vital role in HER2-positive cancer cell survival, and metastasis [85]. In the era prior to trastuzumab availability, HER2-positive tumors were associated with high LRF rate comparable with TNBC [14, 20, 37]. The 5-year LRF ranged from 4.6 to 12.5% in the BCT setting (Table 3) and from 12.6 to 14.7% in the TM setting (Table 4), a rate that is relatively higher compared with the Luminal subtypes. The relatively high LRF observed in HER2 subtype hinted to a potential radioresistance mechanism. Indeed, Piertas el al. demonstrated that, cell lines transfected with HER2 display 25% increase in the dose required to reduce cell survival to 10%; denoting increased in their radioresistant ability with the presence of HER2 [86]. Another confirmatory study demonstrated that breast cancer cells, in which HER2 amplification is silenced by small interfering RNA, exhibited reduction in HER2 and p-AKT with subsequent enhancement of radiosensitivity [87]. Congruently, targeting HER2 with trastuzumab reduced HER2 levels and down-regulated the phosphorylation levels of Akt and MAPK in HER2-positive MCF7 cell lines, and sensitized these cells to RT. Specifically, administration of 8 Gy resulted in 0.02, 0.14 and 0.03 survival fraction in MCF7, MCF7-HER and MCF7-HER treated with trastuzumab [88].

These translational studies were confirmed in the clinical setting. In a matched control cohort (32 patients with local relapse versus control patients without recur-

rence matched with respect to age, tumor size, and radiation dose), investigators sought to evaluate the prognostic impact of HER2 overexpression on locoregional control after BCT. Patients whose tumor overexpressed HER2 sustained more LRF (56% vs. 18%; $p=0.03$) [89]. In spite of the radioresistant properties of the HER2-positive subtype, patients with this subtype do appear to benefit from adjuvant RT compared to chemotherapy alone [90].

In the BCT setting, a meta-analysis of 15 studies addressing locoregional control by reconstructed breast subtype revealed that the relative risk (RR) of LRF at 5 years was 0.34 (95% confidence interval [CI]: 0.26–0.45) in Luminal versus HER2-positive subtypes. Comparing with the TNBC subtype, HER2-positive breast cancer displayed a significantly higher LRF RR 1.44 (95% CI 1.06–1.95). In the post- mastectomy setting, Luminal subgroup had a lower LRF RR of 0.69 compared to HER2-positive tumors subtype; 0.69 (95% CI 0.54–0.89). Conversely, HER2-positive and TNBC subtypes did not show significant difference in LRF RR 0.91 (95% CI 0.68–1.22) [18].

With the standard use of trastuzumab, approximately 50% reduction in LRF is expected [91]. The more recent studies showed a lower LRF in trastuzumab treated HER2-positive tumors, approaching Luminal subtypes' LRF rates [46, 54]. As shown in Table 4, the 5-year LRF rate varied from 2 to 3.8% in HER2-positive tumors treated with adjuvant systemic therapy containing trastuzumab [46, 54], and 5% when the systemic therapy is administered on neoadjuvant basis [39]. Even in low risk node-negative disease, trastuzumab use improved 3-year locoregional control from 90 to 99% ($p=0.01$) in 192 patients undergoing BCT [92].

As previously described in TNBC, HER2-positive breast cancer is associated with increased LRF and relative radioresistance in association with other risk factors. Consequently, HER2 subtype supplements rather than substitute other risk factors when considering the radiation treatment decision. However the widespread use of anti-HER2 agents in patients overexpressing HER2 likely significantly reduces or eliminates HER2 overexpression as a risk factor for local relapse.

Luminal Subtypes

It was postulated that the estrogen driven acceleration G1 to S phase is the mechanism of Luminal subtypes radiosenstivity [16]. However, compared with HER2-positive and TNBC, the relative paucity of metastatic, invasiveness and radiation resistance pathways is primarily the mechanism explaining the superior outcomes seen with Luminal type tumors, aside from their association with other good risk features; such as older age, less propensity to nodal spread, histologic differentiation, and hormonal therapy response.

LumB subtype is defined by high Ki-67 that is HER2-negative and hormone receptor-positive, or by both hormone receptor-positive and HER2-positive. The distinction between Luminal subtypes is based primarily on Ki-67 (proliferative marker) with a cut point set at 13.25% [93]. By itself, Ki-67 did not predict for worse overall outcome [94] and, therefore, LumB is further differentiated by other

proliferative markers such as Cyclin B1 and MKI67 in addition to EGFR and HER2 expression. Depending on the identification method, LumB constitutes 4.6–21.7% of breast cancer (Table 2). However, the triple positive subtype (LumB-HER2-positive) can range from 7.3 to 29% of breast cancer while the percentage of non-HER2-positive LumB varies from 13.6 to 45.4%. In the BCT setting, the 10 year LRF can reach 8.7% in LumB versus 3.6% in LumA [27]. In the post- mastectomy setting, a similar pattern is present with 5-year LRF ranging from 4 to 9.6% in LumB versus 2.4–3.4% in LumA as shown in Table 4.

Other Subtypes

The Normal breast variant is poorly characterized with very limited data regarding its biologic behavior or interaction with RT. Possibly, accounting for about 5–10% of breast cancer, this type has an intrinsic pattern similar to fibroadenoma and normal breast tissue with absent hormone receptor and HER2 expression and, distinctive from Basal subtype, they are also deficient in CK5 and EFGR. Microdissection of breast cancer cancerous cells was used in an attempt to remove possible contamination from normal breast tissue revealing absence of the normal breast subtype; casting doubt on its true existence [95]. Future studies will potentially shed more light on this inconsistently reported subtype.

Claudin Low is another rare subtype (10% of breast cancer) characterized by poor prognosis despite low expression of proliferative markers [96]. The Claudin Low subtype was recently reported [97] as characterized by low level expression of tight junction genes [98] and high expression of immune response genes, separating it from the Basal type. Apart from intermediate response rate to neoadjuvant chemotherapy (between Basal and Luminal subtypes) [96], Claudin Low's behavior, LRF pattern and radiation response remain to be determined.

As the potential to improve RT outcome is limited by the intrinsic radioresistance of each breast cancer subtype, molecular profiling can improve outcome by assisting in selecting agents that modify molecular pathways; PARP inhibitors in TNBC with normal 53BP1 or anti EGFR agents in other TNBC subtypes, trastuzumab or other HER2-targetting tyrosine kinase inhibitors in HER2 subtype, or anti-angiogenic agents when tumor cells express hypoxia markers.

Stepping Into the Molecular Era

Improved Resolution; Improved Individualization

Resorting to limited number of markers to reconstruct breast cancer subtypes was the practical answer to the costly and logistically prohibitive molecular profiling. Yet, the resolution of breast cancer profiling improves with the incorporation of additional genetic markers.

Maggie et al. compared the prognostic significance of defining Basal subtype based on three versus five markers (CK 5/6 and EGFR in addition to ER, PR, HER2); the five markers definition was more predictable of breast cancer specific survival on MVA [26]. Recent subtyping using PAM50 quantitative real time polymerase chain reaction (qRT-PCR) revealed 30% misclassification rate in patients with TNBC previously identified within the Basal subgroup [99]. These data are consistent with a study using gene expression analysis on 587 TNBC to further classify this subclass to six subtypes [100]. Similarly, an unsupervised clustering of multi-gene signatures distinguished three subclasses within 58 HER2-amplified breast cancer; highlighting the within subtype molecular variability and the need of better prognostication tools [101].

As previously mentioned, high throughput genetic analysis refuted our view of breast cancer as a single disease; the seminal publications employing molecular profiling [6, 23] underlined at least four distinct breast cancer entities characterized by unique clinicopathological features. Establishing the intrinsic molecular subtypes is more consistent in separating Basal from the other subtypes [95], mostly driven by proliferative, hormone receptor and HER2-related genes [71]. Beside ignoring the heterogeneity of cancer due to tumor evolution [102] or due to the inherent intra-tumor variability [103], the lack of complete characterization of the number and definition of each subtype was a major limitation of adopting this technology in clinic [104]. Yet, the majority of studies embarked on defining molecular signatures, composed of a set of candidate genes through supervised class-prediction; thus, producing a commercial tool that permits the separation of breast cancer subtypes into classes (based on these signatures) and forecasts the outcome of patients with breast cancer [105–108]. Many signature based microarray [109, 110], or qRT-PCR [107, 111] were validated and are currently commercially available. In an attempt to find a consensus among these different signatures (including different set of genes), a meta-analysis demonstrated that, based predominantly on proliferative markers, there is general agreement across the different platforms on the classification of poor risk patients with breast cancer and that these genetic signatures complement- rather than substitute- other classic clinicopathologic factors (such as tumor size and positive lymph nodes) [112]. Apart from the two-gene expression signature [108], a comparative study, evaluating five platforms [106, 108, 109, 113], demonstrated their prognostic concordance in accurately predicting PFS and OS; thus providing another confirmation on the validity of these tools in the clinical setting despite the diversity of genes set included in each platform [114]. However, the majority of these platforms were used to predict systemic, rather than local, failure pattern with little characterization of the role of RT on local control.

Molecular Signature and Local Control Prediction

Awaiting further evidence, genotyping based RT consideration is still evolving (Table 5). For example, the genomic predictors of breast cancer locoregional recurrence risk after mastectomy had an overall accuracy of 75–78% in Cheng et al.

Table 5 Characteristics and locoregional outcome of breast cancer patients based on their molecular signature

Author	*n*	MFU	Stage	N0	ER+	Age (%)	BCT (%)	Systemic therapy	RT (%)	High risk signature (%)	LRF (%)
Nuyten 2006 [115]	161	7.6	I–II	56	75	<40 (78)	54.4	36 (11 HT)	Non-comp	60.2	5 vs 29[e]
Cheng 2006 [118]	94	3	I–II	30	69.2	<50 (46)	0	NR	None	35	41.4 vs 18.2[d]
Mamounas 2010 [118]	895	12.5	I–II[a]	100	100	<50 (35)	43	100	Non-comp	27.4	4.3 vs 7.2 vs15.8[c]
Solin 2013 [119]	327		DCIS	0	97.9	<50 (20)	100	29.4	None	6.5%	3.7 vs 12.3 vs19.2[b]
Kreike 2006 [120]	50	3.9	I–II	58	49	<50 (100)	100	40	Non-comp	No profile found	NA
Nimeus 2008 [121]	143	~7	I–II	100	~70	NR	100	18	Non-comp[h]	Signature correlates with LRF after RT	
Tramm 2014 [122]	191	~25	II–III	5	72	<50 (33)	0	100	Comp (50)	74	8 vs 57[f]

BCT Breast conservation therapy with lumpectomy and radiotherapy, *Comp* Comprehensive including regional lymph nodes RT, *ER+* Estrogen receptor Positive, *LRF* Locoregional failure, *MFU* median follow up in years, *n* Number of patients, *NR* Not reported, *Pre* premenopausal

[a] All were node negative, Estrogen receptor positive where 38 and 42% had breast conserving therapy in NSABP B14 and NSABP B2, respectively

[b] Ipsilateral invasive breast events based on low versus intermediate versus high OncotypeDX 21-gene score

[c] Low versus intermediate versus high OncotypeDX 21-gene score

[d] Based on 258 gene signature

[e] Based on wound, hypoxia, 70-gene signatures

[f] Based on 7 LRF genes

[h] Only 53% received adjuvant radiotherapy

~ Approximately

publication [115]. Two sets of signature (258 and 34 genes) were used in DNA microarrays studies conducted on 94 patients undergoing mastectomy as their primary surgical treatment. On MVA, the genomic predictive index below 0.8 set point (HR 22; $p < 0.0001$) and ER-negative status (HR 3.4; $p = 0.04$) were independent predictor of 3-year LRF, reaching 9% versus 60% ($p = 0.008$) in patients with more versus less than 0.8 predictive index. Without significant difference in the predictable power of the two models (258 versus 34-gene model), the later model may select post-mastectomy patients who might benefit from adjuvant RT.

In another study, a classifier, based essentially on wound gene signature, achieved 87.5% sensitivity in predicting 10-year local recurrence rate (5% versus 29% in patient with favorable versus unfavorable profile) after lumpectomy and RT [116]. In this early stage breast cancer population subjected to BCT, 78% were younger than 40 years old. In the patients who experienced local recurrence (10.5%), the seventy-gene profile could not establish high and low risk separation, having low sensitivity and specificity 63 and 50%, respectively. Neither did the hypoxia-response gene profile with 75% sensitivity and 44% specificity on the validation set; displaying a non-significant 10-year recurrence risk of 13% versus 15% in low versus high-risk hypoxia gene profile. When applying the wound response signature, the 10-year recurrence risk was 5% versus 29% ($p = 0.0008$) in low versus high-risk, respectively in the validation set. MVA Cox regression, adjusting for age, tumor size, RT boost use revealed that the wound signature is the only significant prognostic indicator of local relapse (HR 16; $p = 0.01$). Through a possible association between cancer cells and wound healing, governed mainly by fibroblast response, the wound-response gene signature was proposed as a possible surrogate to tumor progression and potential prediction of local failure (Table 5) [117].

Another study used 21-gene based Onco*type* DX recurrence score (RS), essentially used to estimate distant recurrence risk- to predict local recurrence in patients with ER-positive, node-negative breast cancer [118]. The RT-PCR was successful in 1023 and 651 patients enrolled in National Surgical Adjuvant Breast and Bowel Project (NSABP) B14 and B20, respectively. In both studies, patients with low risk (Luminal subtype) breast cancer were randomized to tamoxifen versus placebo in B14 or Tamoxifen with or without cyclophosphamide, methotrexate and 5-fluorouracil (CMF) in the B20 study. Adjuvant RT was used in the lumpectomy treated patients (43%). RS based on 21-gene signature (composed of five reference, five proliferative, four estrogen, two invasion, two HER2, GSTM1, CD68, and BAG1 Genes) was categorized into low, intermediate and high. In the placebo group (355 patients), the 10–year LRF of 10.8, 20 and 18.4% (log-rank $p = 0.022$) in low, intermediate and high RS, respectively. Regarding the 424 patients randomized to chemotherapy plus tamoxifen, RS was significantly associated with LRF risk; 1.6, 2.7 and 7.8 (log-rank $p = 0.028$) in low, intermediate and high RS, respectively. Whereas in the 895 patients randomized to Tamoxifen alone, the MVA Cox regression analysis showed that- after adjusting for age, tumor size, initial local treatment and grade- RS significantly predicts LRF risk (HR 2.16; $p = 0.007$). In this subgroup (895 patients), 390 were treated with BCT and their 10-year LRF was 6.8, 10.8, and 14.6% (log-rank $p = 0.043$) in low, intermediate and high RS, respectively. In

BCT treated patients, age younger than 50 was associated with higher LRF rate. A similar association between RS and age was not found in mastectomy treated patients whose LRF estimates were 2.3, 4.7, and 16.8% for low, intermediate, and high RS, respectively (log-rank $p<0.001$). This seminal study revealed that, even within the low risk Luminal breast cancer subtype, the molecular profile could distinguish a subgroup with a high LRF risk justifying RT recommendation in that setting. Moreover, that the tools for molecular profiling are readily available for prime incorporation in the clinic. As higher score predicts higher local recurrence risk, the same technology can be used in guiding RT indications in patients with node-positive disease.

Recently, Onco*type* DX score was shown to predict local recurrence after lumpectomy for breast ductal carcinoma *in-situ* (DCIS) [119]. Among the patients enrolled on Eastern Cooperative Oncology group (ECOG) E 5194 study, the tissue available for analysis was present in 327 patients. The 10-year ipsilateral breast event risks were 10.6, 26.7, and 25.9 and the 10-year invasive breast cancer events were 3.7, 12.3, and 19.2% in low, intermediate and high DCIS RS, respectively (log-rank $p\leq0.006$). In addition to tumor size and menopausal status, DCIS score was an independent predictor of events occurrence on MVA (HR 2.37; $p=0.02$). After validation of the study findings, this readily available technology may select patients with high recurrence score and who are most likely to benefit from adjuvant RT.

Conversely, Kreike et al. failed to find a gene expression pattern to predict local failure in 50 patients with breast cancer undergoing BCT. Using 18K cDNA microarrays and hierarchal clustering classification, the 19 patients who developed breast recurrence did not display a specific signature to distinguish them from the 31 patients free of recurrence. The short median follow up, small sample size and unadjusting for cofounding variables such as age and grade may have contributed to lack of detecting local recurrence gene set [120].

To avoid previous confounders, Swedish investigators analyzed the gene expression profile in 143 patients treated with BCT with negative lymph nodes and negative surgical margins. In their analysis, they divided they study population into four groups based on local failure occurrence and adjuvant RT delivery. In the 77 patients receiving RT, the gene expression profile (16,895 genes) supplemented other clinical factors (age and grade) to predict LRF pattern in the 30 patients who failed locally despite receiving adjuvant RT; the receiver operating characteristic (ROC) was 0.91 in patients with ER-positive disease and 0.74 in patients with ER-negative, outperforming wound-response signature whose ROC scored 0.75 in both ER-positive and ER-negative breast cancer patients. The author concluded that tumor with this aggressive molecular profile would benefit from other alternatives such as mastectomy due to their high chance of not responding to post-operative RT [121].

In contrast to the Swedish study that identified patients at high-risk of recurrence (even after adjuvant RT), the Danish group employed gene expression profiling to predict patient at low risk of recurrence after mastectomy and thus can safely avoid PMRT [122]. The successful microarray analysis of the fresh frozen tumor samples of the Danish 82b and 82c patients identified 7 key genes associated with high local failure risk (*HLA DQA, RGS1, DNALI1, hCG2023290, IGKC, OR8G2,*

and *ADH1B)*. In the patients' group not randomized to PMRT, the 20-year LRF was 57% vs. 8% ($p<0.0001$) in high-risk versus low-risk genetic signature. The molecular signature was an independent predictor of recurrence after adjusting for lymph node status and primary tumor presentation on MVA. Remarkably, PMRT reduced the LRF from 57 to 12% ($p<0.0001$) in patients with high-risk profile; whereas, patients whose tumors exhibited low risk signature did not benefit from PMRT; LRF 8% vs. 9% ($p=0.93$). The author concluded that molecular subtyping using the seven-gene signature could identify a patient population that would not benefit from PMRT regardless of their traditional high-risk clinicopathologic features.

Markers at Recurrence

At recurrence, the molecular subtype may still impact on overall outcome. A recent study included 185 patients who underwent BCT, with histologically proven ipsilateral breast recurrence, to evaluate the impact of reconstructed subtype (approximated using ER, HER2 and Ki-67 markers) on the overall outcome. The 5-year DFS was 86.3, 57.1, 65.9 and 56.6% in LumA, LumB, HER2 and TNBC subtypes respectively (Log-Rank $p=0.0074$). On MVA, breast cancer subtype was an independent predictor of outcome in addition to other features such as: time to recurrence, tumor location, and lymphovascular invasion [33]. Adapting the management strategy to accommodate for the breast cancer subtype aggressiveness at time of recurrence may impact the survival. Parikh et al. demonstrated that 5-year disease metastasis-free survival drops from 90.8 to 48.6% if the recurrence is of the TNBC subtype. Even after adjusting for other covariates and chemotherapy administration, TNBC subtype breast recurrence was a significant predictor of poor disease metastasis-free survival (HR 5.91; $p<0.01$) [32].

Molecular Profile and Radiation Induced Toxicity

In addition to the information provided by tumor profiling to individualize the treatment decision, the patient genetic make-up (including normal tissue radiation response) may impact this decision. The radiation induced toxicity in breast cancer such as fibrosis and telangiectasia increase over time [123]. The severity of these toxicities is both, treatment dependent (dose, fields, fractionation, overall treatment time) [124] and patient dependent (age, comorbidity, skin sensitivity) [123, 125]. However, genetic profile has been also implicated in the development and in the severity of acute and long-term normal tissue toxicity [126]. Developing radiation induced toxicity molecular profile is challenging due to variation of the toxicity pattern across different sites, time and individuals [127].

A recent study evaluated polymorphisms in genes involved in DNA repair *(APEX1, XRCC1, XRCC2, XRCC3, XPD*) and damage response gene (*TP53, P21*)

in 409 patients with breast cancer; *TP53* variant was associated with a significant increase in the late skin toxicity (telangiectasia odd ratio = 1.97; 95 % CI: 1.11–3.52) [128]. Another study assessed 17 single nucleotide polymorphisms (SNP) in several genetic markers (*TGFB1, SOD2, XRCC1, XRCC3* and *APEX*) [129]. Although, Severe radiation reaction was linked to seven of these SNPs, only *XRCC3* retained significance; potentially used in sparing this specific SNP carriers the morbidity of RT if the benefits don't outweigh the toxicity risk. New studies are needed to link the diverse human genetic profile, radiation dose and toxicity [130].

Conclusion and Future Directions

Data continue to unfold the underlying biology of breast cancer subtypes suggesting that molecular profiling will be integrated into the locoregional management. Ultimately, individualizing the local treatment strategy based on the genetic makeup of the host and the molecular profile of the tumor may prove beneficial in addressing the distinct LRF pattern and optimal approach. Prospective studies and additional validation studies are clearly needed before routinely integrating molecular profiling into the decision making process. In addition, prospective evaluation of targeted agents, to be used in combination with radiation, in those tumors that are less responsive to conventional therapy, is an area ripe for future investigations. While integration of molecular profiling and personalized or precision medicine into local-regional management lags behind its integration into systemic therapy decision making, there has been significant progress and discoveries recently that pave the way for future studies and trials which can incorporate molecular profiling into local-regional management, with the ultimate goal of further improving patient outcomes and quality of life.

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Prognostic and Predictive Biomarkers of Endocrine Responsiveness for Estrogen Receptor Positive Breast Cancer

Cynthia X. Ma, Ron Bose and Matthew J. Ellis

Abstract The estrogen-dependent nature of breast cancer is the fundamental basis for endocrine therapy. The presence of estrogen receptor (ER), the therapeutic target of endocrine therapy, is a prerequisite for this therapeutic approach. However, estrogen-independent growth often exists *de novo* at diagnosis or develops during the course of endocrine therapy. Therefore ER alone is insufficient in predicting endocrine therapy efficacy. Several RNA-based multigene assays are now available in clinical practice to assess distant recurrence risk, with majority of these assays evaluated in patients treated with 5 years of adjuvant endocrine therapy. While MammaPrint and Onco*type* Dx are most predictive of recurrence risk within the first 5 years of diagnosis, Prosigna, Breast Cancer Index (BCI), and EndoPredict Clin have also demonstrated utility in predicting late recurrence. In addition, PAM50, or Prosigna, provides further biological insights by classifying breast cancers into intrinsic molecular subtypes. Additional strategies are under investigation in prospective clinical trials to differentiate endocrine sensitive and resistant tumors and include on-treatment Ki-67 and Preoperative Endocrine Prognostic Index (PEPI) score in the setting of neoadjuvant endocrine therapy. These biomarkers have become important tools in clinical practice for the identification of low risk patients for whom chemotherapy could be avoided. However, there is much work ahead toward the development of a molecular classification that informs the biology and novel therapeutic targets in high-risk disease as chemotherapy has only modest benefit in this population. The recognition of somatic mutations and their relationship to endocrine therapy responsiveness opens important opportunities toward this goal.

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Introduction and Overview

Endocrine therapy is prescribed to virtually all patients diagnosed with estrogen receptor (ER)-positive breast cancer. However, up to 50 % of patients with early stage breast cancer experience disease recurrence despite curative local therapy and long term adjuvant endocrine treatment [1, 2]. Differentiating endocrine sensitive versus resistant tumors is necessary for decision-making in the adjuvant setting so that patients with endocrine sensitive disease can be spared unnecessary chemotherapy. For patients with endocrine resistant disease, chemotherapy is the current standard although its anti-tumor effect on ER-positive breast cancer is modest at best [3, 4]. Further biological investigation and drug development effort in the endocrine resistant population is clearly a priority for the scientific community. In addition, as more patients are considering extended endocrine therapy beyond 5 years based on results from the long-term (10 years versus 5 years) tamoxifen trials, including Adjuvant Tmoxifen: Longer Against Shorter (ATLAS) trial [5] and the Adjuvant Tamoxifen To Offer More (aTTOM) trial [6], and the NCIC MA17 trial which evaluated extended endocrine therapy with 5 years of letrozole following 5 years of tamoxifen [7], there is an increasing demand in tools to assess late recurrence risk. Although endocrine therapy is considered well-tolerated compared to chemotherapy, side effects are common and significant, including vasomotor symptoms, sexual dysfunction, and aromatase inhibitor-associated risk of osteoporosis, fracture, musculoskeletal symptoms, and tamoxifen-related risk of endometrial cancer and venous thrombosis [2]. Prediction of low risk of late recurrence after 5 years of endocrine therapy could spare patients from unnecessary toxicities with extended endocrine therapy.

The traditional clinicopathological features including stage, grade, and ER, progesterone receptor (PgR), and HER2 status do not reflect the significant heterogeneity of ER-positive breast cancer. The introduction of genome-wide microarray analysis of gene expression led to the recognition of the four intrinsic molecular subtypes of breast cancer, including Luminal A, Luminal B, HER2-enriched, and Basal-like, that carry prognostic implications [8, 9]. Since then several RNA based multi-gene assays, including Onco*type* Dx, MammoPrint, Prosigna, BCI, EndoPredict, have been introduced in the clinical practice to categorize ER-positive breast cancer into different risk groups of recurrence, some of which have shown promise in predicting late recurrence (more than 5 years after initial diagnosis and treatment). These assays each showed ability to provide added prognostic information to the standard clinicopathologic parameters and are most helpful in patients who

are classified in the low risk category for whom chemotherapy could be avoided. Patients in the low risk category by BCI, EndoPredict or Prosigna have shown to have extremely low risk of distant late recurrence, therefore perhaps extended adjuvant hormonal therapy could be avoided. While the use of these assays has led to a change in the recommendation of adjuvant chemotherapy in many patients, prospective validations are still ongoing. An in-depth understanding of the underlying biology of the high-risk group is needed to design treatment based on the identification of driver events in these tumors, as they are often resistant to chemotherapy. On the other hand, many patients experience late recurrence despite extended therapy, therefore investigation on the biology leading to late recurrence presents another research priority for the scientific community.

The neoadjuvant setting provides a platform for uncovering mechanisms associated with endocrine resistance, in particular *de novo* resistant tumors. Studies of neoadjuvant endocrine therapy trials indicated that biomarker response such as the degree of reduction in the level of the cell proliferation marker Ki-67 following at least 2 weeks of therapy and the preoperative prognostic index (PEPI) score at surgery based on tumor Ki-67, ER and tumor stage, are predictive of long term patient outcomes [10−12]. The application of next generation sequencing technology on ER-positive breast cancers from patients treated in neoadjuvant clinical trials led to a bioinformatics-based association of mutations such as *TP53, BIRC6, CDKN1B, RUNX1* and the long non-coding *RNA MALAT1* with Luminal B status, while *MAP3K1* and *MAP4K2* and *GATA3* with Luminal A status [13]. Using Ki-67 as a metric, *TP53* mutation was associated with high baseline and on treatment proliferation, a pattern suggesting intrinsic resistance to AO therapy and *MAP3K1* had the opposite pattern. *GATA3* mutations also appeared to potentially predict endocrine therapy sensitivity, because mutation was associated with a higher than average fall in Ki-67 values [13]. Sequencing studies of additional neoadjuvant trials are ongoing to further investigate the prognostic or predictive roles of genes that are less commonly mutated.

In the metastatic setting, although most patients derive benefit from initial endocrine therapy with disease stabilization or tumor shrinkage, eventual development of resistant disease invariably occurs. To uncover acquired endocrine resistance and predictive markers, analysis of recurrent disease is needed. An important discovery in recent sequencing studies of tumors progressed on prior endocrine therapies is the *ESR1* mutation acquired under the pressure of estrogen deprivation therapy [14−18]. These mutations cluster in the ligand-binding domain, leading to constitutive ER activation and estrogen-independent tumor growth. In addition, other genetic alterations, such as amplification, translocation, have been identified in the *ESR1* locus. More studies of tumors in the advanced disease setting are needed to generate additional markers and therapeutic targets. Obviously the development of predictors of endocrine therapy is still a work in progress. In this chapter, we will provide an update on the current status of this topic.

Estrogen Receptor

The relationship between ER positivity and tumor responsiveness to endocrine therapy has been well established. In the meta-analysis of individual patient data from 20 randomized trials (n = 21,457) of about 5 years of tamoxifen versus not, the reduction of breast cancer recurrence by tamoxifen was limited to ER-positive disease (ER ≥ 10 fmol/mg by ligand-binding assay). The recurrence risk reduction was substantial (relative risk [RR] 0.67 [0.08]) even in marginally ER-positive disease (10–19 fmol/mg cytosol protein), although the proportional effect was slightly better at much higher ER (RR 0.52 [0.07] for ER ≥ 200 fmol/mg) [1]. Since there is a high degree of concordance between the contemporary immunohistochemistry (IHC) and ligand binding assays in determining ER positivity [19−21], these data justify the use of endocrine therapy in ER-positive breast cancers with 1 % or more cells staining of ER by IHC.

Subsequent studies in both adjuvant and neoadjuvant setting demonstrated a higher rate of endocrine responsiveness with increasing ER expression. In the neoadjuvant P024 trial, a randomized trial of letrozole versus tamoxifen for postmenopausal women with early stage ER-positive breast cancer, the investigators observed a linear relationship between the pretreatment tumor ER Allred score and the clinical response to either tamoxifen or letrozole [22]. ER Allred score, ranges from 0 to 8, is calculated as the sum of an intensity score (range, 1–3) and a frequency score (range, 0–5), which is widely used in clinical practice to provide semi-quantitative measures of ER [23]. Similar relationship between ER level and clinical response was observed in the IMPACT trial, the Immediate Preoperative Anastrozole, Tamoxifen, or Combined with Tamoxifen, when H score, calculated as the product of intensity of staining (0–3) and percentage of cells (0–100 %), was used to quantify ER expression [24]. In the adjuvant setting, patients receiving adjuvant tamoxifen therapy who had tumors with a higher Allred score had improved disease free survival (DFS) [19].

The importance of ER and PgR expression in predicting endocrine responsiveness led to a routine testing recommendation for all invasive breast cancers to guide therapeutic decisions. The original ligand binding assay (LBA), such as the dextran-coated charcoal assay (DCCA), involves the competitive binding of radiolabeled ligand ([125I]-estradiol) to ER, with results expressed as femtomoles of ER per mg of total cytosol protein [25]. ER positivity is defined as ≥ 10 fmol/mg cytosol protein. The LBAs are technically challenging and expensive, requiring the use of radioactive reagents and a relatively large amounts of fresh frozen tissue, and are insensitive in tissues with low tumor cellularity. Since the 1990s, IHC, which involves the use of specific antibodies to ER, became the assay of choice. IHC is less expensive, more sensitive, and can be performed on fixed tissues. Multiple studies demonstrated that IHC is at least comparable or better than LBAs to predict endocrine responsiveness [19, 26−28]. However, the accuracy of IHC is subjected to both pre-analytical (ie, ischemia time, specimen processing, fixative type and duration), analytical (ie, antibodies, reagents, and method), scoring and assay report-

ing variations (ie, cutoff point for ER positivity) [29]. To reduce assay variability, guideline recommendations for ER and PgR testing have been established by the joint American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) [30, 31].

Progesterone Receptor (PgR)

PgR is a well-established prognostic marker for ER-positive breast cancer. Low PgR expression level was associated with a higher risk of recurrence in patients enrolled in adjuvant trials of tamoxifen and aromatase inhibitors [1, 32, 33]. However, PgR expression level was not predictive of endocrine therapy responsiveness in ER-positive breast cancer. In the Early Breast Cancer Trialists Cooperative Group (EBCTCG) Overview analysis, patients with PgR-positive and patients with PgR-negative tumors showed similar benefit from tamoxifen [1]. Similar results were obtained in adjuvant trials of aromatase inhibitors, including the Arimidex, Tamoxifen, Alone or in Combination (ATAC) adjuvant breast cancer trial and Breast International Group (BIG) 1–98 trial, which demonstrated superiority of aromatase inhibitor to tamoxifen regardless of PgR status [32−34].

ER-negative PgR-positive breast cancers are rare, and the negative ER status in these tumors might be attributed to a false negative assay of ER. Indeed, as assays improve, fewer breast cancers have been reported as ER-negative PgR-positive (4 % in the early 1990s but only 1 % in recent years based on Surveillance, Epidemiology, and End Results (SEER) data) [1]. In the meta-analysis of tamoxifen trials, there appear to be some slight benefit from tamoxifen in this population [1]. Therefore PgR is performed routinely in all invasive breast cancers and endocrine therapy is recommended for patients with ER-negative PgR-positive breast cancer and PgR IHC be performed according to the ASCO/CAP guidelines [30, 31].

HER2/neu

About 10 % of ER-positive breast cancers have *HER2* gene amplification [35]. HER2 positivity has generally been accepted as a marker of endocrine resistance and tumor overexpression of HER2 is associated with poor prognosis [36]. In preclinical studies, HER2 overexpression was able to activate MAPK and down regulate ER, which promoted estrogen-independent growth [37, 38]. In the adjuvant setting, HER2 positivity was associated with reduced benefit to tamoxifen [39, 40]. In the neoadjuvant setting, suppression of tumor Ki-67 by either tamoxifen or letrozole was significantly less in these tumors than that in the ER-positive HER2-negative, suggesting therapeutic resistance [41]. In the BIG 1–98 trial, patients with HER2-positive breast cancer experienced worse DFS regardless of adjuvant treatment with tamoxifen or letrozole [42]. Interestingly, superior DFS was observed in patients

with letrozole compared to tamoxifen, suggesting aromatase inhibitors could be better choices than tamoxifen for ER-positive HER2-positive breast cancer [42]. Nonetheless, these cancers warrant treatment with anti- HER2 agents, which improves outcome in HER2-positive breast cancer regardless of ER status [43].

On-Treatment Ki-67 and PEPI Score

Ki-67 is a nuclear antigen that is present only in proliferating cells [44]. The Ki-67 labeling index—the percentage of cells with positive Ki-67 nuclear staining—correlates well with the S phase fraction and mitotic index [45]. In the neoadjuvant setting persistent Ki-67 elevation despite endocrine therapy (rather than the baseline Ki-67 value) identifies estrogen-independent proliferation that is clearly associated with increased risk of disease recurrence and death [10−12]. In the IMPACT trial, the 5-year recurrence-free survival rates were 85, 75, and 60 % for the lowest, middle, and highest values of 2-week Ki-67 expression, respectively [11]. In the P024 trial, Ki-67 levels at surgery following 4 months of endocrine therapy had a robust association with relapse-free survival, and breast cancer–specific survival [12]. A 10 % cut-off point for on-treatment Ki-67 effectively separated patients with sensitive versus intrinsically resistant disease in the neoadjuvant endocrine trials [46]. Preliminary data from the ACOSOG Z1031 "Cohort B" indicated that about 20 % of patients are in this aromatase inhibitor-resistant category and treatment decision making based on on-treatment Ki-67 is feasible [3].

Furthermore, the effectiveness on Ki-67 suppression with short term treatment in the neoadjuvant setting consistently predicts the success of endocrine therapy agents in large adjuvant trials [47]. For example, the more dramatic suppression of Ki-67 with anastrozole than with tamoxifen alone or tamoxifen in combination with anastrozole following 2 weeks of neoadjuvant treatment observed in the IMPACT trial [10] mirrored the superiority of anastrozole over tamoxifen in DFS in the adjuvant ATAC trial [48]. Similarly, data on Ki-67 suppression at surgery from the P024 trial [49] and the ACOSOG Z1031 trial (A randomized neoadjuvant trial of three aromatase inhibitors) [50] predicted the outcome of the BIG 1–98 trial [51] and MA.27 trial [52], respectively.

In addition to Ki-67, three other factors, including pathologic tumor size, lymph node, and ER status of the resected tumor following neoadjuvant endocrine therapy were independently prognostic for long term outcomes for patients treated in the P024 trial [12]. The PEPI was therefore developed as a prognostic tool to further distinguish endocrine sensitive versus resistant disease [12]. The PEPI score of 0 (pT1-2, N0, tumor Ki-67 $\leq$ 2.7 %, ER-positive) identified patents with very low risk of relapse with adjuvant endocrine therapy alone in the P024 and IMPACT trials [12]. These patients are at such low risk of recurrence that chemotherapy could be avoided.

The PEPI 0 rate in the neoadjuvant endocrine therapy trials conducted so far ranged from 17–37 % [12, 53]. The ongoing Alliance A011106 (ALTERNATE trial:

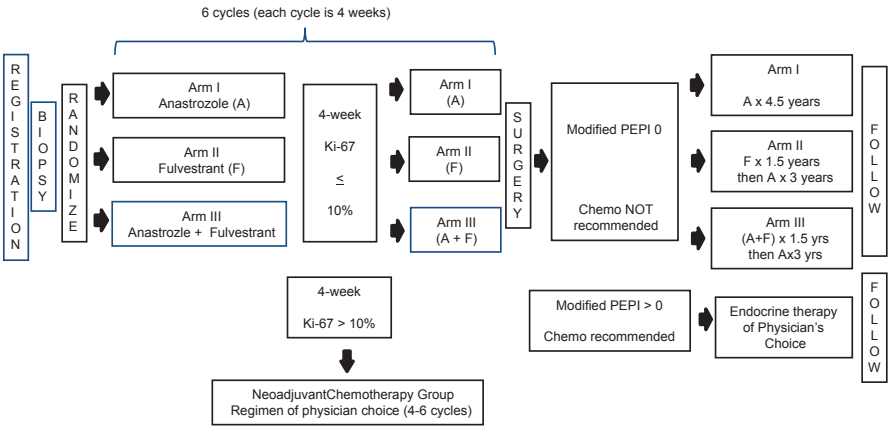

Fig. 1 *ALTERNATE Trial*. The first primary objective is to prospectively validate that modified PEPI 0 predicts > 95 % RFS. The second objective is to determine whether fulvestrant (*F*), or fulvestrant (*F*) plus anastrozole (*A*), is superior to A in inducing a higher rate of modified PEPI 0. Secondary objectives include assessing recurrence-free survival (*RFS*) for patients with endocrine resistant tumor, defined by Ki-67 > 10 % at 4 or 12 weeks, disease progression, or modified PEPI non-0, and pathologic response to neoadjuvant chemotherapy for resistant tumors. During the first phase of the trial, 1200 pts are randomized 1:1:1 to the F, A or F/A. This provides an 82 % chance, 1-sided alpha 0.025 chi-square test to detect at least 10 % difference in modified PEPI 0 rate comparing F or F/A with A. During the second phase, an additional 540 patients in each arm is estimated to obtain 317 patients with PEPI score 0. This will have a 90 % chance, with a one-sided alpha = 0.025 nonparametric Brookmeyer-Crowley type one sample survival test, rejecting that 5-year RFS rate is 95 %. The maximum sample size is 2820 pts

ALTernate approaches for clinical stage II or III Estrogen Receptor positive breast cancer NeoAdjuvant TrEatment in postmenopausal women: A Phase III Study, NCT01953588) (Fig. 1) is prospectively validating the hypothesis that PEPI 0 status is associated with a 5-year relapse risk of less than 5 % without the administration of chemotherapy. In this trial, patients with on-treatment Ki-67 above 10 % at 4 or 12 weeks are also triaged to chemotherapy or an investigational approach and genomic and proteomic investigations of these tumors are ongoing to identify driver events and predictors of endocrine resistance.

On-treatment Ki-67 and PEPI Score are promising approaches to differentiate endocrine sensitive versus resistant ER-positive breast cancer in the early stage setting. However, at present it remains an investigational approach pending results from the ALTERANTE trial. The significant variations in the current analytical practice of Ki-67 IHC could also limit its eventual clinical application. Comprehensive recommendations on pre-analytical and analytical assessment, and interpretation and scoring of Ki-67 have been put forward by the International Ki-67 in Breast Cancer Working Group [54]. Standard operating procedures that incorporate pathologist-guided digital imaging analysis and manual point-counting of selected cases has been developed that allows efficient and consistent scoring of Ki-67 needed in clinical trials, and demonstrated validity in predicting long term outcomes

[55, 56]. This standard operating procedure is currently applied for the central Ki-67 analysis in the ALTERNATE trial and several other neoadjuvant endocrine therapy studies. The result of the ALTERNATE trial is however needed to provide the clinical validity for on-treatment Ki-67 based biomarker approach including PEPI 0 score as a prognostic indicator of long term outcomes.

IHC4+C (4-Marker IHC Panel Plus Clinicopathologic Parameters)

The IHC4+C score is a prognostic tool based on quantitative values of four markers (IHC4: ER, PR, HER2 and Ki-67) and the clinicopathologic parameters (tumor grade, size, nodal burden, patient age, and treatment with aromatase inhibitor or tamoxifen) [57]. IHC4+C was developed in the retrospective analysis of TransATAC trial, in which IHC4+C score was significantly associated with distant recurrence at 9 years in postmenopausal women with node-negative, hormone receptor-positive disease treated with 5 years of adjuvant endocrine therapy [57]. Further validation was provided in an independent cohort of 786 women with early stage breast cancer in Nottingham from 1990 to 1998 [57]. IHC4+C showed similar degree of prognostic information compared to the Onco*type* DX recurrence score [57]. However, the clinical application of IHC4 is limited due to the lack of reproducibility of IHC assays. Although guidelines are available for standardized testing of ER, PR, and HER2 by ASCO/CAP [30, 31, 58], Ki-67 IHC remains a challenge before a uniform standard operating procedure is applied. Further research is needed on the analytical validity and clinical utility of IHC4+C score.

Gene-Expression Profiling

The introduction of genome-wide gene expression profiling technology has allowed the subclassification of ER-positive breast cancer into at least two “intrinsic” subtypes (Luminal subtype A and subtype B) based on unsupervised analysis [8, 9, 59]. The Luminal subtype A tumors demonstrate the highest expression of the ER and ER-associated genes. On the other hand, the Luminal subtype B tumors have low-to-moderate expression of luminal-specific genes but express some of the genes that are characteristic of ER-negative tumor, with more frequent occurrence of *TP53* mutation compared to Luminal subtype A tumors [8, 60]. Patients with Luminal subtype B tumors manifest significantly worse relapse-free and overall survival than those with Luminal subtype A ER-positive tumors [8, 9, 59]. These data suggest that the prognosis of ER-positive breast cancer is determined by multiple genes that work in concert with ER to regulate the response to estrogen and multi-gene assays are needed to predict endocrine therapy responsiveness.

Several RNA-based multigene expression assays including MammaPrint, Oncotype DX, Prosigna, EndoPredict, and BCI have been developed to estimate the individual risk of recurrence of patients with breast cancer (Table 1). Among these, Prosigna, EndoPredict, and BCI have also shown promise in predicting risk of late recurrence (more than 5 years after diagnosis and treatment). This is of particular clinical relevance as approximately half of all disease recurrences of ER-positive breast cancer occur after 5 years of adjuvant antiestrogen therapy.

MammaPrint™ The MammaPrint™ (Agendia, Amsterdam, the Netherlands) is the first commercialized microarray-based multigene assay for prognostic prediction in patients under age 61 with lymph node-negative breast cancer, regardless of ER status. The 70-gene signature predominantly comprises genes related to proliferation, with additional genes involved in invasion, metastasis, and angiogenesis [61, 62]. The test gives a dichotomized result, indicating either a high or low risk of disease recurrence. The signature was developed by supervised analysis of gene expression microarray data on frozen tumor tissues from young patients (age < 55 years) with tumor less than 5 cm, any ER or HER2, lymph node-negative disease [62]. The training set included 34 patients who developed distant metastasis within 5 years (mean time to metastasis was 2.5 years) and 44 patients who were disease free without systemic therapy for at least 5 years after diagnosis (mean follow up was 8.7 years) [62]. MammaPrint was initially validated as an independent predictor of distant recurrence using fresh-frozen tissues from a cohort of 295 young patients (age ≤ 52 years) in the Netherland Cancer Institute with primary invasive breast cancer that was less than 5 cm and lymph node-negative ($n = 151$) or positive ($n = 144$ N) [61]. Among the 295 patients, 180 had a poor prognosis signature and 115 had a good-prognosis signature, and the mean (± standard error) overall 10-year survival rates were 54.6 ± 4.4 and 94.5 ± 2.6 %, respectively. The profile performed best as a predictor of distant metastases during the first 5 years after treatment. This is not surprising as the signature was trained in patients with early recurrence [62]. This initial validation study was criticized by including 61 patients from the initial training set. In addition, 10 patients with node-negative disease and 120 patients with node-positive disease received adjuvant systemic therapy consisted of chemotherapy ($n = 90$), hormonal therapy ($n = 20$), or both ($n = 20$).

Further validation of MammaPrint was conducted using frozen tumor samples in an independent cohort of 307 women younger than 61 years old at diagnosis with T1–T2 (≤ 5 cm), ER-positive or ER-negative, lymph node-negative breast cancer who had not received adjuvant systemic therapy from five European centers (median follow-up of 13.6 years) [63]. The 70-gene signature outperformed the clinicopathologic risk assessment. For node-positive disease, a separate validation study of MammaPrint was conducted in 241 patients with T1-3, one to three node-positive breast cancer who did not receive adjuvant systemic therapy from two institutions [64]. Patients with MammaPrint™ good prognostic signature achieved 91 % distant metastasis-free survival (DMFS) and 96 % breast cancer specific survival (BCSS) at 10 years, while those with poor prognostic signatures had 76 % DMFS and 76 % BCSS [64]. The study concluded that the low risk group by MammaPrint could be

Table 1 Comparison of multi-gene assays

Test	Assay material	Assay type	FDA-cleared	Testing lab	# of genes	Risk category	Clinical parameter	Training set	Validation studies Node neg disease	Validation studies Node pos disease	Predicting relapse (0–10 years)	Predicting early relapse (0–5 years)	Predicting late relapse as separate indication (5–10 years)
MammaPrint	Frozen and FFPE	Microarray	Yes	Agendia	70	Low High	None	Young (<55 years) women, LN negative, no systemic therapy ($n = 117$) [62]	Consecutive patients from Netherland ($n = 295$, including 61 from the initial training set) (151 N0, 144 N+) age ≤ 52 years, chemo pts included [61]		Yes [63, 64]	Yes	No
									Retrospective cohort, T < 5 cm, no systemic therapy ($n = 307$) [63]	Retrospective cohort ($n = 241$) [64]			
Oncotype DX	FFPE	qRT-PCR	No	Genomic Health	21	Low Intermediate High Continuous RS	None	ER-positive breast cancer, including B20 tamoxifen alone ($n = 447$) [68]	NSABP B14 tamoxifen alone arm ($n = 668$) [68]	SWOG-8814 tamoxifen alone ($n = 148$) [69]	Yes [68–70]	Yes [69, 94]	No [69, 94]
									ATAC tamoxifen or anastrozole alone ($n = 872$) [70]	ATAC tamoxifen or anastrozole alone ($n = 306$) [70]			

Table 1 (continued)

Test	Assay material	Assay type	FDA-cleared	Testing lab	# of genes	Risk category	Clinical parameter	Training set	Validation studies Node neg disease	Validation studies Node pos disease	Predicting relapse (0–10 years)	Predicting early relapse (0–5 years)	Predicting late relapse as separate indication (5–10 years)
Prosigna	FFPE	nCounter	Yes	Distributed kits and local lab-based testing	50[a]	Low Intermediate High Continuous ROR and intrinsic subtype[b]	Tumor size T1 versus T2 + integrated into ROR	NKI data set, node negative, no systemic therapy (n = 141) [61, 75] and n = 786 in British Columbia) for ROR weighting for T size and proliferation [81]	Cohorts of node negative, no systemic therapy, mixed ER status (n = 710)		Yes [86, 87]	Yes [86, 87]	Yes ABCSG 8 (n = 1246) [87] and combined ATAC (n = 862) and ABCSG 8 hormonal therapy alone (n = 1275) [89]
									Tamoxifen treated node negative or positive breast cancer (n = 398 in MA12 [82]				
									ATAC tamoxifen or anastrozole alone (n = 739) [86]	ATAC tamoxifen or anastrozole alone (n = 268) [86]			
									ABCSG 8 tamoxifen or tamoxifen followed by anastrozole (n = 1047) [87]	ABCSG 8 tamoxifen or tamoxifen followed by anastrozole alone (n = 431)[87]			

Table 1 (continued)

Test	Assay material	Assay type	FDA-cleared	Testing lab	# of genes	Risk category	Clinical parameter	Training set	Validation studies Node neg disease	Validation studies Node pos disease	Predicting relapse (0–10 years)	Predicting early relapse (0–5 years)	Predicting late relapse as separate indication (5–10 years)
Endo-Predict Clin	FFPE	qRT-PCR	No	Central lab	11	Low High Continuous risk score	Nodal status, tumor size	ER-positive HER2-treated with adjuvant tamoxifen only ($n = 964$)	Combined ABCSG-6 ($n = 378$) and ABCSG-8 hormonal therapy alone ($n = 1324$) (1165 node negative and 537 node positive) [73]		Yes [73]	Yes [73, 74]	Yes Combine ABCSG 6 and ABCSG 8 hormonal therapy alone ($n = 1702$) [74]
Breast cancer index	FFPE	qRT-PCR	No	Central lab	7	Low High Continuous risk index	None	Microarray data set ($n = 410$) [124]	N0 ER-positive, tamoxifen treated cohort ($n = 317$ and $n = 358$) [93] ATAC trial hormonal therapy alone ($n = 665$) [94]	No	Yes [93, 94]	Yes [93, 94]	Yes [93, 94]

ER estrogen receptor, *FFPE* formalin-fixed paraffin-embedded, *HR* hazard ratio, *LN* Lymph node-negative, *ROR* risk of relapse

[a] 46 in the commercial assay

[b] Intrinsic subtype readout available in Europe not USA

spared from chemotherapy because of their excellent prognosis in the absence of systemic treatment.

In conjunction with Adjuvant! Online [65] the utility of the MammaPrint assay in outcome prediction in early stage breast cancer is being prospectively studied in the ongoing Microarray in Node-Negative and one to three node-positive Disease May Avoid Chemotherapy Trial (MINDACT) (Fig. 2) [66]. In this trial, women with node-negative breast cancer will undergo clinical risk assessment and the 70-gene signature. Patients with discordant clinical and genomic predictions are randomly assigned to receive or not receive adjuvant chemotherapy. This trial has completed accrual, and we await results to be presented.

MammaPrint has not been widely used in the United States due to the initial requirement of fresh frozen tissues which are not routinely available. The assay has recently been adapted for use with formalin-fixed paraffin-embedded (FFPE) tissue [67]. Using FFPE analyte, the MammaPrint assay demonstrated an overall equivalence of 91.5 % (95 % CI, 86.9–94.5 %) between the 211 independent matched FFPE and fresh tumor samples [67]. In this study, the precision was 97.3 %, and repeatability was 97.8 %, with highly reproducible results between replicate samples of the same tumor and between two laboratories (concordance, 96 %). However, the adapted FFPE MammaPrint assay has not been validated based on outcome studies.

Oncotype DX The Onco*type* DX assay is a quantitative reverse transcriptase polymerase chain reaction (RT-PCR)–based test that measures 21 genes (16 cancer-related genes and 5 reference genes) in FFPE breast tumors that classifies ER-positive breast cancer into three recurrence score (RS) risk categories, low (RS < 18), intermediate (RS 18–30), and high-risk (RS ≥ 31) [68]. The gene list was derived from an initial 250 candidate genes selected from the published literature and genomic databases and subsequent studies of their relationship with breast cancer recurrence [68]. The training set included three independent clinical studies of breast cancer involving a total of 447 patients, including the tamoxifen-only group of National Surgical Adjuvant Breast and Bowel Project (NSABP) trial B-20. The ability of Onco*type* DX RS to predict the likelihood of distant recurrence was validated in postmenopausal women with node-negative, ER-positive breast cancer treated with 5 years of adjuvant tamoxifen in NSABP B-14 trial [68]. The proportions of patients categorized as having a low, intermediate, or high-risk were 51, 22, and 27 % and 10-year distant recurrence rates of 6.8, 14.3, and 30.5 %, respectively, independent of age and tumor size but not grade [68]. RS was also found to be highly prognostic for DFS in the node-positive ER-positive breast cancer treated with tamoxifen alone in the SWOG 8814, although the risk remains high even in the low RS category [69]. The 10-year DFS estimates were 60, 49, and 43 % for low, intermediate, and high-risk categories, respectively [69]. The prognostic properties of the Oncotype DX assay is most robust for the first 5 years [69].

Data from the ATAC trial further confirmed the performance of Onco*type* DX in postmenopausal women with hormone receptor-positive breast cancer treated with 5 years of tamoxifen or anastrozole [70]. The 9-year distant recurrence rates in low, intermediate, and high RS categories were 4, 12, and 25 %, respectively, in N0

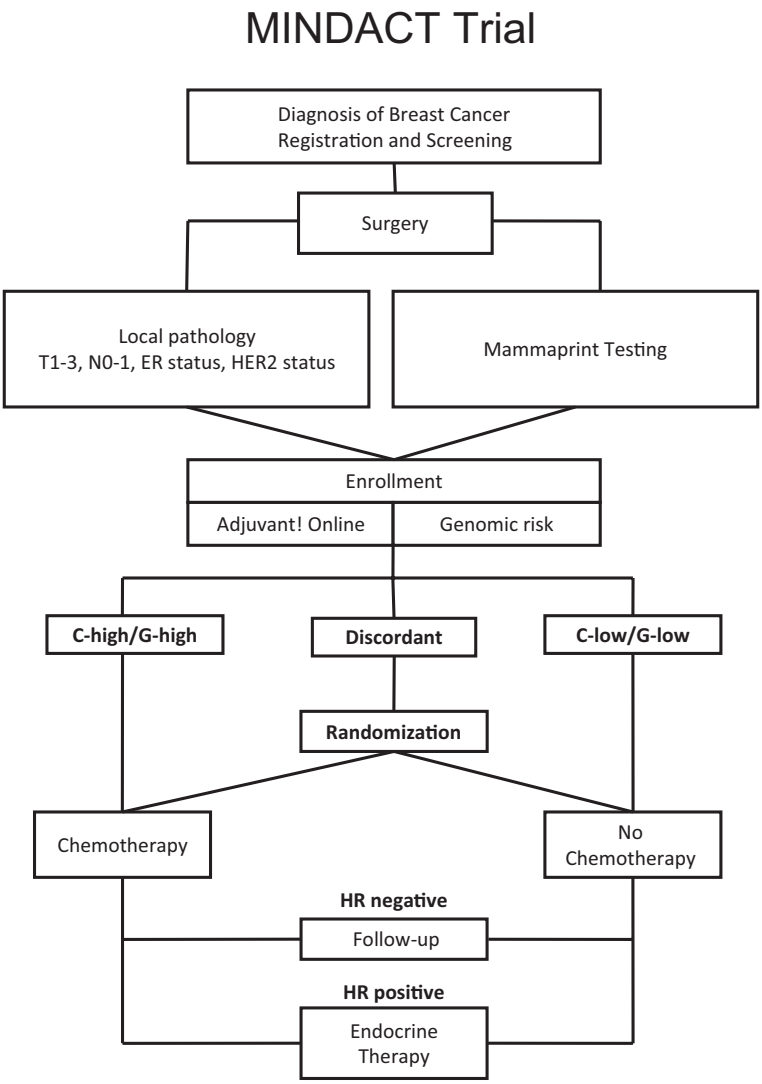

Fig. 2 *MINDACT Trial*. Patients with early stage breast cancer (T1-3, N0-1, ER-positive, or ER-negative, HER2-positive or HER2-negative) will enroll to the study after surgery and pathology and MammaPrint Testing. C-low (low clinical risk) is defined as having a 10-year breast cancer–specific survival rate of greater than 92 % for ER-negative disease or greater than 88 % for ER-positive disease by Adjuvant! Online. C-high (high clinical risk) is defined as having a 10-year breast cancer–specific survival rate of less than 92 % for ER– disease or less than 88 % for ER-positive disease by Adjuvant! Online. G-high (high genomic risk) is determined based on high-risk by the MammaPrint result. G-low (low genomic risk) is determined based on low risk by the MammaPrint result

population and 17, 28, and 49 %, respectively, in women with node-positive breast cancer [70]. The relative risk reduction for anastrozole compared to tamoxifen was similar across different values of the RS.

RS was evaluated to predict clinical benefits from chemotherapy. In the NSABP B-20 trial, which randomized patients with ER-positive, node-negative breast cancer to either tamoxifen or CMF/MF (cyclophosphamide, methotrexate, 5-fluorouracil) chemotherapy, patients with high RS were found to benefit the most from chemotherapy (NSABP B-20 trial) [71]. In the low RS category, there was no difference in 10-year distant relapse free survival (DRFS) between patients treated with tamoxifen alone (DRFS: 96.8 %, $n = 135$) or tamoxifen plus chemotherapy (DRFS: 95.6 %, $n = 218$), while in the high RS category, addition of chemotherapy improved the 10-year DRFS from 60.5 % (95 % CI 46.2–74.8 %, $n = 47$) with tamoxifen alone to 88.1 (95 % CI 82–94.2 %, $n = 117$) ($p < 0.001$). The effect of chemotherapy in the intermediate RS category was less clear, with the 10-year DRSF of 90.9 % ($n = 45$) in the tamoxifen alone arm compared to 89.1 % ($n = 89$) in the combination arm. The study has been criticized by the fact that the NSABP-B20 tamoxifen alone arm was included in the initial training set for the development of the 21-gene assay and the RS [68], therefore potentially confounding the interpretation of the data. In the node-positive ER-positive breast cancer treated with tamoxifen in the SWOG-8814 trial, the benefit of CAF (cyclophosphamide, doxorubicin, and 5-fluorouracil) was observed in the high, not low, RS category [69]. However, the risk of distant recurrence at 10 years in patients with high RS remains high despite chemotherapy, 11.9 % with node-negative disease (NSABP B-20 trial), and 32 % with node-positive disease (SWOG-8841 trial), arguing the need for better treatment approaches in this patient population.

The use of Onco*type* DX to tailor chemotherapy decisions in patients with ER-positive HER2- breast cancer is being prospectively evaluated in the Trial Assigning Individualized Options for Treatment (Rx), or TAILORx trial (Fig. 3a) for node-negative patients [72] and the clinical trial Rx for Positive Node, Endocrine Responsive breast cancer (RxPONDER) (Fig. 3b) in patients with 1–3 positive lymph nodes involvement. However, the primary objective of the TAILORx trial is to determine whether patients in the RS 11–25 group have non-inferior DFS with hormonal therapy than with chemotherapy plus hormonal therapy. The study does not prospectively evaluate chemotherapy benefit in patients with RS < 11 (Arm A) or RS > 25 (Arm D), who are assigned to hormonal therapy alone (Arm A), or chemotherapy plus hormonal therapy (Arm D). The primary objective of the RxPONDER trial is to determine chemotherapy benefit (if exist) depends on the RS score in the RS ≤ 25 group treated with endocrine therapy. Similarly to TAILORx trial, patients with RS > 25 are assigned to chemotherapy plus endocrine therapy, with the assumption that chemotherapy is needed in this population. TAILORx has completed enrollment, and accrual to the RxPONDER trial is ongoing.

EndoPredict EndoPredict (EP) is a quantitative RT-PCR based assay of eight cancer-related and three reference genes in FFPE tumor tissues to calculate a risk score that classifies ER-positive, HER2-negative breast cancer into low and high-risk categories

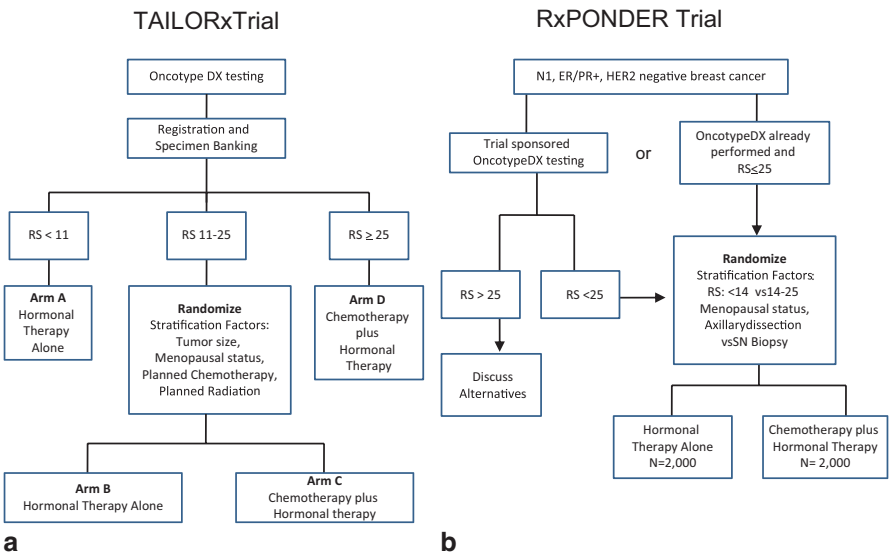

Fig. 3 **a***TAILORx Trial*. The primary endpoint of TAILORx trial is disease-free survival (*DFS*) in patients in the "Primary Study Group (RS 11–25)" ($n = 6860$). Patients in this group are randomly assigned to receive hormonal therapy (Arm B) or chemotherapy and hormonal therapy (Arm C). The study uses a non-inferiority design and was powered to determine whether hormonal therapy is not inferior to chemotherapy plus hormonal therapy in patients in this risk group. A decrease in the 5-year DFS rate from 90 % with chemotherapy to 87.0 % or lower on hormonal therapy alone would be considered unacceptable. A secondary objective is to validate whether patients with RS < 11 (Arm A: all patients are assigned to hormonal therapy alone) have failure rates that are low enough that adjuvant chemotherapy is unlikely to be of much absolute benefit. Patients in the high RS category (RS > 25) are assigned to chemotherapy plus hormonal therapy (Arm D), with the assumption that chemotherapy is needed in this patient population. Other secondary objectives include comparison of outcomes projected by Adjuvant! and to develop more precise estimates of the relationships between RS and chemotherapy treatment effect, if any, at the upper range of the RS 11-25 group. **b***RxPonder Trial*. A phase III randomized clinical trial of standard adjuvant Endocrine therapy + /− chemotherapy in patients with one to three positive nodes, Hormone receptor-positive and HER2-negative breast cancer with recurrence score (*RS*) of 25 or less. The trial plans to screen 9400 women in order to enroll 4000 women with an RS of 25 or less. The primary objective of this trial is to determine the effect of chemotherapy in patients with one to three positive nodes, and hormone receptor (*HR*)-positive, HER2-negative breast cancer with RS ≤ 25 treated with endocrine therapy, whether the difference in disease-free survival (*DFS*) for patients treated with chemotherapy compared to no chemotherapy depends directly on the magnitude of RS. If benefit depends on the RS score, the trial will determine the optimal cutpoint for recommending chemotherapy or not. Secondary objectives include comparison of Onco*type* DX and PAM50 risk of relapse (ROR) scores and measurement of quality-of-life effects

[73]. The EP risk score and its combination with the clinical risk factors, tumor size and nodal status (EPclin), were generated in a large training set of 964 ER-positive, HER2-negative breast cancers from patients treated with adjuvant tamoxifen only. EndoPredict risk scores and cutoff values were validated in two independent external validation cohorts of 1702 patients enrolled in two large randomized Austrian Breast and Colorec-

tal Cancer Study Group (ABCSG) phase III trials [ABCSG-6: *n* = 378, ABCSG-8: *n* = 1324] [73]. EPclin low-risk patients had a 10-year distant recurrence risk of 4 % and EPclin high-risk patients had a 10-year distant recurrence risk of 28 % (ABCSG-6) and 22 % (ABCSG-8) with adjuvant endocrine therapy alone. The EPclin score stratified 64 % of patients at risk after 5 years into a low-risk subgroup with an absolute 1.8 % risk of late distant recurrence at 10 years of follow-up [74]. This is comparable to the PAM50 risk of recurrence (ROR) score for the low risk group in the ABCSG-08 cohort (see below in the section of Prosigna) .

Prosigna Prosigna™ Breast Cancer Gene Signature Assay (NanoString Technologies, Seattle) was developed based on the Prediction Analysis of Microarray (PAM)50 model. It is a FDA 510(k) cleared assay for the assessment of 10-year risk of distant recurrence for postmenopausal women with early stage, hormone receptor-positive, invasive breast cancer using FFPE tumor tissues. It provides a risk category (low, intermediate, high) and a numerical ROR score (0–100). Intrinsic subtype assignment is also reported in countries outside of United States. In contrast to other multi-gene assays, Prosigna is approved for decentralized testing in qualified laboratories by using the nCounter® Dx Analysis System and assay kits from NanoString Technologies, Inc.

The original PAM50 identifies a minimum set of 50 genes with the ability to assign individual breast cancers into intrinsic subtypes, including Luminal A, Luminal B, HER2-enriched, and Basal like [75]. The training set for the PAM50 subtype prediction consisted of 189 breast tumor samples (114 ER-positive and 77 ER-) and 29 normal samples from heterogeneously treated patients [75]. The initial PAM50 ROR models, and cut points for low, intermediate, and high-risk categories, for prognosis were trained in untreated patients with node-negative disease from the cohort of the Netherlands Cancer Institute (NKI, *n* = 141) [61, 75]. The subtype prediction and ROR models were then independently tested for prognosis using data from 761 patients (710 node-negative, 35 node-positive) who received no systemic therapy [61, 76–79] and for chemotherapy response using a separate data set consisted of 133 patients treated with neoadjuvant T/FAC (paclitaxel followed by 5-fluorouracil, doxorubicin, cyclophosphamide) [80]. Of the 626 ER-positive samples, 73 % were Luminal (A or B), 11 % were HER2-enriched, 5 % were Basal-like, and 12 % were normal-like, demonstrating a significant molecular heterogeneity of ER-positive disease. The intrinsic subtypes showed prognostic significance in multivariable analyses that incorporated standard clinical and pathologic parameters including ER status, histologic grade, tumor size, and node status. The ROR score by weighting the expression profile of the 50 genes and pathologic tumor size provided superior prognostic information than clinical factors or subtype model alone [75]. The final model, which incorporates a clinical factor (T size) and a weighting for an index based on the expression of genes associated with proliferation was developed using an qPCR analysis of samples in from women diagnosed in British Columbia between 1986 and 1992 who received 5 years of tamoxifen [81]. Application of the qPCR ROR model to-negative or node-positive tamoxifen-treated premenopausal women enrolled in the NCIC MA.12 trial to ROR score was more prognostic than clinical parameters [82]. Patients with Luminal A tumors likely benefited the most

adjuvant tamoxifen therapy in MA.12 [82]. Furthermore, the intrinsic subtype and ROR score accurately identified patients with tumors with non-responding tumors to neoadjuvant T/FAC [75].

To develop a simplified workflow and an assay which could be performed in local pathology laboratories, Prosigna™ Breast Cancer Gene Signature Assay, the PAM50 was adapted to use the nCounter Analysis System [83], which measures gene expression by multiplexed gene-specific fluorescently-labeled probe pairs, without the PCR amplification step [84, 85]. The analytical performance of NanoString Prosigna test was validated using FFPE breast specimens across multiple clinical testing laboratories [83]. The measured standard deviation (SD) was less than one ROR unit within the analytical precision study and 2.9 ROR units within the reproducibility study [83].

The clinical utility of Prosigna as an independent prognostic model was further validated in both ATAC trial [86] and ABCSG-8 trial [87], which provided level 1b evidence for its clinical application [88]. In the ATAC trial of 1007 patients with ER-positive breast cancer treated with either anastrozole or tamoxifen, ROR (with or without weighing tumor size) provided more prognostic information for distant relapse beyond the clinical treatment score (nodal status, tumor size, histopathologic grade, age, and anastrozole or tamoxifen treatment). ROR (with or without weighing tumor size), provided significantly more information than Onco*type* RS in the overall population and in all four subgroups: node-negative, node-positive, HER2-negative, and HER2-negative/node-negative [87]. ROR provided better differentiation of intermediate- and higher-risk groups than Onco*type* RS; more patients were classified as high-risk and fewer as intermediate risk by ROR than by RS, therefore reduced the proportion of patients for whom chemotherapy benefit is uncertain.

In the ABCSG-8 trial, ROR score added significantly more prognostic information to the clinical predictors (nodal status, tumor grade, tumor size, age, trial treatment) in the set of 1478 postmenopausal women with ER-positive early breast cancer treated with tamoxifen for 5 or 2 years of tamoxifen followed by 3 years of anastrozole who did not receive adjuvant chemotherapy [87]. Among node-negative patients, ROR assigned 47 % to the low-risk group, 32 % to the intermediate risk group, and 21 % to the high-risk group. The 10-year metastasis risk was < 3.5 % in the ROR low risk population.

Importantly, ROR score provided significant additional prognostic information with respect to late distant recurrence free survival beyond 5 years after diagnosis and treatment in the analysis of 1246 patients enrolled in the ABCSG-8 trial [89]. Between years 5 and 15, the absolute risk of distant recurrence was 2.4 % in the low ROR risk group, as compared with 17.5 % in the high ROR risk group. A combined analysis of 2137 patients who did not have a recurrence 5 years after diagnosis from the ATAC and ABCSG-8 trials was recently performed to correlate ROR score with risk of late recurrence at 5–10 years, in which ROR score was significantly prognostic [90]. In the node-negative, HER2–negative subgroup, more prognostic value for late distant recurrence was added by the ROR score compared with the clinical treatment score [90]. These data suggests a potential role of Prosigna as a

tool to assess the need for additional years of endocrine therapy upon completion of 5 years of treatment.

Breast cancer index (BCI) BCI is a RT-PCR based gene expression assay of seven genes analyzed within two biomarkers—the HOXB13 to IL17BR (H:I) ratio and the molecular grade index (MGI)—along with four reference genes, which provide a dichotomous index to classify patients into high versus low risk groups [91]. The test was developed using a cohort of tamoxifen-treated patients from the randomized prospective Stockholm trial of adjuvant tamoxifen and has been shown to significantly predict 0- to 10-year risk of recurrence beyond standard clinicopathologic factors [91, 92]. The BCI model was validated by retrospective analyses of tumor samples from tamoxifen-treated patients from a randomized prospective trial (Stockholm TAM, $n = 317$) and a multi-institutional cohort ($n = 358$) [93]. Within the Stockholm TAM cohort, BCI risk groups stratified the majority (~ 65 %) of patients as low risk with less than 3 % distant recurrence rate for 0–5 years and 5–10 years. In the multi-institutional cohort, which had larger tumors, 55 % of patients were classified as BCI low risk with less than 5 % distant recurrence rate for 0–5 years and 5–10 years. For both cohorts, continuous BCI was the most significant prognostic factor beyond standard clinicopathologic factors for 0–5 years and more than 5 years.

The prognostic ability of the BCI assay, Onco*type* Dx RS, and IHC4 for both early (0–5 years) and late recurrence (5–10 years) was compared in patients with ER-positive, node-negative (N0) disease from the ATAC trial, all assays had significant prognostic ability for early distant recurrence (BCI-L Hazard Ratio [HR] 2.77 [95 % CI 1.63–4.70]; 21-gene RS HR 1.80 [1.42–2.29], $p < 0.0001$; IHC4 HR 2.90 [2.01–4.18], $p < 0.0001$); however, only BCI-L was significant for late distant recurrence (BCI-L HR 1.95 [95 % CI 1.22–3.14], $p = 0.0048$; 21-gene recurrence score HR 1.13 [0.82–1.56], $p = 0.47$; IHC4 HR 1.30 [0.88–1.94], $p = 0.20$). These data indicates that BCI could help to identify patients at high-risk for late distant recurrence who might benefit from extended endocrine or other therapy [94].

Somatic Mutations

- **SMGs in Luminal breast cancer**
  As cancer is largely a disorder of the genome, mutation patterns are rational candidates as predictors of endocrine sensitivity in ER-positive breast cancer. In the last several years, next generation sequencing technologies have allowed in-depth study of somatic mutations in over 1000 breast cancer samples. A striking difference in mutation spectrums exists for the four intrinsic subtypes of breast cancer [60]. Luminal type breast cancers harbored the most diverse and recurrent significantly mutated genes (SMG: genes with mutations occurring more frequent than background mutation rate), despite a lower mutation rate overall compared to the Basal-like and HER2-enriched subtype, suggesting a causative role of these genes in Luminal breast cancers [60].

The most frequent mutation observed in Luminal A breast cancer is PI3K catalytic subunit-α (*PIK3CA*) (45 %) and mutations including *MAP3K1, GATA3, CDH1* and *MAP2K4* which occur almost exclusively in Luminal A type breast cancer. A low mutation rate in *TP53* (12 %) was observed, which is uncommon for epithelial cancers. Approximately 12 % of Luminal A breast cancers carried mutations in either *MAP3K1* or *MAP2K4* in a mutually exclusive manner. Luminal B type breast cancer had a higher rate of mutations in *TP53* and a slight lower rate of mutations in *PIK3CA* (29 % each). Some of these mutations have been associated with endocrine responsiveness, but many are not.

- **Mutations in *TP53*, *MAP3K1* and *GATA3***. To uncover relationships between somatic mutation patterns and the effectiveness of aromatase inhibitor treatment of primary breast cancer massively parallel sequencing of 77 pre-treatment tumor biopsies from patients treated with neoadjuvant aromatase inhibitor therapy was conducted [13]. The clinical significance of three of the highest frequency SMG, *TP53*, *MAP3K1* and *GATA3*, were assessed to correlate with prognosis and treatment response by Ki-67 (Fig. 4). *TP53* was correlated with the poor

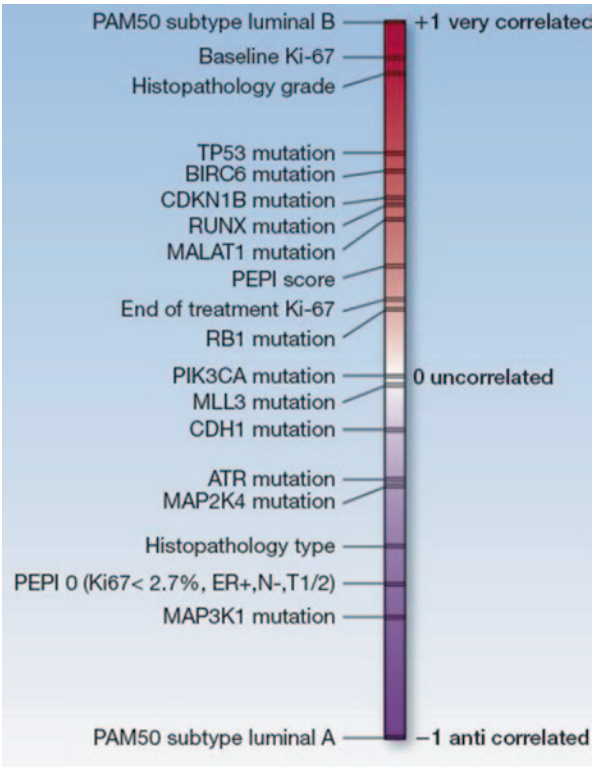

Fig. 4 DiPSC plot (dipstick) illustrates correlations between mutations, biomarkers, and subtypes. (Adapted from Goldstein et al. [101]. Correlation of mutations with Luminal subtype, Ki-67 and PEPI score)

prognosis Luminal B signature and high Ki-67 levels before and after treatment, whereas mutations in *MAP3K1* associated with the Luminal A subtype and low levels of Ki-67 throughout the treatment course [13]. Interestingly *GATA3* mutation correlated with greater Ki-67 suppression after aromatase inhibitor treatment but not baseline Ki-67 levels, suggesting that *GATA3* mutation is a predictive marker for endocrine therapy response [13]. Further verification of these results will require analysis of samples with long-term follow up to confirm the hypotheses that patients carrying tumors with *MAP3K1* or *GATA3* mutations will have a favorable outcome and *TP53* mutation worse outcomes.

- **PIK3CA mutation** Mutations in *PIK3CA*, the alpha catalytic subunit of PI3K, is the most common genetic event in ER-positive breast cancer, occurring at a frequency of 45 and 29 % in Luminal A and B, respectively, which presents an attractive therapeutic target [60]. Up to 80 % of *PIK3CA* mutations are missense mutations clustered in the helical domain (HD) and the kinase domain (KD) and have been shown to be activating mutations [95]. Several studies reported that *PIK3CA* mutation was associated with better prognosis [96–99]. However, *PIK3CA* mutation has not been associated with clinical or Ki-67 response to endocrine treatment in neoadjuvant studies [100].
- ***BIRC6, CDKN1B, RUNX1* and the long non-coding RNA *MALAT1*** Using an informatics tool to dissect pathway activation events (PARADIGM), *MAP2K4* mutations were found to be potentially associated with favorable tumor features (Luminal A, low PEPI scores), which is logical since *MAP2K4* is a substrate for *MAP3K1*. In addition to *TP53, BIRC6, CDKN1B, RUNX1* and the long non-coding RNA *MALAT1* were connected to high Ki-67 values and Luminal B status through pathway informatics (Fig. 4) [101].
- ***ESR1* point mutation, translocation and amplification and alterations in ER pathway genes**
  ESR1 point mutation. Estrogen receptor 1 (*ESR1*; which encodes ERα) mutation in the C terminal ligand binding domain which renders ligand-independent activation of ER is an acquired aromatase inhibitor resistance mechanism. *ESR1* mutation is identified at a frequency of 11–55 % in ER-positive breast cancers progressed on endocrine therapy [14–18], in contrast to the rare occurrence in treatment-naïve primary breast cancers [60, 102, 103]. Most patients with tumors harboring *ESR1* mutations experienced a protracted clinical course prior to sample collection for sequencing and the *ESR1* mutations were absent in the matched primary tumors at diagnosis, supporting the id*ea* that *ESR1* mutation is largely an acquired resistance mechanism that emerges after long-term treatment with endocrine therapy. The mutations cluster in the ligand-binding domain (LBD) with Y537S, C or N and D538G being the most common. These mutations confer ligand (estrogen)-independent target gene activation and cell proliferation in preclinical studies [14–17]. Structural modeling of the mutant ERs demonstrate a constitutive agonist conformation through the formation of hydrogen bonds between S537 or G538 and N351 in helix 12 [14]. In preclinical studies, treatment with tamoxifen and fulvestrant was effective, but higher drug concentrations were required.

ESR1 translocation. An additional recently uncovered genetic endocrine therapy resistance mechanism is *ESR1* chromosomal translocation. Several in-frame fusion genes preserving the first 6–7 exons of *ESR1* (e6 or e7), including the DNA binding domain and hinge region, spliced in-frame into the C-terminus of another gene have been identified to date. Examples include Yes-associated protein 1 (*ESR1-e6>YAP1*), DNA polymerase η (*ESR1-e7>POLH*), and A kinase anchor protein 12 (*ESR1-e6>AKAP12*) [16, 104]. The *ESR1-e6>YAP1* fusion protein is best documented since this fusion gene was identified in an endocrine therapy-resistant patient-derived xenograft (PDX) model derived from the breast tumor of a patient presenting with primary endocrine resistant stage IV breast cancer. In transfection studies *ESR1-e6>YAP1* induced strong hormone independent growth and activation of classic estradiol regulated genes (TFF1 and PgR) [16]. Thus, the YAP1 sequences effectively mimic the ligand activated transactivation domain in the C-terminus of *ESR1*.

Another class of translocation involving the *ESR1* gene are localized gene rearrangements on chromosome 6 between *ESR1* and the "coiled-coil domain containing 170" (*CCDC170*) gene. *CCDC170* resides immediately centromeric to *ESR1*. These fusions join the 5'-untranslated region of *ESR1* to the coding region of *CCDC170* generating the overexpression of amino-terminally truncated ΔCCDC170 proteins. These gene fusion events were found in 8 of 200 primary ER-positive breast cancers and was enriched in more aggressive Luminal B tumors [105]. ΔCCDC170 may engage the GRB2-associated binding protein 1 (GAB1) signalosome to potentiate growth factor signaling and reduce endocrine sensitivity [105].

ESR1 amplification. Amplification of *ESR1* has also been reported as an acquired aromatase inhibitor resistance mechanism. For example, an *ESR1* amplification event was identified in a PDX model and in the corresponding human ER-positive cancer progressed on aromatase inhibitor therapy [16]. The amplicon in this study extended across both the promoter and coding regions of *ESR1* and was associated with high levels of ER expression. Similar to the clinical response in the patient who provided the tumor specimen, treatment of the PDX with estradiol paradoxically induced tumor regression rather than tumor growth [106] While *ESR1* amplification is likely an adaptation to estrogen deprivation, and high level amplification was also detected in MCF7 cells after long-term endocrine therapy, the mechanism of estradiol induced regression remains under investigation. Interestingly estradiol induced apoptosis can be blocked with a SRC inhibitor in experimental models with restoration of estradiol-induced growth [107]. The hypothesis provoked by this observation that a SRC inhibitor could be used to "restore" endocrine therapy sensitivity is supported by a Phase 2 clinical trial of the SRC inhibitor dasatinib [108]. The prevalence of *ESR1* amplification in breast cancer has been a subject of controversy, reported from rare to over 20 % [109−119]. The variability of the data could be a result of non-standardized detection methodologies, which have inconsistent sensitivity, specificity and cut-off-point issues that remain unresolved [115, 120].

Overall, *ESR1* alteration, whether point mutation, translocation or amplification, lead to driver roles in acquired resistance to aromatase inhibitor treatment, less commonly intrinsic resistance, which could serve as predictors of endocrine therapy response. In addition, genetic alteration of ER co-regulators could render endocrine therapy resistance, which remains to be confirmed as these events are uncommon.

- **Other genetic or epigenetic alterations**
  Theoretically, genetic or epigenetic alterations in genes that could lead to deregulated growth factor receptor signaling, PI3K, MAPK pathway activation, cell cycle progression, resistant to apoptosis and senescence could contribute to endocrine resistance. For example amplification or overexpression of cyclin D, or Myc, or deletion of PTEN or negative regulators of cell cycle machinery could lead to uncontrolled tumor growth that is independent of estrogen [121−123]. However, further evaluation of these candidate genes are needed to confirm their prognostic and predictive role in ER-positive breast cancer and endocrine responsiveness.

In general the genomic studies discussed above support the hypothesis that aromatase inhibitor resistance is encoded by the mutation patterns present in individual tumor genomes, but detailed and validated information has yet to emerge. The mutational map developed from the neoadjuvant aromatase inhibitor studies emphasizes the genomic heterogeneity that underlies the clinical heterogeneity of the disease. The next phase of this research is to screen a much larger number of samples to determine the 'endocrine phenotype' of recurrent somatic mutations in Luminal-type breast cancer and understand how their interactions drive prognosis, patterns of metastasis and drug response.

Conclusion

Progress has been made in recent years with the introduction of several RNA-based multi-gene assays to risk stratify patients when treated with adjuvant endocrine therapy. These assays have shown clinical utility in identify patients with sufficiently low risk ER-positive disease on endocrine therapy alone that chemotherapy could be avoided. In addition, several assays have shown promise in predicting late recurrence for selection of patients for extended endocrine therapy. However, there is uncertainty regarding chemotherapy decision making in patients classified in the intermediate risk category. In addition, although chemotherapy is likely beneficial in the high-risk category by these assay, these patients remain at high-risk for recurrence despite adjuvant chemotherapy. There is a significant unmet clinical need to understand the biology of these tumors for novel the development of nrw therapeutic approaches. Genomic sequencing studies have shed light on the potential association of somatic mutations as resistant or sensitive mechanisms of endocrine therapy. These studies have the potential to improve precision in the prediction of

endocrine sensitivity and also provide therapeutic hypothesis for the design of investigational agents. The neoadjuvant setting provides a platform for clinical identification of endocrine resistant tumors and for genomic discoveries, while molecular analysis of recurrent tumors are required to uncover biomarkers related to acquired endocrine resistance. Large scale studies in both settings are in progress. We envision the eventual development of assays that integrate the current multi-gene assays with mutational or genomic profiles that allow a more complete understanding of the key molecular drivers of outcome in ER-positive breast cancer.

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Biomarkers for Predicting Response to Anti-HER2 Agents

Vinay Varadan, Maria Sandoval and Lyndsay N. Harris

Abstract The HER2 receptor is amplified or overexpressed in approximately 20% of all breast cancers, but despite significant efforts of the clinical research community and a growing number of anti-HER2 agents, a significant number of patients with HER2-positive breast cancer either progress or suffer disease relapse within 5–10 years. The development of robust biomarkers that predict response to anti-HER2 agents is therefore an important clinical need to prevent overtreatment and to enable earlier assignment of patients to more optimal therapies. Here we review some of the recent advances in the field by focusing on pathways mediating resistance to anti-HER2 therapies, and the role of the immune system and cancer stem cells in therapy response. We also review preoperative treatment strategies and research paradigms that show promise in identifying novel biomarkers of response while also enabling the delineation of the mechanisms underlying clinical benefit from anti-HER2 therapies.

Keywords HER2 · Targeted therapy · Preoperative therapy · Genomics · Proteomics · Cancer stem cells · ErbB2 · Immune system

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Introduction

The HER2 receptor is amplified or overexpressed in approximately 20% of all breast cancers [1, 2]. Early stage HER2 driven cancers are rapidly proliferative and prone to metastatic spread, with a worse outcome in the absence of targeted therapy [1, 3]. Trastuzumab, a monoclonal antibody that binds to the extracellular domain (Subdomain IV) of HER2, was evaluated in more than 13,000 women enrolled in five prospective adjuvant Phase III trials with combination systemic chemotherapy and trastuzumab and showed a reduction in recurrence rate and improvement in overall survival [4]. As a result, systemic chemotherapy plus trastuzumab, is the standard of care for early-stage breast cancer. However, strikingly, when a 10 year follow up of the North Central Cancer Treatment Group (NCCTG)/N831 and National Surgical Adjuvant Breast and Bowel Project (NSABP) combined trials was recently reported, up to 23% of women with early-stage HER2-positive disease relapse within 5–10 years [5]. Genomic profiling of HER2-positive breast cancer has shown that these tumors are clinically and biologically heterogeneous. Additional HER2-targeted therapies (lapatinib, pertuzumab, and T-DM1) have been developed in order to improve outcome and have been evaluated in combination with or after trastuzumab in the preoperative, adjuvant and metastatic settings [6]. The increasing number of anti-HER2 therapy options (Fig. 1) and the innate heterogeneity of HER2-positive breast cancer points to the essential need for the discovery and translation of biomarkers that predict a patient's response to anti-HER2 therapy to improve outcome and limit toxicity in HER2-positive breast cancer.

In this chapter, we review the current state-of-the-art in the quest for predictive biomarkers for anti-HER2 therapy, ranging from preclinical efforts to studies involving tumor and germline samples derived from patients in clinical trials.

Pathways Mediating Resistance to Anti-HER2 Therapy

Substantial research has been performed to interrogate pathways that are responsible for HER2 signaling [7–13]. These findings have significant implications for mechanism-of-action and therapeutic resistance of anti-HER2 therapy (Fig. 1). Down regulation of PI3K/AKT pathway activity has been proposed as one of the mechanisms of action of trastuzumab [14]. Pre-clinical models suggest that trastuzumab represses PI3K/AKT pathway activity through down-regulation of HER2 signaling [15] or by PTEN activation [16]. Furthermore, analysis of The Cancer Genome Atlas (TCGA) breast cancer dataset shows that genes in the PI3K/AKT axis (eg. *PIK3R1, PIK3CA, PTEN* and *AKT1)* are significantly mutated in some HER2-positive tumors [17]. Preclinical and clinical studies have been performed in an effort to elucidate the role of the PI3K/AKT axis and resistance to anti-HER2 therapies.

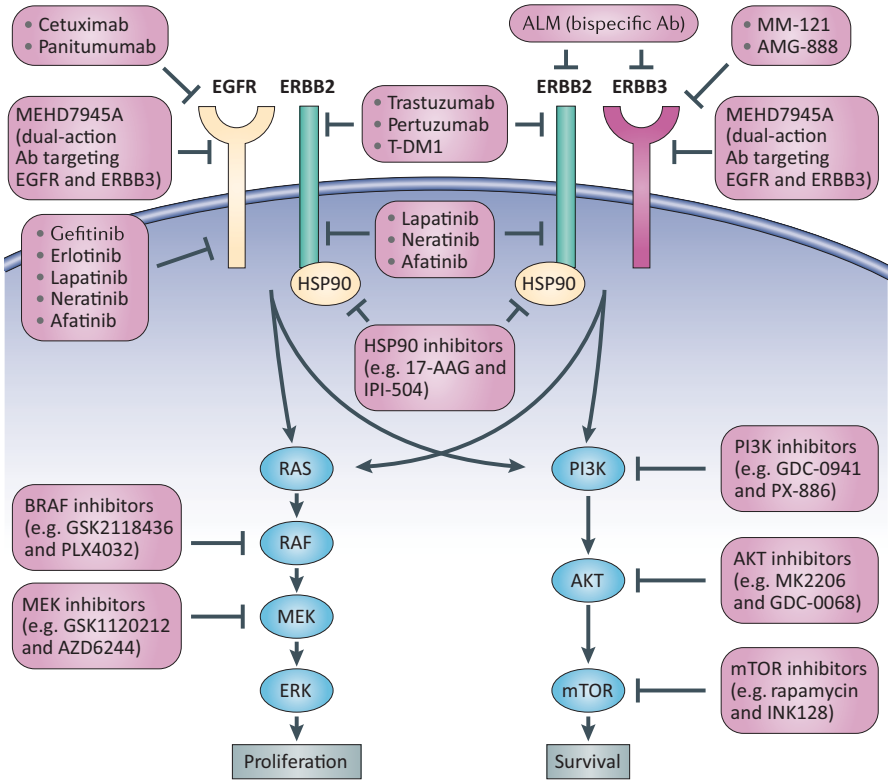

Fig. 1 The HER2 signaling cascade and anti-HER2 targeting agents (Reproduced from "*The ERBBnetwork: at last, cancer therapy meets systems biology*" [7])

Berns et al. used a functional genetic screen to assess the role of mutations in *P13K* and *PTEN* and resistance to trastuzumab [14]. The investigators demonstrated that this pathway was critical for resistance to trastuzumab *in vitro* and supportive evidence was presented in patient samples. In addition, a study evaluating *PTEN* loss using immunohistochemistry (IHC) and *PIK3CA* mutation showed a significant association of PI3K pathway activation and poor response to trastuzumab in the metastatic setting [18]. Furthermore, a study of 63 patients with *HER2*-amplified metastatic breast cancer revealed a higher frequency (77%) of *PTEN* loss and activating mutations in *PIK3CA* amongst the trastuzumab-refractory tumors compared to 44% within an unexposed cohort ($p=0.007$) [19].

Unfortunately, results from studies of early-stage disease have been mixed, further complicating the picture. Somatic hotspot mutations were evaluated in 20 genes in tumors from 700 breast cancer patients in a Phase III adjuvant trial (FinHER) [20]. *PIK3CA* mutations were found in 25.3% of patients and were associated with estrogen receptor positivity and the Luminal-A phenotype but were not significantly associated with prognosis or trastuzumab benefit [20]. In a separate study involving

the oral inhibitor of HER2 lapatinib, tumors coexpressing phosphorylated HER2 (pHER2) and phosphorylated HER3 (pHER3) were more likely to respond to lapatinib whereas loss of PTEN did not preclude response to lapatinib [21].

PTEN expression, on the other hand, was significantly associated with pathologic complete response (pCR) in the preoperative setting [22, 23]. It was observed that patients with higher levels of PTEN responded to trastuzumab better than those with lower levels. To further support this finding, protein level was also investigated and it was observed that increased localization of PTEN to the cell membrane was correlated with better response to treatment [22, 23].

However, a large adjuvant trial evaluating PTEN expression by IHC [24] did not show an association with response to trastuzumab. While there may be explanations for the difference in these findings, ranging from technical variability to the underlying heterogeneity of HER2-positive tumors, these studies suggest that there are more mechanisms of resistance to trastuzumab than can be explained by one gene or pathway. In this context, it is important to note that crosstalk between HER2 and other pathways such as insulin growth factor signaling could also result in modulating the clinical benefit from anti-HER2 therapies.

Early pre-clinical studies showed that increased levels of IGF-IR signaling seemed to interfere with the action of trastuzumab in breast cancer cell lines that overexpress HER2 [25]. In addition, stimulation of the MCF7 breast cell line with IGF-1 resulted in induction of IGF-1 response genes enriched for transcriptional targets of EGFR and HER2 pathways, and activation of an IGF-1R activity signature showed strong correlation with poor patient outcome [26]. Furthermore, an IGF-1 ligand signature that is inversely correlated with the IGF-1R activation signature showed strong association with good prognosis in multiple studies [27]. Finally, the recent biomarker analyses in the CLEOPATRA trial showed that high levels of IGF-1R membrane expression was associated with resistance to pertuzumab (interaction test $p = 0.041$), although the study did not consider this to be a predictive effect due to overlaps in confidence intervals and potential impact of multiple testing [28]. Taken together, these studies suggest that IGF-1 receptor activity may be associated with the Luminal phenotype and may portend reduced benefit to trastuzumab. However, the baseline IGF-1R signature has not been shown to be sufficiently robust in predicting response to trastuzumab to warrant the development of a clinical biomarker assay.

The knowledge that signal transduction by HER2 occurs through heterodimerization with other receptors of the HER family (HER1, HER3, HER4) has resulted in the hypothesis that the dual inhibition of HER2 and HER3 by targeting the HER2-HER3 complex, using either antibodies such as pertuzumab [29] or dual-inhibition of HER1 and HER2 with tyrosine kinase inhibitors such as lapatinib [30, 31] or neratinib are likely to be more beneficial than HER2 inhibition with one agent. The paradox that HER2-targeting agents have shown higher clinical activity than the tyrosine kinase inhibitors, despite the fact that they are weaker inhibitors of HER2 signaling, has led to increased focus on alternative dimensions underlying the clinical activity of HER2-targeting antibodies [32]. The role of the HER2-targeting antibodies in provoking endogenous immunologic responses has opened

up new avenues of biomarker discovery efforts related to therapy-induced immune response as well as alternative therapeutic approaches to engender effective immunogenic response against the tumor cells.

The Immune System and Response to Anti-HER2 Therapy

The interactions between cancer cells and the microenvironment affect both tumor growth and progression, with an increasing accumulation of preclinical and clinical evidence that the immune system may play a significant role in the therapeutic effects of HER2 targeted agents [33]. Trastuzumab has been shown to inhibit HER2 expressing tumor cells via antibody-dependent cellular cytotoxicity (ADCC), where antigen-specific antibodies help direct natural killer cells to antigen-expressing cancer cells (Fig. 2). Antigen presenting cells then capture the opsonized cancer cell fragments and present them to lymphocytes, thus eliciting the induction of the adaptive immune response (Fig. 2). Indeed, studies have demonstrated that the administration of trastuzumab resulted in the recruitment of natural killer cells at the site of the tumor, leading to ADCC [34, 35].

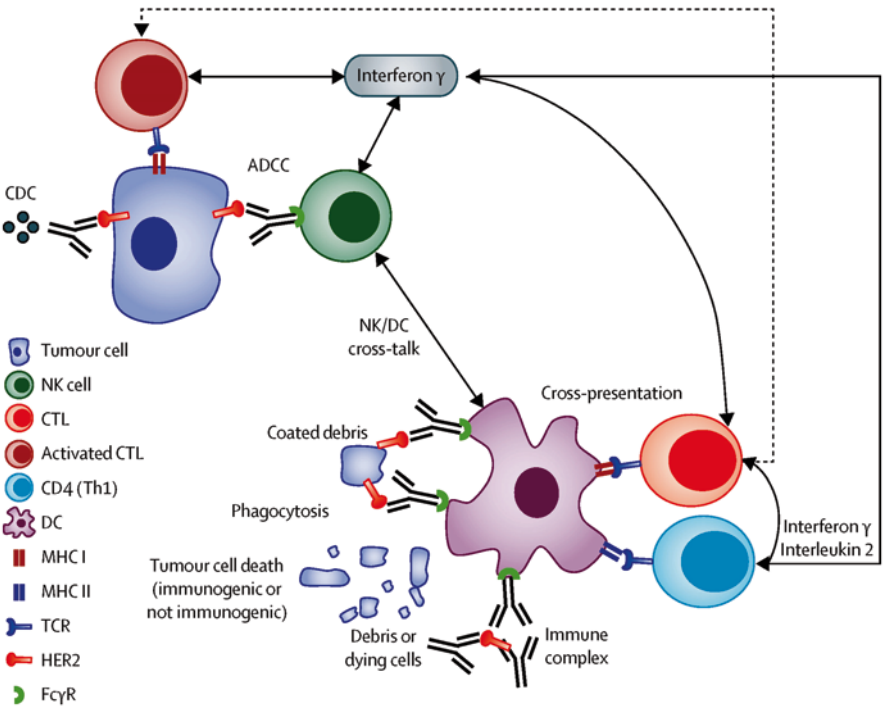

Fig. 2 The role of the immune system in clinical benefit from anti-HER2 therapy (Reproduced from "*The immune system and response to HER2-targeted treatment in breast cancer*" [33])

Several studies have focused on the development of immune markers that can predict the efficacy of treatment using IHC-based assessment of macrophages, natural killer cells, T and B lymphocytes. The BIG 02–98 investigators evaluated the role of tumor-infiltrating lymphocytes (TILs) in the adjuvant setting and reported an association of intratumoral and stromal lymphocytic infiltration with benefit from higher-dose anthracycline [36]. In addition, the predictive value of TILs was seen in a prospective-retrospective study of FinHER, a Phase III adjuvant trial of chemotherapy with or without trastuzumab [37]. In the HER2-positive breast cancer subgroup, each 10% increase in lymphocytic infiltration was associated with decreased distant recurrence in patients randomized to the trastuzumab arm [37]. Overall survival data in this study are immature, and the cut-off used for lymphocytic infiltration requires validation. However, the FinHER study does suggest the evaluation of immune markers may predict benefit from trastuzumab.

The study of transcriptional programs of immune-related functions has led to the identification of gene signatures predictive of trastuzumab benefit that provide potential biological insights into the mechanisms mediating clinical response. A prospective-retrospective study of mRNA expression in 1282 patients enrolled on to the adjuvant NCCTG-N981 trial [38] identified a signature of immune function genes to be predictive of relapse-free survival in patients treated with trastuzumab plus chemotherapy, but not in patients treated with chemotherapy alone. In addition, the NeOAdjuvant Herceptin (NOAH) trial investigators demonstrated that increased expression of an immunoglobulin metagene was linked to higher frequency of pCR in patients that received trastuzumab and chemotherapy when compared to chemotherapy alone [33]. Furthermore, two serial preoperative clinical trials, Dana-Farber Cancer Institute 03-311 (DFCI 03-311) and Brown University Oncology Group 211B (BrUOG 211B), were able to identify specific subytpes of HER2 tumors that benefit from trastuzumab-containing preoperative therapy. Using a novel, 'run-in' paradigm, Harris et al. found that a single dose of trastuzumab can define which patients will benefit most from the subsequent HER2 targeted regimen [39, 40]. Consistent with other studies [41], the DFCI 03-311 and the BrUOG 211B trials showed increased pCR rates in the HER2-enriched PAM50 subtype [39]. However, in these two trials, a predefined 140-gene expression-based ImmuneScore [42], was significantly induced after one dose of trastuzumab only in the HER2-enriched intrinsic subtype and not in the Luminal B or Basal subtypes [39]. More importantly, this induction was only seen in tumors that eventually achieved a pCR ($p=0.03$) suggesting it may be an important mechanism of response to trastuzumab-based therapy and not simply a feature of the HER2-enriched subtype. Most importantly, this same signature was validated as predictive of pCR in the 211B trial ($p=0.05$), showing the unique value of this paradigm. Of note, a single dose of nab-paclitaxel did not produce these associations, suggesting this was a trastuzumab-specific effect. Taken together, these data suggest that trastuzumab modulates activity of immune-specific transcriptional programs, which may be responsible for the mechanism of benefit of trastuzumab-containing therapy in specific subtypes of HER2-positive breast cancer. This unique preoperative paradigm is worthy of pursuit as it may provide patients with HER2-positive tumors and their providers the

ability to predict if therapy is going to be effective and the opportunity to change to (or add) another HER2 targeted therapy to improve outcome.

Another avenue for the development of predictive biomarkers for trastuzumab benefit are polymorphisms found on the Fc Gamma receptors that are found on the surface of macrophages and natural killer cells. Based on the hypothesis that ADCC is one of the mechanisms of action trastuzumab that occurs when the Fc portion of the tumor-bound antibody is recognized by the Fc Gamma receptors, polymorphisms within Fc Gamma receptors may be associated with impaired regulatory activity. Norton et al. [43] evaluated specific polymorphisms within these receptors and found a significant interaction between a polymorphism in the inhibitory gene *FCGR2B* with trastuzumab benefit. These data suggest that mechansims other than ADCC may be at play in response to antibody-based anti-HER2 therapy.

The Preoperative Paradigm

Despite extensive efforts of the breast cancer research community, biomarkers derived from single biopsies have not reliably predicted response to anti-HER2 therapy, much less guided the subsequent treatment choices. Part of this lies in the fact that biomarker studies of a robust nature are difficult to conduct due to limited availability of adequately sized cohorts and optimally collected tissues. If positive findings are reported, further validation sets are often not available or the results are negative and tend to be underreported. The underlying reason why findings are not validated is complex but may be largely due to the heterogeneity of the 'HER2 tumor subtype,' which is a misnomer as HER2 is in fact an amplicon and may arise in several breast tumor lineages and host backgrounds [44]. From a patient perspective, what is more important is 'is the therapy going to kill my cancer?' and from this point of view our approach needs to consider what we can learn from the tumor during therapy. Hence, we and others have pursued the approach of requesting a second biopsy after one dose of targeted therapy to determine the likelihood of benefit from a particular regimen (Fig. 3a). There is strong evidence that this paradigm has and will provide useful results and should be pursued for patient benefit [39, 40].

As discussed in the context of tumor immunity, a significant change in immune activity can be seen in certain subgroups of HER2-positive tumors and this predicted pCR to trastuzumab in a test and validation set [39]. In addition, previous studies of DFCI 03-311 found that IGF1 and AKT pathway activity upon brief exposure to trastuzumab predicts pCR using a predefined set of signatures [27, 45] (Fig. 3b). The activity signatures were again only predictive after brief-exposure and suggests not only that this paradigm is useful but provides a testable hypothesis to be pursed in the laboratory and in subsequent studies. These results have not yet been validated the overall dataset and will require further confirmation by next generation RNA sequencing.

The opposite end of the spectrum from treatment sensitivity, is treatment resistance, and phenotypes found in these two extremes are markedly different. We have

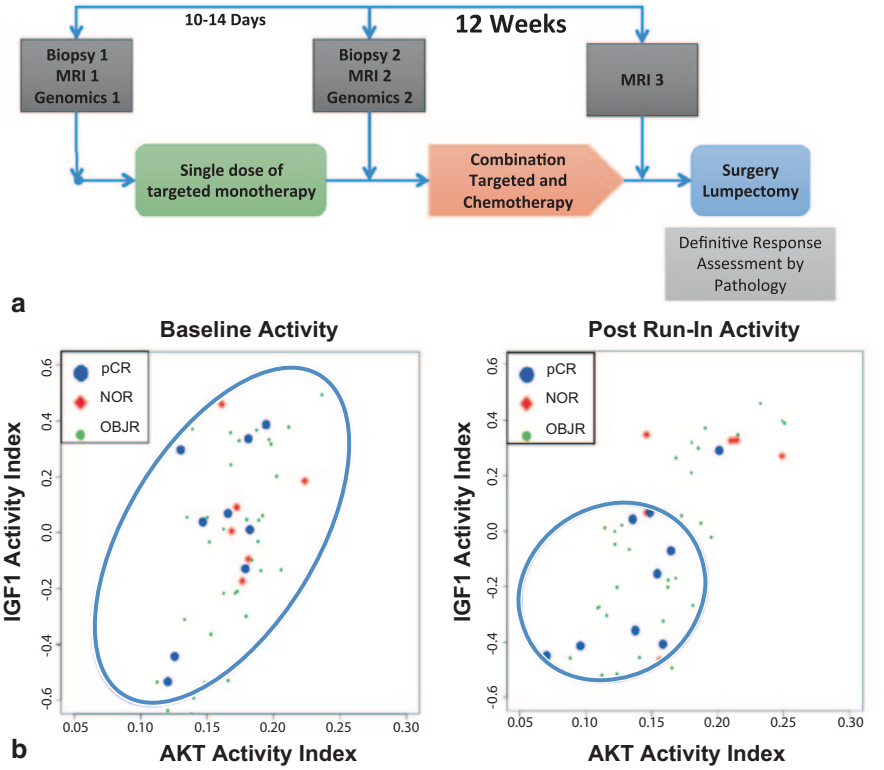

Fig. 3 (**a**) Novel brief-exposure to targeted pre-operative therapy paradigm to identify early predictors of therapy benefit (**b**) IGF1 and AKT activity signatures at baseline and after a single dose of preoperative trastuzumab across patients in the 03-311 preoperative trial

examined transcriptional profiling in trastuzumab-resistant tumors and found that these signaling networks tend to be much more complex, involving multiple growth factor pathways, cytokines and downstream effectors [6]. In addition, but perhaps not surprisingly, genes that were characteristic of Basal-like breast cancer were seen in non-responding tumors, suggesting that defects in DNA repair and genomic instability might be at play in this phenotype. In addition to lineage markers (*CK14, CK15, CK5, CK17, GABRP* and *BOC*) the signature included anti-apoptotic genes (alpha B crystallin), Wnt family members and other indicators of the Basal phenotype and suggest potential targets for therapy. Both lineage and genomic instability are important features of HER2 tumor biology and may play a pivotal role yet in the development of drug resistance over time. In addition, the presence of breast cancer stem/progenitor cells contributes not only to progression but to likelihood of response to specific therapies (discussed below).

Although enormous strides have been made in establishing preoperative therapy as a 'standard of care' the brief exposure paradigm is still somewhat new and requires further study and emphasis. Intriguingly, a number of predictive biomarkers have been identified, and some have been validated in second trials [39, 40]. Nevertheless, the validity and clinical significance of these markers needs validation in large prospective-retrospective trials such as the neoALTTO cohort. These studies lay the groundwork for such an undertaking and justify their being carried out in the near-term.

Cancer Stem Cells as Predictive Factors to Treatment Response

In the past decade, treatment strategies have been guided by molecular profiles that have divided breast cancer into four categories: Luminal A, Luminal B, HER2-amplified and Basal-like. However, issues such as resistance to treatment and disease progression have highlighted the necessity of looking for additional therapeutic targets. Recent data suggest that subpopulations of cancer cells with stem cell-like characteristics, (CSCs) are present in breast tumors and that these cells are more resistant to treatment.

The first report identifying CSCs in breast cancer combined expression of two cell surface markers: $CD44^+$ and $CD24^-$, and was found primarily in the basal-like subtype [46]. This population exhibited *in vitro* and in vivo stem cell-like properties such as mammosphere-forming ability, the capacity to regenerate a heterogeneous tumor in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice and invasive capability. After Ginestier and colleagues characterized a breast cancer specific marker, the enzyme aldehyde dehydrogenase 1 (ALDH1) [47], Charafe-Jauffret showed that the ALDH1 population could metastasize in vivo and carried a worse prognosis [48] and Creighton found that the $CD44^+/CD24^-$ -MS signature was seen in therapy-resistant patient samples.

To explore the $CD44^+/CD24^-$ phenotype in HER2-overexpressing breast cancer, Chang and colleagues compared the effect of chemotherapy and lapatinib on this population. The $CD44^+/CD24^-$ population was upregulated with chemotherapy (4.7–13.6%) but this was blocked in the lapatinib arm (10–7.5%), although non-significantly. Of note, the baseline level of $CD44^+/CD24^-$ population was higher in the HER2-positive patients (10% versus 4.7%), which suggests a CSC phenotype is more prevalent in this population and potentially a good target for anti-HER2 therapy [49]. Nevertheless, a non-significant result is not definitive and the role of the CSC population in HER2-positive tumors requires further study.

A study from Duru et al. suggest that the HER2-NF-kB-HER2 loop radioresistance to HER2-positive tumors (Fig. 4) as patients with recurrent invasive tumors tended to be HER2-positive with worse outcome [50]. These data propose a mechanism for radioresistance in CSC from HER2 tumors, requiring further confirmation.

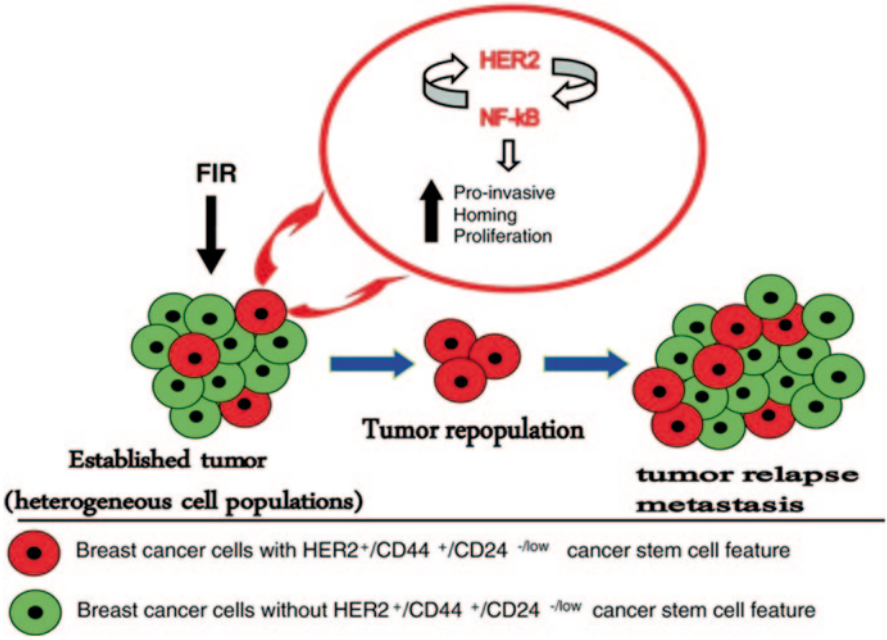

Fig. 4 The role of cancer stem cells in clinical benefit from anti-HER2 therapy (Reproduced from "*Breast cancer adaptive resistance: HER2 and cancer stem cell repopulation in a heterogeneous tumor society*" [50])

Liu and colleagues [51] developed a 17-gene signature that is specific for HER2-amplified breast cancer (HER2-Tumor Initiating Cells enriched signature; HTICS). Using a mouse model, they utilized serial dilution and single cell transplantation assays to purify the CSC population identified in $CD24^{+}/JAG1^{-}$ fraction that was then found to contain stem cell-like properties. In a subsequent cohort, the HTICS was associated with resistance to chemotherapy however they responded to combination chemotherapy with trastuzumab. This signature was found to be a powerful predictor of clinical response and was independent of additional clinical variables like age, tumor grade, size, lymph node involvement [51]. Taken together, there is mounting evidence of CSC populations in HER2-positive tumors that are more resistant to therapy that could be potentially overcome by anti-HER2 agents. Future studies will need to ratify these findings in order to move the potential of CSC therapy into clinical practice.

Conclusions

The development of anti-HER2 targeted agents for treatment of HER2-amplified breast cancer has been a success story as introduction of agents such as trastuzumab has completely altered the course of this disease changing it from a death sentence

to a cure in many patients. However, despite the advances, the clinical benefit from anti-HER2 agents remains heterogeneous and biomarkers to identify which patients most benefit from specific anti-HER2 therapies are sorely needed. Despite the extensive efforts of the research community, no good predictors of response to trastuzumab are currently available for clinical use. Ongoing efforts are needed to validate promising markers, to standardize the assays and to make them available to patients once analytic and clinical validity and clinical utility are proven. In addition, and perhaps most importantly, novel research strategies such as the brief exposure preoperative paradigm are needed to identify the specific therapy that will be most likely to cure the patient. These approaches will allow us to reach the ultimate goal, which is the highest cure rate possible for the patient with a HER2-positive tumor.

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Intratumor Heterogeneity in Breast Cancer

Francisco Beca and Kornelia Polyak

Abstract Intratumor heterogeneity is the main obstacle to effective cancer treatment and personalized medicine. Both genetic and epigenetic sources of intratumor heterogeneity are well recognized and several technologies have been developed for their characterization. With the technological advances in recent years, investigators are now elucidating intratumor heterogeneity at the single cell level and *in situ*. However, translating the accumulated knowledge about intratumor heterogeneity to clinical practice has been slow. We are certain that better understanding of the composition and evolution of tumors during disease progression and treatment will improve cancer diagnosis and the design of therapies. Here we review some of the most important considerations related to intratumor heterogeneity. We discuss both genetic and epigenetic sources of intratumor heterogeneity and review experimental approaches that are commonly used to quantify it. We also discuss the impact of intratumor heterogeneity on cancer diagnosis and treatment and share our perspectives on the future of this field.

Keywords Heterogeneity · Breast cancer · Evolution · Selection · Clonality · Phenotype · Therapy · Resistance · Progression

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Introduction

While the topic of heterogeneity is receiving substantial interest by both researchers and clinicians, heterogeneity within tumors has been recognized for a very long time. Rudolph Carl Virchow, one of the fathers of modern pathology and an influential biomedical scientist of the nineteenth century, had already pointed to the existence of distinct cellular phenotypes within tumors (historic perspective in [1]). Specifically in breast cancer, and also in the nineteenth century, the same phenomenon was almost immediately recognized when the first core needle biopsies were performed (historic review in [2]). Later on, in the first half of the twentieth century, breast cancer phenotypic heterogeneity was casted with the publication of the first classification of breast tumors based on histological types [3]. Intratumor heterogeneity at the functional and genetic level was also beginning to be appreciated in melanoma models [4]. Using more in-depth analysis in the second half of the twentieth century, pioneer studies of tumor heterogeneity were conducted where the existence of distinct subpopulations of cancer cells within tumors with different tumorigenicity, resistance to treatment, and ability to metastasize were described [4–6]. The clinical implications of tumor heterogeneity were recognized almost simultaneously. Breast cancer was one of the first solid tumor types in which the clinical and treatment implications of heterogeneity for cellular phenotypes was established while analyzing the expression of the estrogen receptors [7]. Specifically, researchers determined that variability for this biomarker could classify tumors into distinct subtypes guiding treatment decisions, but its variability within tumors posed a challenge in the clinical management of some of these patients [7]. Breast cancer research was also pioneering with the identification of intrinsic molecular subtypes based on global gene expression profiling studies and the rapid translation of this knowledge into clinical practice [8–10].

Due to advances in molecular biology methods, research in cancer stem cells (CSC), and the appreciation of its clinical impact, tumor heterogeneity is currently one of the most highly investigated areas in cancer research. It has also been acknowledged that tumor heterogeneity is more than just a reflection of genetic diversity within tumors resulting from genomic instability and clonal evolution. Newer tools and accumulating knowledge have led to the identification of important sources of non-genetic tumor heterogeneity such as epigenetic heterogeneity due to differentiation hierarchies and stochastic mechanisms as well as non-hereditary cellular heterogeneity for various phenotypes [11, 12]. Today we recognize innumerous sources of phenotypic features that display substantial cell-to-cell intratumor variability. These include activation of signaling pathways, evasion of antitumor immunity, induction of senescence, production of secreted factors, migration, metastatic potential, angiogenic capacity, genetic makeup, response to anticancer drugs and activation of metabolic pathways. However, even though this increased knowledge has led to the more in-depth understanding of tumor biology, there is still a significant lag with its clinical translation and incorporation into diagnostic, prognostic, and therapeutic strategies. Though contemporary sounding, conclusions such as

"*The possible existence of highly metastatic variant cells within a primary tumor suggests that we no longer should consider a neoplasm to be a uniform entity*" were written almost 40 years ago [5]. Despite this recognition, no substantial clinical advances have been made considering the phenomena of intratumor heterogeneity.

In this chapter, we will review and discuss some of the sources of genetic and non-genetic intratumor heterogeneity with a special emphasis on breast cancer. Additionally, we will discuss current technologies that are applied in intratumor heterogeneity studies and the most pertinent clinical implications of intratumor heterogeneity.

Sources of Intratumor Heterogeneity

Genetic Heterogeneity

For decades, cancer biology and oncology have been dominated by a gene-centric view. Normal tissues exhibit low genetic heterogeneity being all phenotypic diversity attributable to non-genetic sources. In contrast, in cancer, phenotypic differences were thought to be due to defined genetic alterations. According to this perspective, genetic changes would accompany and drive the development of a neoplasm ensuing progression to an increasingly malignant phenotype [13]. Descendants of the same cell are defined as a clone. Strictly speaking, the whole cancer is a clone, since tumors almost always initiate from a single transformed cell. Every time a cancer or a normal cell divides mutations may be acquired and the number of mutations that distinguish two cells marks the time from their common ancestry. Based on global mutational data, the clonal (or subclonal) architecture of the tumor can be defined. This clonal heterogeneity is overlaid by cellular genetic diversity, since due to high genomic instability every cancer cell can be genetically different within the same tumor. In a phenomenon analogous to speciation, in large tumors, cells located in distant regions harbor more differences than neighboring cells [14]. Similarly, larger tumors (as with larger populations) usually exhibit greater genotypic diversity [15], highlighting the importance of multiple rounds of cell divisions for tumor development and diversification [16]. Phenotypic heterogeneity among clones leads to the selection for the ones with higher fitness advantage by a Darwinian selection process leading to a unique pattern of clonal architecture within tumors. As a direct consequence of this process, during tumor evolution only a fraction of all clones will be associated with distinct phenotypic traits and an even smaller fraction will have biological impact. In other words, only a small proportion of mutations will have direct phenotypic manifestations. However this is a rather simplistic view of clonal selection. Today we know that tumor growth can be driven even by a minor subpopulation, which enhances the proliferation of all cells within a tumor by overcoming environmental constraints [17]. Additionally, mutations that are phenotypically silent in one condition can manifest in another due to interactions with other

silent mutations [18] or altered heat-shock responses, for instance [19]. Even in cases when the mutational landscape is well known, predictions on tumor evolution taking into account only the genetic sources of intratumor heterogeneity are still very inaccurate. Accounting for non-genetic intratumor heterogeneity sources is utterly needed for understanding the full complexity of intratumor heterogeneity.

Epigenetic Heterogeneity: Differentiation Hierarchies

Epigenetic alterations can be defined as changes in gene expression or phenotype caused by mechanisms other than changes in the DNA sequence. Since epigenetic alteration can condition the cancer cell phenotype and potentially provide fitness benefit, epigenetic mechanisms are an additional source of intratumor heterogeneity. In breast cancer, several epigenetic alterations have been described and usually occur within the larger context of extensive changes to chromatin structure related to altered patterns of histone modification, and methylation gains and losses on CpG dinucleotides within DNA sequences [20, 21]. These mechanisms, usually through *epigenetic silencing*, are responsible for the repression of multiple tumor suppressor genes and condition numerous important phenotypic traits. Hypermethylation profiles have been associated with hormone receptor and HER2 status [22, 23]. Either by estrogen receptor (ER) silencing or through silencing of its promoter, hypermethylation is involved in the regulation of ER expression. *ESR1* promoter methylation has been shown to be a better predictor of clinical response to adjuvant tamoxifen than hormone receptor status determination by immunohistochemistry (IHC) [24]. Epigenetic events have also been implicated in breast cancer progression including the epigenetic silencing of $p16^{INK4A}$ [25] and *RASSF1A* [26, 27]. Besides establishing important phenotypic traits, epigenetic mechanisms add another layer of complexity to tumor heterogeneity as they are reversible. Nevertheless, the phenotypic traits set by epigenetic changes are not immutable, but rather stable or semi-stable and therefore still regarded as the "gray zone of tumor evolution" [11]. The magnitude of intratumor epigenetic diversity is even greater than genetic diversity. However, there is still a significant gap in our understanding of the causes and consequences of epigenetic sources of intratumor heterogeneity. Despite currently available knowledge on epigenetic modifications with enormous potential for clinical application, translation to clinical use with the identification and validation of prognostic markers as well as drugs targeting epigenetic mechanisms is still in its infancy.

The idea that phenotypic differences in tumors are somewhat representative of differentiation hierarchies observed in normal tissues has been around for a long time [28]. More recently, the concept of differentiation hierarchies in cancer was fueled by the discovery of a subpopulation of cells in acute myeloid leukemia (AML) with stem cell-like characteristics [29] and the "boom" in CSC research that followed it. In breast cancer, phenotypic heterogeneity mapped to distinct differentiation states seems to be a better predictor of clinical behavior than that based on

mutational profiles [30] since basal and luminal features are strongly associated with invasive and metastatic potential [31]. But even though this is an appealing perspective, several caveats can be identified. First, the parallel between normal and tumor differentiation hierarchies is not straightforward owing to the massive epigenetic abnormalities acquired by cancer cells and multiple mutations that can result in unique deterministic phenotypes without a normal counterpart [12]. Second, while the ability to dedifferentiate is not limited to cancer cells, oncogenic transformation increases the probability of this dedifferentiation and thus, increases epigenetic and phenotypic plasticity [32]. Although conceptually useful for understanding tumor biology, the idea of differentiation hierarchy within tumors does not provide on its own a comprehensive explanation for all forms of intratumor epigenetic heterogeneity (Fig. 1).

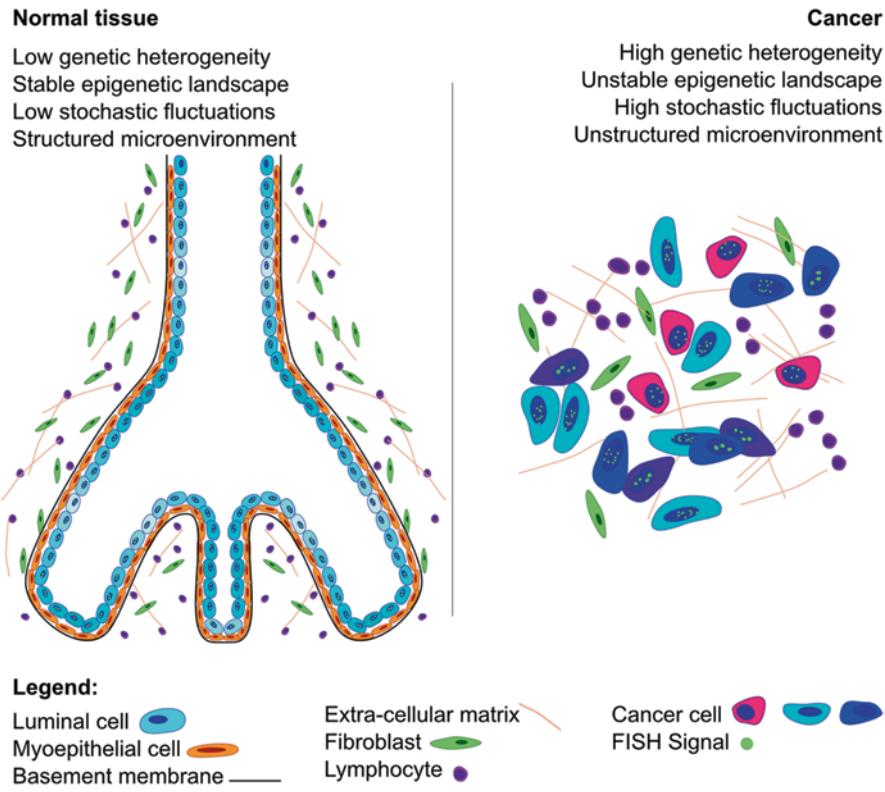

Fig. 1 Genetic, epigenetic, and microenvironmental heterogeneity in cancer. Cellular phenotypes represent the integration of several inputs. In normal tissues, genotypes are homogeneous, there is a defined (stable) epigenetic landscape with low stochastic fluctuations and the microenvironment is highly structured, resulting in limited number of distinct niches. In contrast, in most tumors there is significant genetic heterogeneity, an unstable epigenetic landscape, and the microenvironment is disorganized leading to a larger number of less well-defined niches. The integration of these influences result in a large repertoire of highly variable cellular phenotypes in neoplastic compared to normal tissues.

Epigenetic Heterogeneity: Stochastic Mechanisms

Additional sources of intratumor epigenetic heterogeneity are via stochastic mechanisms. Due to recent advances in single-cell analysis, it has been demonstrated that even members of a genetically identical group of cells or organisms in identical environments can exhibit different phenotypes [33]. In other words, even in isogenic cells that share the same deterministic phenotypic state, there are transient phenotypic variants. These variants are the result of stochastic changes in the biochemical processes within the cells. The best studied processes of stochastic intratumor heterogeneity are the variable gene expression and the "burst-like" way in which most eukaryotic genes are transcribed [11]. These processes are still puzzling and might involve changes in chromatin states and/or modulation of the turnover of mRNAs [34, 35]. Despite not being fully understood, stochastic mechanisms of intratumor heterogeneity appear to be influential in the differential sensitivity of cancer cells to cytotoxic therapies[36]. Spencer et al. showed that naturally occurring differences in the levels or states of proteins regulating receptor-mediated apoptosis are the primary causes of cell-to-cell variability in the timing and probability of cell death in human cell lines [36]. These stochastic mechanisms may also be responsible for transitions between cellular differentiation states and result in subpopulations of human breast cancer cells with distinct properties despite shared clonal origin and culture conditions [37]. For example, Fillmore and colleagues showed using eight different human breast cancer cell lines that cells with $CD44^{+}CD24^{-}ESA^{+}$ (epithelial-specific antigen) phenotype had significant tumor-initiating and self-renewing abilities *in vitro* and that these cells would give rise to phenotypically diverse progeny with increased resistance to chemotherapy. Despite the stochastic gene expression, proteome fluctuations, and cell-to-cell variability in phenotypes, the full impact of cellular heterogeneity on pharmacologic responses and the treatment of human disease is largely unknown. Additional advances in mathematical modeling methods and study designs are required to account for the stochasticity of biochemical processes in intratumor heterogeneity studies.

Characterization of Intratumor Heterogeneity

Genome-Wide Studies and Bulk Tumor Sequencing

Many of the first genomic studies of human cancer were conducted to evaluate heterogeneity between tumors rather than between subpopulations of cancer cells within tumors. In breast cancer the first studies investigating differences in gene expression and genomic profiles (mostly copy number variations (CNV)) were conducted many years ago in the mid-late 1990s). However, in-depth genome-wide studies have been made possible only more recently with the development of new technologies. One of the most significant studies was performed by The Cancer Genome

Atlas (TCGA) Network initiative. New insights were provided into the previously defined intrinsic breast cancer subtypes based on comprehensive integrated view of CNVs, DNA methylation, exome sequencing, RNA-seq, microRNA sequencing, and reverse-phase protein array data [38]. Specifically, the authors confirmed the existence of four main breast cancer intrinsic subtypes, each showing significant molecular heterogeneity with common (>10% incidence) somatic point mutations occurring only in three genes (*TP53, PIK3CA*, and *GATA3*) across all breast cancers. Another large scale study subjecting 2000 breast tumors to integrated genomic and transcriptomic profiling, proposed a more detailed classification of breast cancer into 10 subtypes based on presumed molecular drivers [39, 40]. However, since these studies rely on sequencing of bulk tissues, only an average estimation per tumor/sample is provided thus reflecting only the broad mutational landscape of the majority of cells in the tumor [41]. As such, the possible underlying clonal complexity is averaged and the clonal frequencies of mutations and CNVs have to be inferred using computational methods. These methods estimate tumor purity and ploidy from the observed copy number profile and mutational landscape or using hierarchical Bayesian modeling [42, 43]. To better define intratumor clonal heterogeneity within tumors genome-wide analysis of topologically distinct areas of the same tumor have also been performed. Combining the use of macro-dissection with nuclei isolation by fluorescence-activated cell sorting (FACS) and performing array comparative genomic hybridization (aCGH) in different areas of breast carcinomas, attempts were made to more precisely study intratumor genetic heterogeneity in breast cancer [44]. Based on the analysis of different sectors of the same tumor, different clones were found to be either topologically segregated throughout the tumor or intermingled within the same sector [44].

Genome-wide studies have also been used to investigate clonal relations in breast cancer between primary tumors and metastasis. For example Ding et al. [45], performed genomic analyses of four DNA samples from different sites (peripheral blood, primary tumor, a brain metastasis and a xenograft derived from the primary tumor) from an African-American patient with Basal-like breast cancer. Using this approach, the authors showed that, in this Basal breast cancer, despite occurrences of additional somatic mutations, copy number alterations and structural variations during the clinical course of the disease, most of the original mutations and structural variants present in the primary tumor are propagated during disease progression. However, the differential mutation frequencies and structural variation patterns in metastasis and xenograft compared with the primary tumor indicated that metastatic lesions might arise from a minority of cells within the primary tumor rather than from the dominant clone [45].

In summary, bulk tumor sample profiling can provide useful information about the tumor as a whole but cannot determine the cellular origin of the signal, topology within tumors or the degree of intratumor heterogeneity. Additionally, although to some extent the detection of subclonal populations using bulk tumor data is possible due to recently developed computational tools, these are limited by the error rate of the sequencing platform at the commonly used sequencing depths and aberrations in rare cells that may escape detection [46]. Despite all these challenges and

caveats, genome-wide studies of bulk tumors still continues to remain a popular choice given its cost-effectiveness and straightforward study design implementation when compared to other methods.

Genome-Wide Studies in Single Cells

In recent years, technological advances have made it possible to conduct studies using single cell sequencing. Single cells can be obtained from a multitude of samples, but most commonly from fresh tumor samples or circulating tumor cells (CTC) in peripheral blood. Techniques applied to single cell studies in breast cancer can also vary from whole-genome and exome sequencing [47, 48] to high-resolution oligonucleotide aCGH and targeted sequencing of candidate genes [49]. Single cell sequencing is the most objective way to assess intratumor clonal heterogeneity, since it allows for the direct inference of clonal genotypes [41]. However, this technique has several limitations, the most important being the need for whole-genome amplification. While structural variations can be reliably assessed by single cell sequencing, genome-wide assessment of mutations is still challenging due to artifacts produced by whole genome amplification and sequencing errors [50]. To address this problem, Wang et al. [48] recently developed a high-coverage whole genome and exome single cell sequencing method called nuc-seq. Exploiting the fact that single cells duplicate their genome during S phase, Nick Nevins et al. isolated nuclei from cells in the G2/M phase of the cell cycle and were able to achieve high-coverage data with low error rates. This technique enabled the demonstration that aneuploid rearrangements occur early in tumor evolution and remain highly stable as the tumor masses clonally expand. In contrast, point mutations seem to evolve more gradually, generating extensive clonal diversity [48]. The authors also showed that triple-negative tumors have an increased mutation rate compared to ER-positive ones.

Despite major advances in single cell sequencing technology, the current cost and time required make this technique prohibitive for routine clinical use. More importantly, the clinical relevance of single cell sequencing methods is still unclear since data from single cells may not provide information on the remaining of the tumor population and much of its interest relies on the potential use for characterizations of CTCs. Nonetheless, it remains to be demonstrated whether CTCs are representative of the whole tumor (and all of its complexity) and if they are the cells that mediate the metastatic process [51].

In Situ Analysis of Intratumor Heterogeneity

Due to extensive topologic heterogeneity within tumors, the favored methods should allow for the evaluation of intratumor heterogeneity *in situ*. By preservation

of the tissue context, *in situ* techniques provide detailed topological information that is complementary to the information provided by bulk and single cell sequencing. Additionally, most of them can be applied to formalin-fixed paraffin-embedded (FFPE) samples, available in large cohorts in many pathology departments and tumor banks.

Today, *in situ* techniques range from traditional IHC to *in situ* polymerase chain reaction (PCR) [52] and *in situ* target-primed rolling cycle amplification [53, 54]. The detection and evaluation of antigens in FFPE tissues can easily be achieved by IHC and immunofluorescence (IF). IHC is the standard ancillary technique in all pathology departments throughout the world and is the basis of ER, progesterone recpetor, and initial HER2 assessment in breast cancer. IHC is particularly useful for rapid analysis and semi-quantitative assessments of expression levels of the proteins of interest, while preserving topological information close to a haematoxylin and eosin (H&E) stained section. Nowadays IHC is a robust technique that can be easily automated and allows for the study of immunophenotypic heterogeneity of tumors. The multiplexing capabilities of routine IHC are rather limited and it is not easy to visualize more than two proteins simultaneously. Thus, the evaluation of multiple targets is better performed with IF. IF can be easily multiplex to simultaneous detect two to three targets, but several techniques have been developed to increase the multiplexing capacity. As an example, Gerdes et al. [55] recently reported the development of a novel method that allows multiplexed quantitative single-cell IF microscopy and used it to detect 61 protein antigens in a single FFPE slide. Thus, IF can be used to investigate co-expression of multiple proteins and specific signaling pathways in different cell populations while keeping topological information intact and using quantitative methods [56].

In situ hybridization techniques have been used to detect RNA and DNA in frozen and FFPE samples to provide information on copy number alterations, mutation, and expression levels. Both DNA and RNA can be used as probes and labeled for detections with radioactive isotopes, enzymes or fluorochromes. Additionally, in many pathology departments, the technical component of the routinely used *in situ* hybridization techniques (i.e., HER2 by fluorescence *in situ* hybridization (FISH) to assess HER2 amplification) can now be performed automatically [57]. Furthermore, many of these techniques can be successfully combined. Using a combination of FISH and IF (immunoFISH or iFISH), Park et al. [58], investigated the association of commonly used markers of cellular differentiation states and genetic alterations in human breast carcinomas of different stages. According to the authors, there was a high degree of genetic heterogeneity both within and between distinct tumor cell populations that were defined based on markers of cellular phenotypes including stem cell–like characteristics, with the combined use of CD24 and CD44. Indeed, in several breast tumors, cell populations expressing CD44, a stem cell–like marker and cell populations expressing the more luminal differentiated marker, CD24, were genetically distinct [58]. Also using iFISH, Almendro et al. [59] assessed breast cancer intratumor heterogeneity during the course of neoadjuvant chemotherapy and showed that intratumor genetic diversity was tumor subtype specific and lower pre-treatment cellular genetic diversity was significantly associated

with pathologic complete response. Additionally intratumor genetic diversity did not change significantly in tumors with partial or no response to treatment but phenotypic diversity was different between pre- and post-treatment samples [59]. Immuno-FISH was also used to study intratumor heterogeneity at the single cell level and during metastatic breast cancer progression [60]. In this study, it was shown that genetic diversity was the highest in distant metastases compared to primary tumors and lymph node metastases and was generally concordant across lesions within the same patient. However, in treatment naïve patients, the cellular genetic heterogeneity indices of primary tumors and matched lymph node metastases were frequently more divergent. In contrast, cellular phenotypes were more discordant between distant metastases than between primary tumors and matched lymph node metastases. These two studies highlight the importance of integrated genotype and phenotype analysis of single cells *in situ* in intact tissues to infer tumor growth and evolutionary dynamics.

Lastly, other techniques traditionally not used *in situ* are now finding its way to such applications. PCR and reverse-transcription PCR (RT-PCR) can now be performed *in situ*, combining the resolution to detect point mutations with topological information [61, 62] even in whole mount preparations [63]. Additionally, novel techniques such as *in situ* target-primed rolling cycle amplification [53] and DNA paint [64] are unlocking the potential to multiplex the detection of mutations in sections of fixed tumor samples and even combine *in situ* mRNA genotyping while simultaneously obtaining information on protein interactions or post-translational modifications [54].

In conclusion, with the *in situ* techniques available today, it is already feasible to trace back and integrate with topology many of the "hits" found by bulk or single cell sequencing approaches. Thus, the integrated analysis of tumors would not only provide information on the genetic events but also on the local interactions between tumor cells and the local microenvironment.

Clinical Impact

The Impact of Intratumor Heterogeneity on Diagnosis and Biomarker Studies

Diagnostic classification of tumors is still mostly based on immunophenotypic characteristics. In breast cancer, histological type and tumor grade are still some of the most informative prognostic characteristics. Additionally with the evaluation of ER, PgR, and HER2, all the necessary clinically relevant information is available for the selection of first line therapy in a newly diagnosed breast cancer case. Interestingly, despite its simplicity, the classification based solely on these markers, reflects meaningful biological differences with well-known distinct clinical prognosis and response to treatment [8, 9]. After the seminal discovery of the intrinsic

molecular subtypes, several efforts were made to clinically implement this classification [10]. Current guidelines are designed to assign each patient to a specific subtype and maximizing patients that would benefit from target therapies. Perhaps the best example for this is the use of a 1 % cut-off of tumor cell positivity required to classify a tumor as ER expressing and to recommend the use of endocrine therapy [65]. Although aimed at maximizing benefits, this approach completely ignores intratumor heterogeneity, which in turn limits the success of the classification, especially when distinct populations are unequally distributed within tumors [66]. In the case of HER2 quantification, similar situation occurs. Despite efforts to introduce a clinical definition of HER2 heterogeneity [67] and demonstration of differential disease-free survival in heterogeneous HER2 amplified cases [68], in the most recent guidelines of the American Society of Clinical Oncology/College of American Pathologist (ASCO/CAP) the criteria for heterogeneity has been simplified [69]. In this updated guideline, cases with heterogeneous areas of HER2 amplification are considered, and each of the areas are to be scored and reported separately, but the overall case is interpreted as amplified if any one of the areas meets the standard criteria for HER2 amplification, which is only needed in > 10 % of invasive tumor cells [69]. Of course it is arguable whether using the 10 % of adjacent invasive tumor cells is a good cut-off, especially considering that HER2 frequently displays a "cell-to-cell" mosaic variation [67]. Most importantly, this approach maximizes eligible patients for targeted therapy but does not consider the clinical implications of intratumor heterogeneity. It remains to be proven whether HER2 heterogeneity as assessed using this new guideline's criteria is clinically meaningful. Consequently, and considering that many of the routinely used breast cancer biomarkers can be analyzed at the single cell level, efforts to evolve to a more quantitative reporting of heterogeneity should be undertaken. Quantitative diversity measurements such as the Shannon index [70], should be further tested for its clinical usefulness as there is promising preliminary data available using FFPE patient samples in breast cancer [58–60]. If proven feasible and useful in routine clinical practice, standardized reporting of readily available and implemented biomarkers' heterogeneity could provide valuable information in the short term to guide therapeutic decisions.

One additional challenge posed by intratumor heterogeneity, related to diagnosis, is the discordance in biomarker expression assessments between primary tumors and metastasis. Progression to metastatic disease has been predominantly viewed as a linear progression model. In this model, cancer cells pass through multiple successive rounds of mutation and selection for competitive fitness in the context of the primary tumor [71]. After a certain number of rounds, the fittest to metastasize would seed secondary growths. Therefore, metastasis development is considered a late consequence of the evolution of the primary tumor and would recapitulate much of the genetic landscape of the primary tumor with eventual observed differences attributed to epigenetic regulation [71]. However, since the 1950s, this view has been challenged by an alternative parallel evolution model [72, 73]. This alternative view bas been slowly gaining acceptance and states that metastatic dissemination can occur at early stages of the disease, and primary and metastatic tumors may co-evolve in parallel. Regardless of the progression model, each individual

tumor location has its own unique phenotype. In the case of breast cancer where tumor biomarker characteristics such as ER, PR, and HER2 determine the choice of therapy, discordance between primary tumor and metastasis can have major clinical implications. Such discrepancies between primary breast cancer and metastasis have been shown to be as frequent as up to 30% for the hormonal receptors and up to 10% for HER2 [74, 75]. Amir et al. [74] demonstrated that divergence between primary and metastatic lesions would alter the choice of therapy in 14% of patients. Using samples from metastatic sites of breast cancer and FISH, Wilking et al. [75] showed that intra-patient agreement for HER2 status was only 76%. Importantly, the authors also showed that patients with change in HER2 status during metastatic progression had significantly worse outcome compared to those with concordant HER2 positivity; once again demonstrating the impact of intratumor heterogeneity on clinical outcomes and its importance in clinical practice.

While tumor evolutionary mechanisms are not fully understood and accurate predictions not possible, both primary tumors and metastases would need to be repeatedly assessed during the course of the disease, with more frequent and repeated biopsy than currently applies [41]. To make tumor monitoring less invasive, some authors forward the hypothesis of using CTC or circulating tumor DNA (ctDNA), with some interesting examples of this approach already been tested in metastatic colon cancer [76, 77], breast [77], and ovarian cancer [78]. Setting aside the already mentioned potential problem of how representative of the whole tumor and all of its complexity CTC are, the possibility of using ctDNA to monitor tumors is very exciting, transforming a blood sample into a truly "liquid biopsy" [78]. However, the widespread routine clinical use of this approach in solid malignancies is still several years ahead. For the time being, due to its minimal invasiveness, safety, cost-effectiveness and possibility to be coupled with modern ancillary technics, fine-needle aspiration (FNA) is probably the best method to routinely address the need of tumor re-biopsy for monitoring purposes (Fig. 2) [79, 80].

> "*...each patient's cancer may require individual specific therapy, and even this may be thwarted by emergence of a genetically variant subline resistant to the treatment.*"
>
> Peter Nowell 1976

The Impact of Intratumor Heterogeneity on Treatment Design and Resistance

Heterogeneous tumors are composed of multiple subpopulations, some which can be resistant to treatment. The prevailing view is that in treatment naïve cancers, chemo-sensitive cells, by having lower energy needs than chemo-resistant cells, have a fitness advantage and dominate the tumor mass. However, during the course of treatment as chemo-sensitive cells are eliminated, chemo-resistant cells become the most fitted population in this environment due to a new equilibrium in selective pressures.

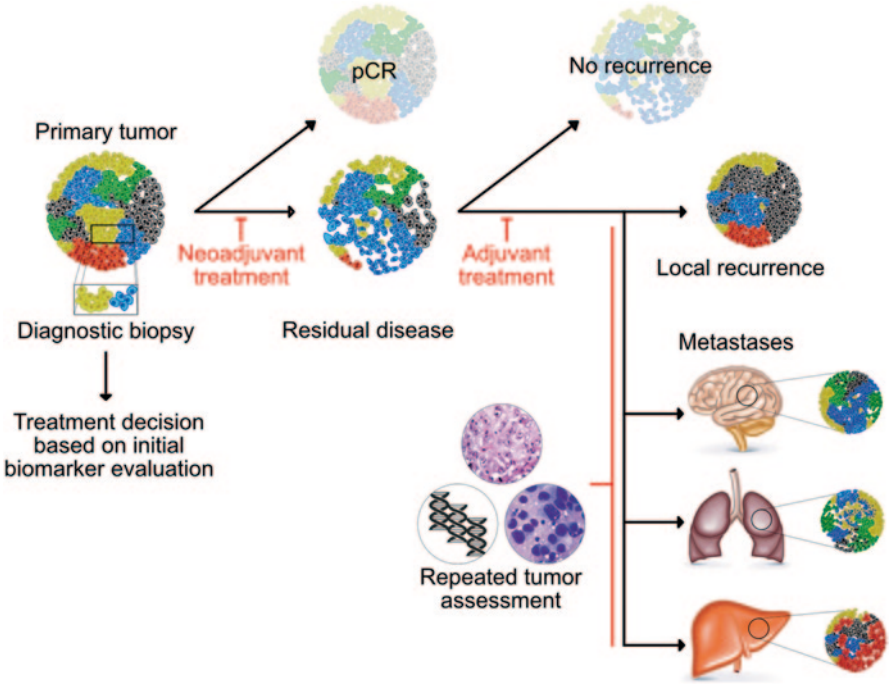

Fig. 2 Intratumor heterogeneity in cancer diagnosis and treatment. Intratumor heterogeneity of cellular phenotypes can complicate definitive cancer diagnostics and therapeutic decision-making. Due to spatial heterogeneity within tumors, a single biopsy might not provide an adequate reflection of the phenotypic composition of the tumor as a whole. Additionally, due to continuous tumor evolution during treatment, treatment design for recurrent tumors made based on scoring the dominant phenotype at the time of diagnosis might be misleading. Repeated assessment of tumor characteristics by serial sampling of tumors using biopsies, FNA (fine needle aspiration), or ctDNA is necessary to guide more rational treatment decisions. Robust biomarkers that accurately reflect intratumor heterogeneity may also provide better predictive estimates of therapeutic responses and probable pathways of tumor evolution. *pCR* pathological complete response.

Acknowledging this problem and in an attempt to optimize chemotherapy and limit development of resistance, a number of approaches have been developed from which we highlight metronomic therapy and adaptive therapy in breast cancer. Metronomic therapy refers to the scheduling of repetitive, low doses of chemotherapy drugs administered at close regular intervals with no extended interruption [81]. The first study on metronomic therapy in breast cancer was published in 2002 [82] and other trials have already combined this approach with the use of targeted therapies [83]. The prevailing view is that metronomic therapy is an option for breast cancer patients with a low toxicity profile and efficacy in most patients [81]. However, the impact of metronomic therapy on intratumor heterogeneity has not yet been thoroughly studied. An alternative approach is the adaptive therapy [84]. What

sets apart this strategy from the more traditional high density dose strategy (based on the Norton-Simon model [85, 86]) or from metronomic therapy strategy is the recognition that optimal therapeutic strategy may evolve and change in response to intratumor dynamics. According to this model, optimal treatment strategy would modulate therapy to maintain a stable population of chemosensitive cells that can, in turn, suppress the growth of resistant populations under normal tumor conditions and therefore increase survival [84]. However, most of the data regarding this strategy has not yet been translated into clinical trial design. Additionally for adaptive therapy use, both physicians and patients have to reconcile "cure" as an implicit or explicit objective, and rather focus on controlling the tumor, which can make it difficult to accept the widespread use of this strategy.

Different from conventional chemotherapy, two other strategies have been gaining relevance in the context of highly heterogeneous tumors. First is the identification of targets that play key roles in the generation of intratumor heterogeneity [87]. One of this targets that is quickly finding its way into the clinics are chaperones such as Heat Shock Proteins (HSPs), namely HSP90 [88]. In normal cells HSPs buffer mutated proteins, this way acting as a molecular checkpoint of proteins. In cancer cells, HSP's promote survival through tolerance to increased altered proteins and may promote environmental adaptation via regulation of phenotypic diversity [89]. Therefore, targeting HSPs has the potential to decrease tumor evolution and progression to treatment resistant disease with promising results already available in several preclinical models of breast cancer and even in a phase II study [90]. "Epigenetic homogenization" of the tumor to a common therapy-sensitive state via targeting epigenetic regulators is another potential approach that may decrease intratumor heterogeneity and improve therapeutic responses.

Immunotherapy is yet another strategy potentially useful in highly heterogeneous tumors. The principle of this strategy is that as highly heterogeneous tumors may produce large numbers of mutated proteins, these may turn to be tumor antigens inducing a simultaneous sustained anti-tumor response to large number of antigenic targets [91]. However, if the immunologic reaction cannot cope adaptively with tumor evolution—and the possible emergence of clones not presenting the target antigen—immunotherapy could be useless. Therefore, clear understanding of how immune-host-tumor interactions work is fundamental to the design of effective immunotherapy and how this can be useful in highly heterogeneous and evolving tumors.

Several strategies aiming to coping with intratumor heterogeneity by preventing it or reducing, by both "homogenizing" a tumor when a druggable target is available or reversing acquired drug resistance, are currently under development at both preclinical and clinical levels [92]. Apart from the development of new drugs, we expect in the near future that the better understanding of the impact of intratumor heterogeneity will possibly unlock the effective use of already approved drugs in new dosing schemes or combinations for a more effective individualized therapy.

Conclusion and Future Directions

In this chapter we have reviewed the sources of intratumor phenotypic heterogeneity, and the most commonly used technologies to study this phenomenon. Additionally we have commented on the clinical impact of intratumor heterogeneity in both diagnosis and therapy.

Tumor cell phenotypes are the result of a complex crosstalk between inputs from genome, environment, and stochastic processes. Diversity of cellular phenotypes within a tumor that arises from genetic and epigenetic abnormalities and the additional rounds of natural selection, poses the biggest contemporary obstacle to the understanding and clinical management of cancer. The better comprehension of intratumor heterogeneity can only be achieved by the recognition that tumors contain phenotypically distinct populations of both tumor and stromal cells that interact in a dynamic and reciprocal manner.

Intratumor phenotypic heterogeneity is already recognized and accounted for in many preclinical studies of breast cancer. Despite being recognized in clinical setting, translation of this knowledge and tools from the preclinical to the clinical setting is still slow and many important interrogations persist. In breast cancer, it seems clear that the degree of intratumor genetic heterogeneity has been associated with aggressiveness and poor prognosis [58–60]. However, how this information can be used for clinical decisions is still unanswered. Furthermore, given the contribution of nongenetic sources to intratumor phenotypic heterogeneity, we strongly favor the view that the link between non-genetic phenotypic diversity and clinical outcomes should be better explored.

Regarding the tools to study intratumor heterogeneity, single cell sequencing technologies are evolving at an extraordinary pace. However, as we already mentioned, only by coupling sequencing information with topological information is it possible to decipher the intricate network of interactions between the tumor cells, stromal cells and non-cellular components of microenvironments. This can never be achieved from the analysis of each of these individual components alone.

Concerning the clinical and therapeutic challenges, we believe there is potential for intratumor heterogeneity reduction strategies towards drug sensitive states. A considerable number of epigenetic states modifier drugs, as histone deacetylase inhibitors and other compounds, are already in late states of clinical development and have the potential to change the therapeutic landscape. Moreover, strategies aimed at reducing phenotypic heterogeneity through modulating the tumor microenvironment, such as anti-angiogenic therapies, are starting to be better understood and patients' subgroups that potentially benefit from them better identified. Additionally, while other new classes of drugs make their way into clinical use, better understanding of the evolutionary dynamics of tumors could lead to the development of novel therapeutic approaches through innovative administration and dosing schemes of the already used drugs. Unfortunately, so far, much of these insights are mostly based on pure mathematical modeling, and animal models to interrogate

evolutionary dynamics in tumor progression and test new therapeutic approaches are mostly non-existent.

We are confident that the deeper understanding of the evolutionary dynamics of tumors will allow the correct prediction of tumor evolutionary pathway and thus allow the design of novel and more effective therapeutic interventions that will improve patient prognosis.

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Pharmacogenetic Predictors of Response

Daniel L. Hertz and James M. Rae

Abstract Pharmacogenetics attempts to predict treatment response using a patient's "germline" genome as the biomarker of interest. This chapter on pharmacogenetic predictors of breast cancer response is divided into four sections. The first introduces readers to genetic variation and describes how variation in the germline genome can affect biology or pharmacology. The second section introduces the translational pathway for pharmacogenetic research and discusses the specific challenges to identifying pharmacogenetic predictors of breast cancer response. The third section is divided into three subsections, each of which discusses a distinct category of pharmacogenetic response predictors; pharmacokinetics, cancer cell sensitivity, and effector cell activation. Within each subsection a specific pharmacogenetic association is described in detail; *CYP2D6*-tamoxifen, *BRCA*-PARP inhibitors, and *FCGRA*-trastuzumab, respectively, followed by a general discussion of other less well-established examples or areas for further research. The chapter concludes with a summary of the current status of pharmacogenetic predictors of breast cancer response and a few predictions for the future of this field.

Keywords Pharmacogenetics · Germline polymorphism · Pharmacokinetic predictors · *CYP2D6* · Cancer-cell sensitivity · *BRCA* · Effector-cell activation · *FCGR*

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Variation in the Somatic and Germline Genome

The simplest and most common genetic variation is the substitution of a single DNA base. Other types of variation including insertions/deletions of a base, a segment of bases, or even large genomic regions, or translocations in which distant segments of the genome are fused, are far less common. Genetic variation can have functional consequence if it affects protein activity, by influencing protein expression and/or function (Fig. 1). Our ability to interpret consequences of genetic variation is extremely limited outside of the coding regions and the areas immediately adjacent to these regions, which represent an exceedingly small portion of the overall genome; however, substantial progress is being made toward understanding the influence of the remainder of the genetic code.

Variation in the genetic regions that encode proteins, the exons, is the most straightforward to understand. A variant in an exon can change the amino acid that is coded for, either to a stop codon, producing an incomplete protein that typically

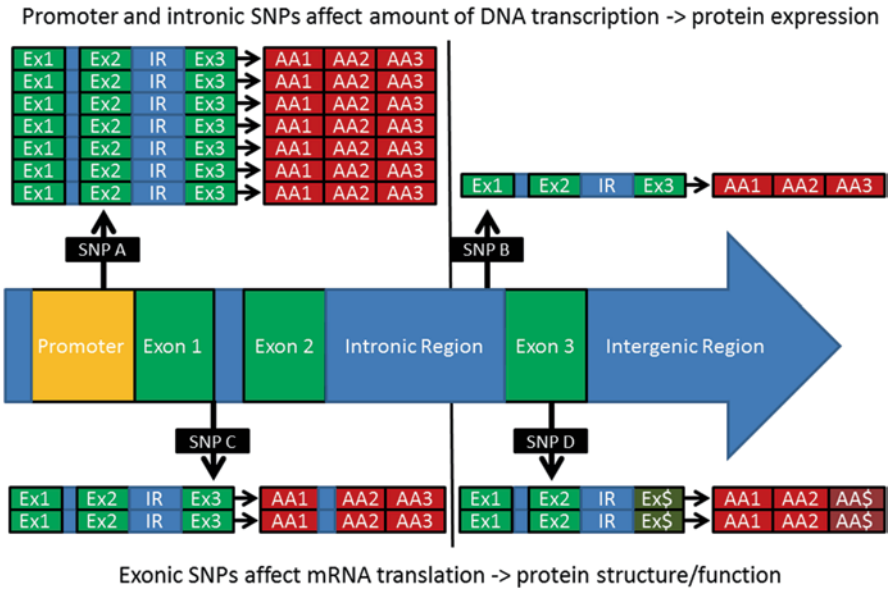


Fig. 1 Genetic variation can affect protein activity by influencing either protein expression or protein function. Single nucleotide polymorphisms (SNPs) in the promoter (SNP A) or intronic regions (SNP B) can affect the amount of DNA transcription that occurs. This has a downstream effect on the expression of the protein, but not the protein structure. In this image SNP A has increased expression of the normal protein (AA1-AA2-AA3) and SNP B has decreased expression. Alternatively, SNPs on exonic splice sites (SNP C) or within exons (SNP D) affect protein function by affecting the amino acid structure, but do not influence expression. SNP C caused a loss of the mRNA splice site between exons 1 and 2, yielding a protein with an extra segment of amino acids. SNP D changed the sequence in exon 3 non-synonymously, causing the third protein segment to also change structure. These structural changes can affect protein function, which in turn affects overall protein activity

has no functionality [1], or to a different amino acid, referred to as a non-synonymous variant. By changing protein structure non-synonymous variants are often assumed to diminish protein function but this is not always the case, many times non-synonymous variants retain normal activity or sometimes they increase activity. Alternatively, a synonymous variant codes for the same amino acid. These are typically assumed to be non-consequential, but this may also not always be the case [2]. Exonic variants can influence protein activity in other ways such as introducing or eliminating an mRNA splice site or shifting the amino-acid reading frame.

The regions surrounding exons also have known function, making interpretation of variation straightforward in some cases. In contrast to the exonic variants that typically modify protein structure and function, variants outside of the exons are more likely to affect protein expression. The best understood functionally consequential non-exonic variants are found in gene promoters and directly influence transcription and downstream protein expression [3]. Genetic variants outside of the promoter can also affect the expression of nearby, or even distant, genes, however the mechanisms by which this occurs are not completely understood. These variants are being catalogued within projects such as Single Nucleotide Polymorphisms and Copy Number Annotation (SCAN) [4] and the Genome Tissue Expression (GTEx) [5] while large-scale projects to assign functional mechanisms to variation in the non-coding genome, such as the the Encyclopedia of DNA Elements (ENCODE) [6], continue.

In cancer there are two genomes of interest, that of the patient (germline) and the tumor (somatic) [7]. Variation in these genomes can influence the patient's risk of developing cancer, the prognosis of a cancer they develop, or their likelihood of responding or experiencing toxicity during treatment. The somatic genome exists within the cancer cells and has changed from the germline genome in a way that circumvents the typical mechanisms that control cellular replication. During the last decade The Cancer Genome Atlas (TCGA) catalogued the somatic genomes of many tumor types [8], including breast cancer [9]. This immensely important work identified the genetic events that most often cause oncogenic transformation and defined a tractable number of targetable pathways for development of extremely effective cancer therapies [10]. Relative to other tumor types breast cancer has perhaps benefitted less from the genomic revolution because most of the oncogenic drivers had been identified prior to this work. The prognostic and predictive importance of estrogen or HER2 receptor expression has withstood the development of more sophisticated genetically-informed tools [11, 12]. Meanwhile, effective agents have yet to be developed that target the oncogenic pathways most commonly activated in breast cancer including PI(3)K and p53. While the somatic genome is immensely important in identifying biomarkers of cancer prognosis and treatment prediction, it is not the focus of this chapter.

An individual's germline genome is inherited from their parents and the genomes of any two unrelated people differ in millions of discrete ways [13, 14]. The vast majority of variants are single nucleotide polymorphisms (SNPs), common substitutions of individual DNA bases that have a population frequency 1%. The vast majority of these SNPs likely have no, or at most negligible, functional conse-

quence. However, a small minority of SNPs exerts a profound effect on the activity of a single, or in some cases many, proteins. Because the germline genome exists within the cells in all organs of the body, the consequences of germline genetic variation are more diffuse. The germline genome is extremely important in predicting a patient's risk of cancer occurrence; however, the field of disease genetics is distinct from pharmacogenetics and is also not covered in this chapter. Pharmacogenetics studies the effect of the germline genome on treatment outcomes, both response and toxicity, and this chapter will focus specifically on pharmacogenetic predictors of breast cancer response. Before discussing specific pharmacogenetic examples, it is important to understand the pathway of pharmacogenetic research, from discovery through clinical translation, and the particular challenges of identifying pharmacogenetic biomarkers of breast cancer response.

Identifying Pharmacogenetic Predictors of Breast Cancer Response

The immense potential for pharmacogenetics to improve the care of cancer patients was recognized relatively recently [15]. It is critically important that patients receive effective therapy to prevent cancer-related mortality, and nearly equally as important to avoid superfluous therapy due to the morbidity, and sometimes mortality, associated with treatment. Validated pharmacogenetic predictors of efficacy or toxicity can inform selection of the most effective agent, estimation of a dosing regimen that optimizes therapy, or avoidance of agents that are most likely to cause toxicity [16]. Unfortunately, the pathway from discovery to clinical implementation for biomarkers, including pharmacogenetics, is a long, complicated, challenging process (Fig. 2).

The first step in pharmacogenetic research is the initial discovery of an association. The vast majority of pharmacogenetic discovery studies are conducted retrospectively in "convenience cohorts," databases of patients with heterogeneous disease, treatment, and outcome data collection. These cohorts are widely available and these analyses are relatively quick and cheap. The problem is that in pharmacogenetics, as in other biomarker fields, most of the published discoveries cannot be replicated [17]. Retrospective correlative analyses are vulnerable to biases, including publication bias, and statistical confounding. Also, there is a general disregard for proper statistical methodology both by investigators and journal reviewers [18], which has inundated the pharmacogenetics literature with false positive discoveries [19]. Literature curation to differentiate true associations from false positives, such as Pharmacogenetics Knowledge Base (PharmGKB) [20], is extremely helpful but true validation requires replication in an independent dataset with an *a priori* defined analysis plan.

Independent validation of the association between the genotype and a clinical outcome, known as clinical validity [21], may be adequate for making genotype-guided treatment decisions if the genetic information previously exists [22, 23]. The

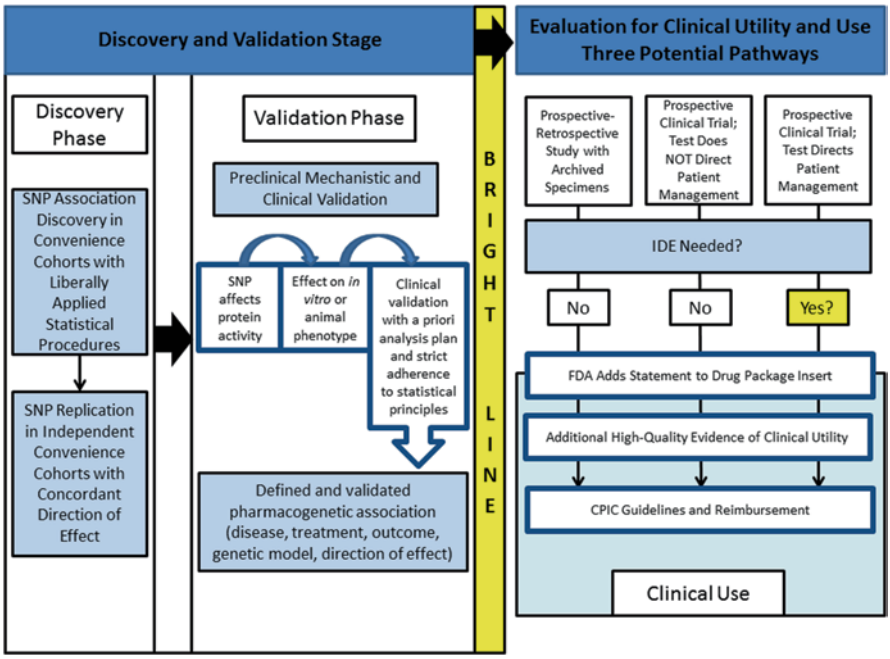


Fig. 2 The pathway from initial discovery to clinical use for pharmacogenetic associations. Initial discovery and replication of pharmacogenetic associations occurs in convenience cohorts with liberally applied statistical procedures. Validation could then take place mechanistically using *in vitro* and animal models in addition to clinical validation in independent patient cohorts with strict statistical procedures. After validation, high priority pharmacogenetic biomarkers should be moved into prospective validation of clinical utility, followed by development of CPIC guidelines and clinical use. This figure is adapted from an Institute of Medicine report describing the general translational pathway for omics biomarkers [28]

Clinical Pharmacogenetics Implementation Consortium (CPIC) publishes consensus guidelines for making genetically informed treatment decisions in this situation [24]. Genetic information exists for some patients due to direct-to-consumer genotyping or from previous pharmacogenetic analyses. Cancer patients are particularly likely to have existing genetic information from somatic genetic analyses that are becoming more common [25], creating an efficient setting for pharmacogenetic implementation [26]. In situations where genetic information does not exist, and pre-emptive genotyping is necessary, clinical validity is necessary but not adequate for clinical translation of pharmacogenetics.

Demonstration that genetically informed treatment decisions improve treatment outcomes, known as clinical utility, is typically considered a requirement for pharmacogenetic implementation. Very few pharmacogenetic associations have adequately demonstrated clinical utility in prospective, randomized, genotype-guided clinical studies, which require large patient cohorts and are extremely expensive but lack a financial stakeholder willing to provide funding [27]. The other option,

which has been established as the evidentiary threshold for biomarkers in general, is the prospective-retrospective study [28]. In this design, a previously conducted prospective clinical trial is used to validate the clinical utility of a biomarker. Prospective clinical trials are superior to convenience cohorts because they have defined criteria for patient inclusion, uniform treatment, and systematic collection of outcomes. These analyses are still subject to biases and confounding, but they have proven to be extremely useful datasets for attempting validation of pharmacogenetic associations that had previously been replicated multiple times, as can be seen in several of the examples in the next section.

Several factors have contributed to the lack of clinically useful pharmacogenetic biomarkers of breast cancer response. First, as described earlier, the somatic genome is the cause of cancer growth and the primary source for response biomarkers for either targeted or chemotherapeutic agents [29]. Second, breast cancer pharmacogenetic research is complicated by the use of combination therapy, which can mask pharmacogenetic associations [30] and make it challenging to distinguish which agent is responsible for treatment efficacy. Third, breast cancer recurrence in the adjuvant setting is relatively uncommon, severely limiting statistical power. Finally, it is unclear whether pharmacogenetic biomarkers of response that have been validated in other tumor types or treatment regimens are generalizable to breast cancer patients. In part due to these challenges pharmacogenetic biomarkers currently have limited utility for predicting breast cancer response, but the next section will explain why that is unlikely to be the case indefinitely.

Pharmacogenetic Predictors of Breast Cancer Response

This section is divided into three subsections, each describing a category of pharmacogenetic predictors of cancer response (Table 1). The first subsection focuses on predictors of drug exposure, or pharmacokinetics, which could be referred to as "classical pharmacogenetics." Variants in these genes indirectly influence treatment efficacy, and toxicity in many cases, through a direct effect on pharmacokinetics. These associations are probably particularly important for non-targeted drugs, primarily cytotoxic agents, which has been the backbone of systemic treatment until the recent past. The second subsection focuses on pharmacogenetic predictors that dictate the sensitivity of cancer cells to treatment, which exist in both the tumor and patient genome and occupy a gray area between pharmacogenetics and somatic genetics. These are mostly, but not exclusively, relevant to targeted therapies that are replacing chemotherapy in many tumor types. The last subsection focuses on a relatively new area, pharmacogenetic predictors of the activation of immune effector cells. Therapies that activate the immune system to fight cancer have finally reached clinical application, and the host genome is likely to be a critical predictor of the immune response to these therapies. In an interesting way these categories mimic the past, present, and future of both pharmacogenetics and cancer treatment. Because of this the information available regarding the drugs, and the pharmacoge-

Table 1 Three categories of pharmacogenetic predictors of cancer response

Pharmacogenetic biomarker category	Most relevant cancer treatment approach	Types of relevant protein	Examples in breast cancer	Preclinical mechanistic models		Biomarker for clinical studies	Treatment approach for patients
				Cellular	Animal		
Pharmacokinetic	Chemotherapy	Enzymes and transporters	*CYP2D6*-Tamoxifen *SLCO1B1*-Methotrexate	Drug metabolism in liver cells	Drug exposure in healthy animal	Systemic drug concentration	Change dose to optimize drug exposure
Cancer cell sensitivity	Targeted therapy (including hormonal)	Drug targets and intra-cellular signaling pathways	*BRCA*-Olaparib *ERCC1/2*-Carboplatin	Cancer cell cytotoxicity	Tumor response in animal cancer model	Tumoral kinase phosphorylation?	Select agent based on mechanism/target
Effector cell activation	Immunotherapy	Immunogenic activation and inter-cellular signaling	*FCGR2/3A*-Trastuzumab *CTLA4*-Ipilimumab	Cancer cell cytotoxicity when co-incubated with effector cells	Inflammatory response in animal model	Systemic inflammatory markers	Select immunotherapy as treatment approach

netic associations with their effectiveness, tends to decrease from the first subsection to the last. These subsections are also differentiated by the preclinical model systems used to mechanistically validate associations and the translational approach to treating patients who carry validated variants, topics that are commented on where appropriate.

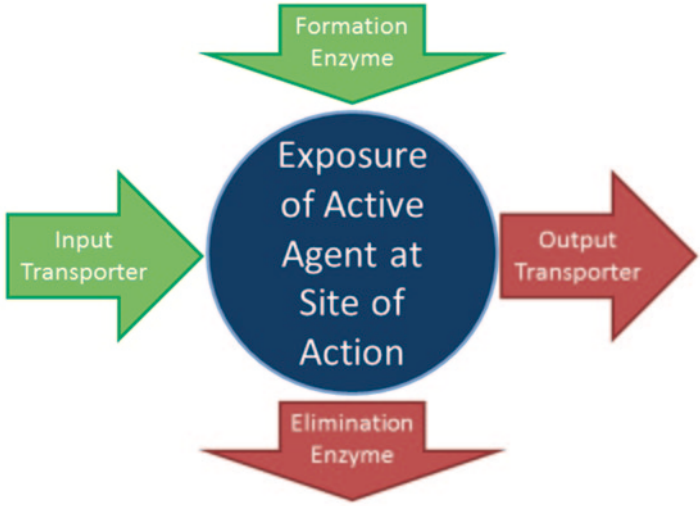
Pharmacogenetics of Drug Pharmacokinetics

The vast majority of pharmacogenetic research, across disease states, has focused on non-synonymous SNPs in genes encoding enzymes and transporters that have a putative influence on drug pharmacokinetics, and a downstream effect on treatment outcome. These have been the object of intense discovery efforts because drug metabolism and transport are relatively well understood and a small number of enzymes and transporters are responsible for the majority of the metabolism, distribution, and elimination of most drugs. Much of the common genetic variation within these genes has been identified and the findings for one substrate are often generalizable to other substrates, thus, the effort of pharmacogenetics researchers across many drugs and disease states feeds into a repository of shared knowledge. Genetic variants that affect the expression or function of enzymes or transporters can be mechanistically validated in established *ex vivo* model systems. Cellular models are useful for validating the direct effect on protein expression or activity and rodent models can be used to investigate the downstream influence on pharmacokinetics and treatment outcome.

The result of all of this effort is double-edged, many variants that influence pharmacokinetics have been discovered and validated, but many false positives have also been published for these genes. A distinct advantage for pharmacogenetic predictors of pharmacokinetics, that has been inadequately leveraged, is that the relevant *in vivo* phenotype, drug concentration, can be directly measured with relative ease. Demonstration that patients who carry a particular genetic variant have different exposure to the active agent should be used as an initial step of validation prior to attempted translation of these associations.

There is an interesting debate as to whether an association with pharmacokinetics should be considered "clinical validity". The association between pharmacokinetics and efficacy is generally accepted for chemotherapy, hence maximum-tolerated dosing, but is often quite weak for other drug classes. The other situation for which pharmacogenetic determinants of pharmacokinetics seems to be particularly critical is for pro-drugs, drugs that are inactive in their administered form and require bioactivation [31]. The association between cytochrome P450 2D6 (*CYP2D6*) and tamoxifen that is described in this subsection fits into this category, yet, there is intense debate as to the clinical validity of this association. Those who are more conservative about changing practice require validation that the genotype is associated with an actual clinical outcome, which has also been a tremendous challenge.

Patients who carry genetic variants that are validated to influence pharmacokinetics should be dose-adjusted to achieve optimal drug exposure. This requires an



Protein	Protein Activity	Resulting Exposure
Formation Enzyme or Input Transporter	⇩	⇩
	⇧	⇧
Elimination Enzyme or Output Transporter	⇩	⇧
	⇧	⇩

Fig. 3 The downstream consequence on drug exposure of pharmacogenetic variants depends on the interaction between the protein and the drug and the effect of the variant on protein activity. If the protein of interest is an enzyme that forms the active agent (i.e. prodrug activation) or a transporter that brings the drug to the site of action (i.e. intestinal absorption) then the resulting exposure will be directly related to the change in protein activity. However, if the enzyme eliminates the active agent (i.e. metabolic inactivation) or removes the drug from the site of action (i.e. hepatic elimination) then the resulting exposure will be inversely related to the change in protein activity

understanding of the drug, the role of the enzyme/transporter, and the consequence of the genetic variation (Fig. 3). One interesting consideration for these associations is that pharmacokinetics often determines both efficacy and toxicity. There is understandable reluctance from clinicians to pre-emptively decrease dosing in patients with genetic variants that increase toxicity risk, as decreasing dosing would be expected to decrease treatment effectiveness in addition to toxicity.

CYP2D6 and Tamoxifen

The possible association between *CYP2D6* genotype and tamoxifen effectiveness in adjuvant breast cancer is one of the most researched and debated pharmacogenetic associations [32]. Tamoxifen is a highly effective selective-estrogen receptor modulator (SERM) that is used to treat and prevent estrogen receptor (ER)-positive

breast cancers. The mechanism of tamoxifen activity is its antagonism of ER in cancer cells that are reliant on estrogenic signaling for growth and replication [33]. However, tamoxifen itself is a weak anti-estrogenic prodrug that requires metabolic activation. The metabolism of tamoxifen includes dozens of metabolites formed through various discrete and overlapping metabolic pathways [34]. Tamoxifen and its most abundant metabolite, n-desmethyl-tamoxifen, are each substrates for CYP2D6-catalyzed bioactivation to 4-hydroxy-tamoxifen and endoxifen, respectively [35]. These metabolites are far more potent ER antagonists, and are hypothesized to be responsible for the anti-estrogenic efficacy of tamoxifen.

The *CYP2D6* gene is one of the most important pharmacogenetic predictors of drug pharmacokinetics. It is a highly polymorphic enzyme with more than a hundred curated variants, plus common gene duplications and deletions [36]. This genetic variability causes dramatic variation in CYP2D6 activity across patients. Complex systems have been created for characterizing the metabolic activity of patients based on their *CYP2D6* genotype [37] and pharmacogenetic *CYP2D6* associations for other drugs such as codeine have been translated into clinical practice [38]. The CPIC guidelines classify more than 20 variants as non-functional, another 12 as reduced-function, and about 10 others as normal-function, enabling classification of each patient as a poor (PM), intermediate (IM), extensive (EM), or ultra-rapid (UM) metabolizer, in ascending order of enzyme activity.

The reliance of tamoxifen bioactivation on CYP2D6 suggests that there could be a role for genotype-directed tamoxifen treatment in breast cancer patients. Several clinical analyses have validated that CYP2D6 activity, as estimated by genotype, is a strong predictor of endoxifen concentration [39, 40]. What remains unclear is whether this pharmacokinetic difference is relevant to treatment effectiveness. Two studies have reported that patients at the bottom end of the distribution of endoxifen concentration have inferior outcomes in the adjuvant treatment setting [41, 42]. In contrast, the first prospective study testing for possible correlations between endoxifen concentrations and breast cancer recurrence found that higher endoxifen concentrations correlate with higher risk of recurrence [43]. Therefore, consensus has not been reached as to whether endoxifen concentration is associated with breast cancer outcomes and, if so, what the target concentration should be. More studies that collect endoxifen concentrations and treatment outcomes are necessary to adequately answer these questions.

Despite several dozen studies, consensus has not been reached as to whether there is an association between *CYP2D6* genotype and tamoxifen effectiveness. Most of the analyses reported to date are typical discovery efforts, retrospective analyses in small patient cohorts, that have reported discordant results, including positive associations in the opposite direction [32, 44]. It was anticipated that prospective-retrospective analyses in large clinical trial cohorts including Arimidex and Tamoxifen Alone or in Combination (ATAC) and BIG 1-98 would provide a definitive answer. Neither of these analyses supported the hypothesis that patients with low-activity *CYP2D6* phenotypes had inferior efficacy from tamoxifen treatment [45, 46], however, the validity of these results and those of other studies continues to be debated [47–50].

Due to the ongoing debate regarding the clinical validity of *CYP2D6* genotype as a predictor of tamoxifen response, little has been done for prospective translation into clinical practice. One prospective clinical study dose-escalated only the *CYP2D6* PM and IM patients to 40 mg/day and measured all patient's endoxifen concentration at enrollment and after 4 months. After dose escalation the IM patients achieved endoxifen concentrations similar to that of EMs who remained on 20 mg/day, however, the PM patients on 40 mg/day were still significantly below this relative target [51]. In theory, increasing the dose of tamoxifen in *CYP2D6* PM patients could improve drug efficacy regardless of whether they achieved endoxifen levels similar to those in EM patients if, as reported in prior studies, there is a threshold endoxifen concentration below which tamoxifen efficacy is compromised [41, 42]. The other consideration, as previously mentioned, is that increasing the dose of tamoxifen could simultaneously increase treatment-related toxicities. In the genotype-guided prospective study there was a modest, but statistically significant increase in treatment-related toxicity in IM patients dose-escalated to 40 mg/day [52]. An ongoing prospective *CYP2D6*-guided dose escalation study in Europe that is collecting outcomes should eventually answer some of the remaining questions regarding the clinical validity and utility of the pharmacogenetic association between *CYP2D6* and tamoxifen. Unless these data demonstrate clinical benefit, the existing data on enhanced toxicity, combined with debatable clinical validity, argue against wide-scale implementation of *CYP2D6*-guided tamoxifen dose escalation for PM patients. This is the current position of the most influential clinical practice guidelines committees [53, 54], the Food and Drug Administration (FDA) which has chosen not to include *CYP2D6* genetic information in the tamoxifen package insert, and an implicit decision by CPIC who has not released any guidelines for dose-adjusting tamoxifen based on *CYP2D6* genotype.

Other Pharmacokinetic Pharmacogenetic Examples

There are a few notable pharmacokinetic pharmacogenetic associations for breast cancer drugs. Methotrexate was commonly used in adjuvant breast cancer in combination with cyclophosphamide and 5-Fluorouracil (CMF) until randomized studies demonstrated superior efficacy or tolerability of anthracycline-based regimens [55]. Methotrexate is still used in some patients and there are strong data supporting an association between methotrexate pharmacokinetics and variants in *SLCO1B1*, which encodes the OAPT1B1 transporter responsible for hepatic methotrexate uptake [56, 57]. The non-synonymous *SLCO1B1**5 variant (rs4149056, V174A) creates a decreased function transporter, increasing systemic concentrations of substrates including simvastatin [58, 59] and, interestingly in breast cancer, estrone conjugates [60]. This variant is strongly predictive of methotrexate pharmacokinetics [61–64], specifically in pediatric tumor types in which methotrexate is administered at doses 100-fold larger than those given in breast cancer. Several studies of high dose methotrexate have reported associations with toxicity, the dose of leucovorin necessary

to prevent toxicity [64–66], or event-free survival [67], but again these studies are in high-dose therapy and may not be relevant to breast cancer.

5-fluorouracil (5-FU) is another component of CMF now used much less frequently in breast cancer. 5-FU has an established and clinically useful pharmacokinetic association with dihydropyrimidine dehydrogenase, encoded by *DPYD* [68]. Low-activity *DPYD* polymorphisms increase 5-FU systemic concentration dramatically [69–71]. Most of the clinical pharmacogenetic studies focus on the severe, often life-threatening toxicity in patients with *DPYD* variants treated at full dose, but it is possible that sufficiently decreased doses could be safe [72, 73]. Doses that normalize the 5-FU concentration with that of *DPYD* wild-type patients may be exceptionally effective in these patients, whose tumors should be exquisitely sensitive to 5-FU treatment [74, 75]. The association with 5-FU may be of limited relevance in breast cancer but these data should apply similarly to capecitabine, an inactive prodrug of 5-FU. There are surprisingly little data evaluating polymorphisms in carboxylesterases (*CES1* and *CES2*) [76, 77], which are necessary for the bioactivation of capecitabine to 5-FU, and the systemic 5-FU concentration in capecitabine treated patients, though there is one report of an association with treatment efficacy [78].

Cyclophosphamide, another prodrug requiring enzymatic bioactivation, has also been somewhat overlooked in pharmacogenetic predictors of pharmacokinetics. Few studies have analyzed the effect of these SNPs on cyclophosphamide bioactivation or efficacy, though one study has reported increased concentration of the inactive parent in carriers of the low-activity *CYP2C19*17* allele [79]. Non-synonymous *CYP2C8* variants with diminished paclitaxel metabolic activity have been reported [80], and at least once associated with pharmacokinetics [81]. However, larger analyses have failed to replicate this association or discover other enzyme or transporter SNPs with strong predictive effects [82], suggesting that paclitaxel pharmacokinetic variability is not strongly affected by a single polymorphism. Finally, there has been some work discovering pharmacogenetic predictors of aromatase inhibitor pharmacokinetics including a *CYP2A6*-letrozole association [83], but the link between anti-estrogen drug concentration and effective estrogen depletion or efficacy has been difficult to establish [84], as described earlier for tamoxifen.

Pharmacogenetics of Cancer Cell Sensitivity

As discussed previously, response to cancer treatment is primarily based on the somatic (tumor) genetics. This next group of pharmacogenetic predictors could be considered somatic genetics; they are found in both genomes and their importance is derived from their existence in the somatic genome. They dictate the sensitivity of the cancer cell to the particular mechanism of action of cancer drugs. Breast cancer drugs have a variety of mechanisms of action; including DNA damaging agents (platins, cyclophosphamide), microtubule targeting agents (taxanes, vincas), anti-es-

trogens (tamoxifen, aromatase inhibitors), and HER2 targeting agents (trastuzumab, pertuzumab, lapatinib). Variants that affect the drug target or the balance of pro- and anti-apoptotic signaling could dictate sensitivity to these drugs. These variants can be mechanistically validated *in vitro* via genetic modification of cancer cells and comparison of drug sensitivity followed by comparison of treatment response in rodent cancer models.

There are two distinct research pathways for pharmacogenetic biomarkers in this category. The first is the typical retrospective effort to identify variants relevant to the mechanisms of existing, untargeted chemotherapy agents. It is critical to recognize how limited our mechanistic understanding is of many of the chemotherapeutic drugs that are used to treat cancer. For example, paclitaxel has been used for decades with a putative mechanism of action of microtubule assembly causing mitotic arrest [85], but within the past year a new mechanism of action was reported to predominate at physiological paclitaxel concentrations [86]. Similarly, the mechanism of action for trastuzumab, one of the first and most effective targeted agents for breast cancer treatment, continues to be debated, as will be discussed in the next section. Our limited understanding of drug mechanism precludes effective selection of candidate genes for pharmacogenetic association testing. The second research effort is quite different from the retrospective discovery and validation strategy. The understanding of cellular biology and oncogenesis has evolved tremendously during the past few decades, enabling rational design of drug mechanisms. Drugs with specific mechanisms are being developed to take advantage of the vulnerabilities of cancer cells, some of which exist in the germline genome, such as *BRCA* described in this section. These germline pharmacogenetic biomarkers of targeted agents are thought of as drug targets, and these drugs are often approved with companion diagnostics for somatic genetic evaluation to inform selection of the appropriate agent. Clinicians have become comfortable with the concept that a tumor diagnostic, such as receptor expression, can be used to select an effective agent, as opposed to the somewhat unfamiliar concept of using patient genetics to inform selection of a drug dose. This next section describes a clinically relevant example of germline genetic variation in the tumor genome that dictates cancer cell sensitivity, *BRCA* and PARP inhibitors, and surveys other ongoing but less validated pharmacogenetic associations with breast cancer sensitivity for untargeted drugs.

BRCA and PARP Inhibitors

The breast cancer susceptibility genes (*BRCA1* and *BRCA2*) are responsible for homologous recombination, one of two complimentary cellular DNA repair pathways. These genes are very well known for their critical importance in determining susceptibility to several tumor types, most notably breast and ovarian cancer [87]. Germline *BRCA* variation increases cancer risk because loss of homologous recombination increases the error rates during DNA replication. Eventually one of these errors occurs in an oncogene or tumor suppressor gene, causing oncogenic conver-

sion of the cell. The importance of germline *BRCA* mutations extends beyond cancer risk as they are also informative of cancer prognosis [88] and, most relevant to this chapter, prediction of treatment effectiveness.

Cells with germline *BRCA* mutations are unable to perform homologous recombination, leaving them susceptible to DNA damage. This knowledge was used to develop a class of targeted agents that interfere with the complimentary pathway for DNA repair, base-excision repair. Base-excision repair is performed by a protein complex that includes the enzyme Poly(ADP-ribose) polymerase (PARP). A PARP inhibitor has little activity when administered to a cell that can perform homologous recombination and a cell that loses homologous recombination due to *BRCA* mutation can survive as long as base-excision repair is still functional. However, when a PARP inhibitor is administered to a cell that lacks homologous recombination the combination has synthetic lethality [89]. In preclinical studies PARP inhibition is highly effective in *BRCA* mutant cell lines [90] and early clinical trials of the first generation PARP inhibitor olaparib in breast cancer patients demonstrated impressive efficacy [91]. Unfortunately, the first PARP inhibitor to complete a Phase III trial in breast cancer, iniparib, failed to improve progression free or overall survival [92]. It has been suggested that these disappointing results are due to inadequate PARP inhibition for this specific compound [93], and clinical development of PARP inhibitors in *BRCA* deficient patients continues. The recent approval of olaparib in *BRCA* mutant ovarian cancer proves the potential efficacy of agents designed to target cancer cell vulnerabilities caused by germline genetic variation.

Other Cancer Sensitivity Pharmacogenetic Examples

A logical strategy for treatment of tumors that cannot perform DNA repair is to use a DNA damaging agent such as a platinum. In retrospective studies *BRCA* mutations are biomarkers of effectiveness of platinum containing regimens [94, 95]. The combination of platinums and PARP inhibitors is currently being tested in *BRCA* deficient breast and ovarian tumors [96]. There is strong evidence that variants in other genes performing DNA repair including *ERCC1*/2 and *XRCC* predict sensitivity to DNA damaging agents and efficacy of platinums in other tumor types [97–99]. There are relatively little data for SNPs in these genes and response in breast cancer specifically, though the sensitizers to treatment should theoretically be generalizable across tumor types.

There are other examples of germline genetic variants that may predict cancer cell sensitivity but as of yet none have moved into prospective validation. As with all pharmacogenetic biomarkers, for nearly every drug a successful biomarker study has been reported. There have been reports of germline variants that regulate expression of B-tubulin that are associated with paclitaxel treatment efficacy [100], however, this has not been replicated. Similarly, thymidylate synthase (*TYMS*), the target of 5-FU and capecitabine, and methylenetretrahydrofolate reductase (*MTHFR*) have been extensively studied as biomarkers of sensitivity to several agents

across tumor types, with intriguing results [101, 102]. Finally, there are several reports that variants in *NQO1/2*, which are involved in scavenging and detoxification of free radicals, may be associated with doxorubicin efficacy [103, 104].

Pharmacogenetics of Effector Cell Activation

The last category of pharmacogenetic associations is relatively underexplored but is poised to be critically important. The drugs currently used in cancer primarily work through direct activity within the cancer cell. The previous two subsections described genetic variation that influences the amount of the active drug that reaches the cancer cell and the sensitivity of the cancer cell to the drug. Recently there has been tremendous progress in activating the patient's immune system to help fight the cancer, and the field of immune therapy is likely to be the next evolution in cancer treatment. This third group of pharmacogenetic associations is the genetic variants that dictate the activation of the immune cells that attack the tumor.

Specific aspects of genetic variation of immune system activation have been explored in detail. One that is highly relevant to pharmacogenetics is germline variation in the HLA system. Some rare, severe treatment related hypersensitivity reactions that were previously unexplainable can now be predicted based on HLA genotype [105–107], including lapatinib-induced liver toxicity [108, 109]. Another example, that is more relevant to efficacy of immune-mediated treatment, is the effectiveness of hepatitis C treatment in patients carrying SNPs in *IL28B* [110, 111]. These results demonstrate, unsurprisingly, that the patient genome can be extremely important in dictating the host's response to immune activation.

The era of cancer immunotherapy has just begun, so there is somewhat limited understanding of the mechanisms of these drugs and the interaction between the immune system and the tumor. However, there is tremendous existing knowledge about the inter-cell signaling and immune system activation, which should be applicable to selection of candidate genes for pharmacogenetic biomarker discovery. These fields also have established *in vitro* and animal models of immune activation that should be adaptable for mechanistic validation of genetic variation. Another advantage to this field is that immune activity has established *in vivo* surrogate markers, cytokines and interleukins. While this is not the direct phenotype of interest, like drug concentration is to pharmacokinetic associations, this is a superior marker to what is available for biomarkers of cell sensitivity.

Pharmacogenetic predictors of breast cancer response to immunotherapy will be discovered retrospectively and during drug development, as pharmacogenetics is now being integrated into most clinical trials. This should streamline the process for clinical validation and perhaps enable prospective demonstration of clinical utility during pivotal clinical studies. These pharmacogenetic biomarkers will likely be used to select an immune-active agent for patients predicted to mount a robust immune response, similar to the selection of targeted drugs in tumors sensitive to

specific drug mechanisms. While it is very early days for this field, there is some existing data for trastuzumab, which may work at least in part through immune activation, and the *FCGR* genes.

FCGR and Trastuzumab

The HER2 receptor is overexpressed in approximately 20% of breast tumors and is a marker of aggressiveness and poor prognosis. Trastuzumab is a humanized IgG1 monoclonal antibody designed to target HER2 that is very effective in these tumors [112]. It is standard practice that all breast cancers are evaluated for HER2 expression to determine whether the patient should receive HER2 directed therapy [113]. Additional HER2 targeted agents including pertuzumab, lapatinib, and trastuzumab-emtansine have been approved for use in HER2 overexpressing tumors [114]. Although there are several effective treatment options, many HER2-positive tumors have inadequate response to trastuzumab and these other targeted agents.

There is some debate regarding the mechanism of action for trastuzumab. It was originally believed that the effectiveness of the monoclonal antibody was due to inhibition of HER2 dimerization and prevention of cellular replication signaling. However, this may not be the only, or even the predominant, mechanism of action. Trastuzumab and other monoclonal antibodies including cetuximab (anti EGFR) and rituximab (anti CD-20) may work through antibody-dependent cell cytotoxicity (ADCC) [115]. ADCC occurs when the monoclonal antibody attaches on one end to the cancer cell antigen, HER2 in the case of trastuzumab, and on the other end with an effector cell of the immune system. Several effector cells of the immune system bind to the Fc fragment of antibodies including natural killer cells, dendritic cells, and macrophages. This interaction activates the effector cell which then signals other immune cells to locate cancer cells expressing the antigen and destroy them.

Binding of the effector cell to trastuzumab occurs via the fragment-c gamma receptor (FcγR) [116]. The hypothesis that ADCC contributes to the activity of monoclonal antibodies is supported by *in vitro* mechanistic work demonstrating less efficacious treatment in FcγR-null mice [117]. FcγR has several subtypes including two that are known to have non-synonymous polymorphisms, *FCGR2A* (H131R) and *FCGR3A* (V158F). *In vitro*, cells with the variant receptor bind antibodies less strongly and cause less immune activation [118–120]. Several small, retrospective biomarker studies reported that rituximab efficacy was dependent on *FCGR* genotype [121–123], however, subsequent larger analyses were not able to confirm the predictive role for these specific variants [124–126]. Similarly, there were several reports that the *FCGR* polymorphisms predict efficacy of trastuzumab [127, 128], one of which was accompanied by *ex vivo* evidence of greater cytotoxicity in the mononuclear cells from patients with the wild-type genotype [129]. However, once again, when validation was attempted in a prospective-retrospective analysis of a large clinical trial cohort with systematic enrollment, treatment, and outcome data the association could not be confirmed [130].

Other Effector Cell Pharmacogenetic Examples

Clinically relevant pharmacogenetic biomarkers of immune-therapy treatment efficacy are likely to be found in the germline genome. Immunotherapies including PD-1/PD-L1 inhibitors, such as nivolumab which was recently approved in melanoma [131], are currently being tested in many tumor types including breast cancer (see NCT02129556, NCT02309177, NCT01848834, NCT01375842) [132]. No pharmacogenetic biomarkers of PD1/PD-L1 efficacy have been validated. There are preliminary reports of germline polymorphisms in *CTLA4* that predict response to CTLA-4 inhibitors including ipilimuab, used in melanoma [133, 134], and in early clinical studies in breast cancer (see NCT00083278).

Conclusions and Areas for Further Research

The challenges for discovery, validation, and translation of biomarkers have limited the clinical usefulness for pharmacogenetics in predicting breast cancer response. The germline genome influences the pharmacokinetics of many drugs; however, it is unclear whether there are any SNPs that are informative for selection of doses of the drugs commonly used in breast cancer. More studies should collect drug concentrations, particularly concentrations of active metabolites of prodrugs, to find those SNPs most likely to be clinically useful. Biomarkers of efficacy for untargeted and targeted cancer treatment are more likely to exist in the somatic genome, and some of these may originate in the germline genome and be useful for drug selection. A more detailed understanding of the mechanisms by which drugs work would be instrumental in improving the ability to select candidate genes, and identify candidate SNPs, for pharmacogenetic analyses. Finally, as the field of cancer immunotherapy progresses, investigators should recognize that the predictors of immune activation are extremely likely to reside in the patient's genome. Systematic collection of germline genetics and *in vivo* markers of immune activation, such as cytokines and interleukins, during clinical development will ensure efficient discovery of pharmacogenetic biomarkers and validation in adequately powered prospective-retrospective analyses. Meanwhile, continued development of cellular and animal models for interrogation of the effect of genetic variation on pharmacokinetics, cancer cell sensitivity, and effector cell activation would enable mechanistic validation of pharmacogenetic discoveries for clinical translation. As chemotherapy has given way to targeted therapy this era in cancer treatment will be remembered as the age of the somatic genome; and as immunotherapy replaces targeted therapy the next era in cancer treatment may well be the age of the germline genome.

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Metabolomics in Breast Cancer: Current Status and Perspectives

Christopher D. Hart, Leonardo Tenori, Claudio Luchinat and Angelo Di Leo

Abstract Metabolomics refers to the study of the whole set of metabolites in a biological sample that constitute a reflection of cellular functions. Cancer cells display significantly altered cellular processes, and thus metabolites, compared to normal cells. This can be detected in a number of ways, and is already exploited to a limited extent in the diagnosis of cancer. The host response to the tumor is perhaps equally important, as it either rejects or permits tumor growth, and this may also potentially result in a measurable metabolite signature. Analysis then of entire pools of metabolites may yield critical information about both tumor presence and host response, and represent a possible novel collective biomarker for cancer behaviour that could allow prediction of relapse, response to therapy, or progression. Isolating meaningful differences in the sea of metabolites and within the context of significant metabolic heterogeneity both within and between patients remains a great challenge. This chapter will review current metabolomic research in breast cancer, with a focus on efforts to translate the technology into clinical practice.

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Introduction: What is Metabolomics?

At its most basic level, metabolomics refers to the study of some or all of the metabolites in a biological sample, be it tissue, cells, serum, or other bodily fluid [1]. Whilst not a new science, advances in detection methods, statistical analysis and computing power have led to renewed interest in this area and its potential in the field of cancer. It forms a distinct branch of the 'omics' sciences, along with genomics, proteomics or transcriptomics. Genomic analysis identifies the genes present, including mutations of functional genes. Yet only a subset will actually be expressed [2], meaning that the remainder may be of limited or no clinical significance. Furthermore, it will not identify the normal genes that are being overexpressed by other processes. The transcriptome, as defined by the measurable RNA present, represents then the output of the genome, while the proteins produced (the proteome) are the most relevant product, being a step closer again to clinical effect. However, the interplay between these proteins, their relative enzymatic activity and the direct clinical effects can still vary. For example, the presence of altered PI3K signalling molecules from a *PIK3CA* gene mutation does not necessarily result in increased downstream signalling of the AKT mTOR pathway, and can depend on PTEN concentration [3, 4]. The metabolome, by contrast, represents the step 'after the fact.' It is the collection of molecules that exists as a result of cellular processes, which are themselves a result of the enzymatic processes catalysed by products of the genome. It is thus direct evidence of what actually exists or existed, ie the phenotype, as opposed to what could exist, and offers a complementary and multidimensional picture of both the tumor and the host.

All cellular processes produce metabolites, whether as a specific function (hepatocytes) or as products of normal cellular activities such as maintenance of homeostasis, replication, and activation of signalling pathways. These in turn are also influenced by many factors including diet, toxins, diseases and drugs [5]. These metabolites therefore can represent any number of molecular classes, from small molecules or amino acids, to lipids or carbohydrates, or any of their breakdown products [6]. Collectively they are referred to as the metabolome, which is representative of all the processes occurring in a cell, an organ or the entire body at a particular time, and which necessarily varies over time according to the multitude of influences on the body, both normal and pathological.

Metabolites can be detected in any biological sample, ranging from blood (serum or plasma) to tissue, urine, sweat, tears, saliva, or even exhaled breath condensate [7, 8]. This represents a significant clinical advantage, as acquiring samples such as serum is straightforward yet may provide significant tumor-specific information, potentially representing a liquid biopsy and sparing the patient a more invasive procedure. The caveat to this is the sensitivity of the samples to incorrect handling—the

metabolic profile may change after sampling depending on a number of factors including temperature and changes in pH [9]—as well as the modulating effect of a number of variables discussed later.

Cancer Metabolism

In cancer, a number of metabolic processes are altered, either within the cancer cell, the tumor milieu, or in other parts of the body as a result of the cancer. Where this results in a measurable change in metabolites, such changes represent a potential biomarker of cancer presence or activity. Significantly altered metabolic pathways within cancer cells are well recognised. For example, many cancer cells employ aerobic glycolysis in place of the usual mitochondrial oxidative phosphorylation to generate adenosine triphosphate (ATP), a phenomenon known as the "Warburg effect" which is believed to confer a survival advantage in hypoxic conditions [10, 11]. This feature of malignant cells is already exploited in cancer imaging: fluorodeoxyglucose (FDG)-positron emission tomography (PET) relies on the enhanced uptake of radio-labelled glucose by cancer cells to define tumors on imaging studies. Other common metabolic shifts in cancer result in changes in choline and fatty acid metabolism [12]. Choline is typically absent or at very low concentrations in normal tissue, and found in higher concentrations in tumor. Magnetic resonance imaging (MRI) can be adapted to include spectroscopic interrogation of parts of the image down to a single voxel to detect choline levels; areas of high choline concentration are very likely to represent presence of malignancy. This is currently employed in brain imaging of gliomas, and screening for early breast cancer in high-risk populations.

Whilst metabolomic studies are used to detect individual metabolites that might serve as predictive biomarkers, this is not the only application. Furthermore, although several metabolites have been identified that correlate with the progression and development of breast cancer, this has not resulted in any significant clinical gains. Current metabolomics research aims to take this considerably further by looking at groups of metabolites or indeed the metabolome as a whole. These collections of data will contain patterns that then represent the metabolic signature of the sample, which can be compared to the patterns of other samples without the need to identify any of the individual molecules. This has the advantage of incorporating known and unknown metabolites of all the upstream events: gene expression and activated cellular pathways from the tumor; reactive and immunological responses from the host; as well as integrated signalling pathway cross talk and environmental influences, by far a more comprehensive picture, albeit embedded in a vast sea of other metabolite data.

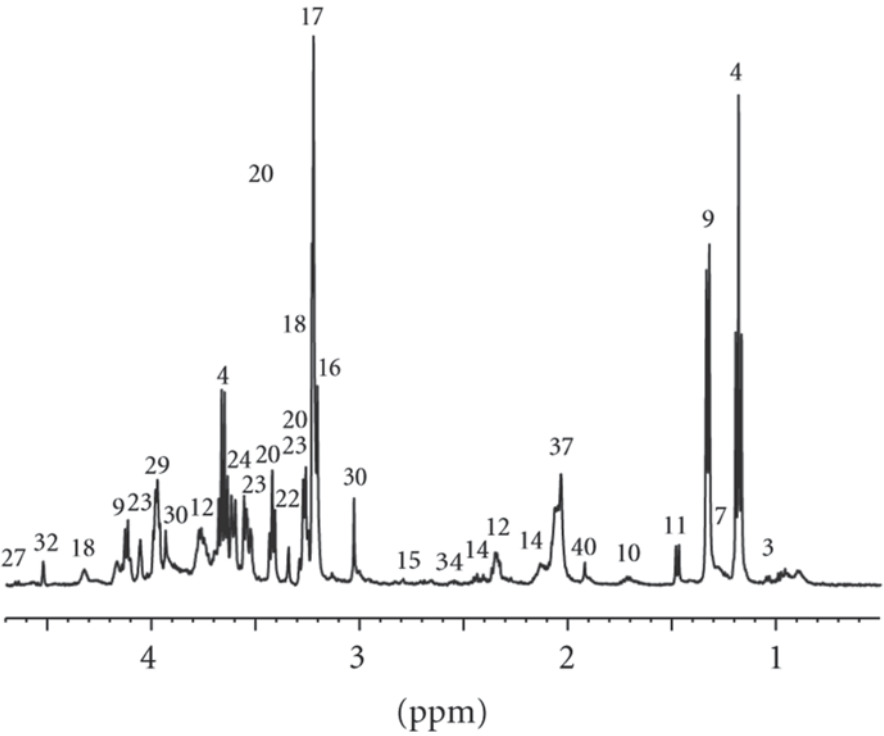

Fig. 1 NMR MAS spectrum of ovarian cancer tissue. Each of the numbered spikes represents a seperate metabolite, with the relative heights (signal strength) related to concentration. Fourty have been identified here, but a sample may contain hundreds. (Adapted from Ben Sellem et al., "Metabolomic Characterization of Ovarian Epithelial Carcinomas by HRMAS-NMR Spectroscopy," Journal of Oncology, vol. 2011, Article ID 174019, 9 pages, 2011. doi:10.1155/2011/174019. Permission for reproduction available under the Creative Commons Attribution License 3.0 (http://creativecommons.org/licenses/by/3.0/)

Metabolomic Techniques

Two standard techniques for metabolomic analysis are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). MS has higher sensitivity than NMR, and requires lower amounts of samples.

NMR is faster, less expensive and more reproducible [13]. Another advantage of NMR is that the sample requires only a minimal handling prior to the analysis. Because NMR does not damage analytes, it is particularly useful for studying metabolite levels in intact tissues, such as tumor biopsy samples, which can then be used in further experiments. In recent years, the development of high resolution ^{1}H magic angle spinning (MAS) made the acquisition of data on small slices of tissue without any treatment feasible: with the rapid spinning of the sample at the magic angle of 54.7°, the line broadening effects and the associated loss of information are reduced [14–16], resulting in high resolution spectra (Fig. 1).

Both techniques have their role in metabolomic research, depending on the aim of the investigation. In particular NMR can be used for rapid, untargeted screening; then, once metabolic pathways of interest are discovered, MS can be used in a targeted way to detect specific metabolites that could not be revealed in the NMR spectra due to the low concentration.

Analysis

Metabolomic data are high-dimensional in nature. As many as several hundred metabolite (relative) concentrations may be measured by means of NMR or MS platforms, usually on a limited number of samples. Biological information is retrieved from these data by means of univariate and multivariate statistical methods [17, 18]. Multivariate methods use the relationships among the variables, in contrast to univariate methods that focus solely on the mean and variance of a single variable. Commonly used univariate methods are t-test and analysis of variance [19]. Multivariate methods constitute a broad category that can be further divided into two types of data analysis: supervised and unsupervised.

Unsupervised analysis looks at the measured data on their own, to try to identify patterns. As such, the analysis is unbiased to the results, and is more open to discovery of novel metabolites or patterns of metabolite presence or concentration. It can be used to look for inherent patterns or intrinsic clustering that occurs within the samples, without knowing any outcome data, and may be more appropriate in exploratory experiments. On the other hand, it often involves extremely large quantities of data, requiring complicated mining methods to extract meaningful peaks or patterns. Once patterns have been established, they can be tested in a sample with known characteristic or outcomes, to see if the patterns offer genuine discriminating power, eg for diagnosis, prognosis, or prediction of response to treatment. Some examples are principal component analysis (PCA) [20], and the recently published KODAMA [21].

Supervised analysis involves obtaining data referenced to a known established control. This might be any number of previously identified metabolites. Statistical methods like multiple regression [22] or partial least squares discriminant analysis (PLS-DA) [23] and machine-learning techniques like artificial neural networks [24], random forest [25] and support vector machines [26] are used as supervised techniques in metabolomics [17, 27, 28].

One concern is that using established prognosis calculators to supervise and thus define the profile may risk developing yet another calculator of similar power, and thus no enhanced utility. Current prognostication based on tumor grade, size, biomarker status and nodal status, such as Adjuvant! Online, or even gene expression profiling, still misclassifies a significant proportion of patients, and it is for this very reason that improved techniques are being sought. Thus, unsupervised analysis must be the initial technique, rather than supervising with established risk factors. Then, to validate the result, the gold standard is to design large cohort prospective studies.

The science of measuring and interpreting correlations in metobolomics to infer significance and true inter-relatedness is in itself an evolving science [29]. As more metabolomic data are obtained and understanding of pathways is improved, these can be shared on public networks to try to offer a comprehensive picture of human metabolism [30, 31]. The Human Metabolome Database, for example, is one of several databases, and lists approximately 7900 metabolites [32].

Challenges

The metabolic profile of an individual is not static, but rather in constant flux according to the constant variation in cellular process in response to a number of factors, including normal homeostasis, exercise, diurnal rhythm, diet, hormones, and drugs [13]. This introduces many variables that can be difficult to control for. For example, certain metabolites can vary depending on how recently a person ate, or what time they took their regular medications. This creates increased noise in data acquisition, rendering these difficult to interpret. Furthermore, if these data are controlled carefully in experimental stage, the reproducibility in the real world may be difficult, where patients may be less likely to cooperate with dietary or other lifestyle factors [33, 34].

The metabolome of an individual [35, 36] will also vary significantly from that of another, regardless of the presence or not of malignant disease [5]. This is because it can reflect any number of small differences inherent, including race, sex, age, comorbidities, gut microflora, as well as factors mentioned above [37].

Thus we see that there can be both intra-patient and inter-patient variability (Fig. 2). Any putative biomarker, be it a single metabolite or a metabolic signature,

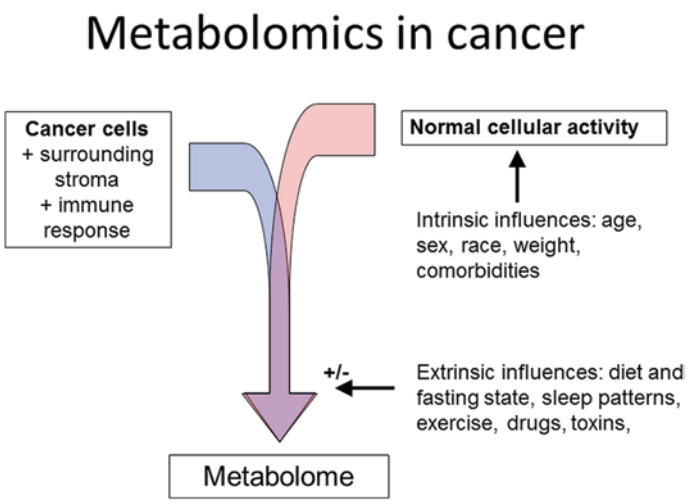

Fig. 2 The metabolome consists of metabolites from all cellular process, which is influenced by intrinsic and extrinsic factors. Metabolites produced by cancer cells are superimposed on this landscape

must be reliably discernible through this background variation if it is to become a useful and robust tool.

No standard reference exists yet for metabolomics, due to the great inherent variability from one patient population to the next, and the complex variety of chemometric techniques that can be employed in analysis. As such, each new experiment in metabolomics that looks to differentiate two groups first requires a training set to establish the specific patterns and levels that are associated with the outcome of interest, such as disease relapse following adjuvant chemotherapy. Once this is achieved, it must then be tested against the remaining data, or against multiple subsets of the data, to validate these patterns as having genuine correlation with the outcome of interest. Examples of this in breast cancer research will be detailed in the next section.

Metabolomics in Breast Cancer

In breast cancer, as in other tumor streams, metobolomic research remains in the experimental stage, with as yet little translation into clinical application. A number of potential applications have been and continue to be explored (Table 1).

Metabolites as Biomarkers

Metabolomic analyses have detected a number of potential biomarkers which could proceed to further validation. An example is the ratio of glutamine to glutamate in tumor tissue, where it has been shown to correlate with estrogen receptor (ER) status, tumor grade and overall survival [38]. This illustrates how a broader analysis allowed appreciation of the importance of examining more than one metabolite at once. Glutamine or glutamate levels individually bear only rough and unreliable correlation with cancer presence, yet this study demonstrates that their levels relative to one another become more informative. Whether this will lead to enhanced predictive or prognostic ability is yet to be assessed, but the hypothesis-generating ability is in itself valuable.

Prediction of Stage

Studies of NMR spectra of fine needle aspirates of suspected early breast cancer showed that malignant tissue, nodal involvement and tumor vascular invasion could

Table 1 Applications of metabolomics in breast cancer

Novel metabolite discovery
Determination of prognostic factors
Prediction of treatment effect or toxicity
Early diagnosis

be predicted with high accuracy [39], and could be used to predict grade, ER and progesterone receptor (PgR) status, or axillary spread [40, 41]. Larger numbers are needed to validate these results, and unless the profiles can be shown to offer superior prognostication to current methods, then clinical utility is debatable. Nevertheless, it is evidence that the metabolic signature tells of the aggressiveness of the phenotype.

Prediction of Treatment Effect

Prediction of response to neoadjuvant chemotherapy using metabolomic data has been achieved using combined MS and NMR data [42]. Levels of four metabolites, threonine, glutamine, isoleucine and linolenic acid, were identified that correlated strongly with pathologic complete response (pCR) following neoadjuvant chemotherapy. What remains unclear, however, are the metabolic pathways implicated in the changing metabolite levels, and their roles in cancer development and treatment response. Furthermore, the predictive benefit needs to be compared to that already offered by clinicopathological features to ensure it increases prediction power and confers a clinical advantage.

Early Detection of Recurrence

Compared to standard approaches, recurrence can be predicted earlier with metabolomics, shown in a study by Asiago et al. [43]. The investigators combined both NMR and MS techniques to analyze stored patient sera from resected early breast cancer patients. Multiple samples over time were available for each patient. A number of metabolites were found to be strongly associated with relapse, and a model was developed that predicted for relapse with sensitivity of 86 % and a specificity of 84 %. Compared to detection by standard clinical means, the profile was able to detect recurrence 13 months earlier on average in 55 % of patients. Whilst to date there is no proven clinical utility for early detection of metastatic disease, early diagnosis of local recurrence is associated with a survival advantage [44], and these results are exciting. This could form a basis for further studies into the benefits of early initiation of treatment for relapsed disease. It could also allow early recognition of failure of adjuvant endocrine therapy, preventing continuation of futile treatment or indicating new intervention to counteract resistance.

Predicting Recurrence Risk in Early Breast Cancer

Several studies have been performed to test whether metabolomic profiles have any prognostic power in early breast cancer, in terms of predicting relapse. It is worth

going into the details of some of these trials to illustrate the techniques required for metabolomic analysis, the limitations of the studies, and the potential benefits.

In the field of early breast cancer, the improvement in prognostication remains a priority. This is because current practice favors over-treatment of women with systemic therapy due to an inability to identify and isolate those for whom adjuvant treatment is more likely to be beneficial. We know from early studies that even in high-risk node-positive disease, a subset of these women will be cured with local therapy alone. Seminal studies performed by the Milan group [45] comparing the CMF combination (cyclophosphamide, methotrexate, 5-fluorouracil) to no adjuvant therapy in women with node-positive early breast cancer, with over 25 years clinical follow up, demonstrated that 22 % of these clinico-pathologically high-risk women who had no adjuvant therapy remained disease free. Women with node-negative, ER-negative disease receiving no adjuvant treatment had higher survival of 40 %, with 20 years follow up [45]. Even allowing for the improved risk stratification offered by modern gene expression profiling, there is room for improvement: in the National Surgical Adjuvant Breast and Bowel Project (NSABP) B20 study comparing chemotherapy plus tamoxifen to tamoxifen alone in women with node-negative, ER-positive, resected early breast cancer, those with tumors classified as high-risk by the Onco*type*DX 21 gene recurrence score had long term survival well over 60 % with tamoxifen alone [46, 47]. Today, many of those women would almost invariably be offered systemic therapy, and likely chemotherapy, with all the inherent risks and cost.

The search for biomarkers to improve stratification of patients with early breast cancer to detect those who will benefit from chemotherapy, and those for whom the toxicity outweighs the benefits, is vital. Current risk stratification relies on data taken from the biopsy and resected tumor: ER, PR, HER2, Ki-67, tumor grade, and extent of nodal involvement. Genome expression profiling has refined this, particularly in the node-negative cohort (Onco*type* DX, 70 gene recurrence score), yet still a large proportion of women who were cured by surgery alone are not identified and are subsequently treated unnecessarily.

Common to these approaches is risk assessment based on features of the primary cancer alone, once it has been removed. Whilst offering clear prognostic benefit as surrogate markers, these may not reflect the biology of residual disease. In the post operative setting in breast cancer, the decision to offer adjuvant therapy is based on the likelihood of relapse, which in turn is linked to the presence of micrometastatic disease, the residual tumor cells which may be genetically or phenotypically different from the primary cancer, and thus the cells that need to be addressed. Circulating tumor cells (CTC) or disseminated tumor cells may offer a more targeted approach, and are known to confer a worse prognosis [48]. However, detection and collection in non-metastatic setting is difficult, such cells may still not be representative of all remaining cancer cells, and this approach may still fail to appreciate the host response.

Metabolomics offers a unique perspective, as it takes into consideration signals from the host, the tumor microenvironment, and the tumor cells themselves, as well as any interactions between them. This residual pool of cancer cells, and the host response to them, may result in a detectable change in the metabolic profile that

might differentiate those who are likely to be cured by surgery alone from those who are more likely to relapse. It is for this reason that metabolomics may provide complementary and possibly more comprehensive information that could be added to current stratification models and aid in prognostication.

Establishing the Metastatic Metabolomic Signature

An initial test of the hypothesis that such signatures may be detectable and discriminating was performed by our group using one-dimensional proton NMR spectra of serum samples [49]. Fortyfour patients with early breast cancer had serum taken for metabolomic analysis both pre and postoperatively. As a control, 51 patients with advanced breast cancer also had serum taken. The aim was to see if serum metabolic profiles of early breast cancer patients differed from those with advanced disease; whether this changed after surgery; and whether the profiles could be used to generate a risk score that had prognostic power comparable to an existing prognosis calculator (Adjuvant! Online). A further 45 patients with early disease provided a post operative blood sample that would be used as a validation series, ie to determine if risk scores generated in a new post operative group have a similar correlation with prognosis compared to the initial group, demonstrating reproducibility and validity.

Once spectra were obtained from the serum samples, a series of analytical steps was required to allow meaningful comparisons, including data reduction using orthogonal projection to latent structure (OPLS), a technique used to convert each spectrum to a single point on a two dimensional graph to allow simple comparison of the different fingerprints. This demonstrated significant separation of the preoperative and metastatic groups into distinct clusters, illustrating that the fingerprints did indeed differ from one population to the other to varying extents. Double cross validation was then used to assess prediction ability of the model, showing a discrimination sensitivity of 75%, specificity of 69%, and predictive accuracy of 72%, with some patients with metastatic disease being consistently misclassified as early, and some early patients as metastatic.

A ‘metabolomic risk score’ was then established for each early breast cancer patient based on how much their profile resembled the metastatic profile, measured as an inverse function of the distance to the barycentre of the metastatic cluster. In other words, the more the fingerprint resembled that of patients with metastatic disease, the higher the risk score. This is based on the premise that the presence of the primary and/or micrometastatic disease is more likely to yield a metastatic profile, and that its presence makes relapse more likely. High metabolomic risk score in preoperative patients was found to be highly correlated with misclassification as metastatic.

The metabolomic risk based on the preoperative serum was then compared to the 10 year breast cancer mortality estimate from Adjuvant! Online, for each patient, with the arbitrary threshold of 10%, 10-year mortality risk for low-and high-risk. Here, concordance was low. However, once the primary tumor was removed, there was considerable change in metabolomic risk, with 86% of patients initially

assessed as having high metabolomic risk switching to low metabolomic risk, suggesting that the signal was coming entirely from the primary cancer in this group. Interestingly, 8 of 10 patients assessed as both high preoperative metabolomic risk and high Adjuvant! Online risk moved to low metabolomic risk postoperatively. Only 6 out of 21 patients with high Adjuvant! Online risk had high postoperative metabolomic risk.

When the same technique was repeated with the validation set (post operative serum samples), a similar pattern was observed, with high concordance of low metabolomic risk with low Adjuvant! Online risk, but only 32% of high Adjuvant! Online risk patients showing high metabolomic risk. Thus we see that this metabolomic risk score generally classifies more patients as low risk.

Key points from this trial are that a detectable metabolomic signature is present in patients' serum that can indicate the presence of breast cancer, and distinguish early from metastatic disease in a high proportion of patients. The shift in signature from a high-risk (metastatic) to low-risk following removal of the primary tumor in 86% of patients supports this. Where a metastatic signature exists post-operatively, this is more likely to be associated with a high-risk status according to traditional measures, yet fewer post-operative patients overall are classified as high-risk. This has the potential therefore to offer greater discriminatory power in selecting those who are less likely to require adjuvant therapy.

What is missing from this trial however is follow-up data, which would offer far greater evidence of predictive power than comparison with another risk calculator. Simply using established prognosis calculators to validate the profile may risk developing another calculator of similar power, and thus will not enhance utility. Furthermore, the trial requires further validation in different patient cohorts.

Predicting Clinical Outcome

To these ends, Tenori et al. [50] performed a similar study in which they examined serum ^{1}H-NMR metabolic profiles in both early and metastatic breast cancer patients, again with the aim to demonstrate that the spectra could differentiate between the two groups, and also to establish a risk score that might predict relapse. Importantly though, in this case there were clinical follow-up data for the patients with early breast cancer, which had to be available for a minimum of 5 years or until relapse. Serum samples were selected from a biobank at the Memorial Sloan Kettering Cancer Center (MSKCC) in New York in which left-over patient samples are stored for scientific use, with patient consent. Eighty samples from patients with early breast cancer were selected, with the criteria that they must have post-operative serum available, taken up to 90 days post surgery, but prior to commencing adjuvant therapy.

Ninety-five samples from patients with metastatic disease were obtained, and their NMR spectra obtained to create the metastatic fingerprint. The early stage group was split into two groups of 40 samples; the first half was used to generate a reference spectral patterns for early disease and to develop a risk score (training

set), and the other half was used to test the risk score for concordance and accuracy (validation set). The underlying hypothesis was that sera of patients with early breast cancer with micrometastatic disease would have metabolic fingerprints more closely resembling those of the metastatic cohort, and that these patients would be more likely to experience disease relapse. Ten out of 40 patients in the training set, and 11 out of 40 in the validation set, had documented evidence of relapsed disease.

Random Forest (RF) classification was used to classify samples as either metastatic or early, based on the spectra. This is an analytical technique that can take large numbers of variables into consideration, is less prone to error or over-fitting, and does not require cross validation. This was performed on three different spectra for each sample using different NMR techniques: NOESY1D, CPMG, diffusion-edited. Similar to the previous study, there was high accuracy in predicting early or metastatic status, with correct prediction in 84–87 % of cases across the three NMR techniques.

A RF risk score was generated, based on the risk of a patient with early breast cancer specimen being classified as metastatic, and this score was taken as an indicator for clinical relapse. The RF risk scores generated from each of the spectra were then compared to the known outcomes of the patients using eceiver operating characteristic (ROC) analysis. CPMG spectra resulted in the greatest area under the curve (AUC) on the ROC curve (0.863), and were selected for use in the validation set. From here, a cut-off for the RF risk score was determined, aiming for maximum accuracy with appropriate sensitivity and specificity. The RF risk score of ≥ 53 was used, yielding sensitivity, specificity and accuracy of 90, 67 and 73 %, respectively for predicting likelihood of relapse.

This CPMG risk score model was then applied to the validation set in an unsupervised analysis (ie blind to the clinical outcome). Here the correlation between predicted relapse and actual relapse was high, with AUC 0.824, demonstrating that in this cohort the risk calculator was robust. Sensitivity was 82 %, specificity 72 %, and predictive accuracy 75 %. Nevertheless, 25 % of patients were misclassified, and, if used to dictate adjuvant chemotherapy decisions, 18 % of patients who would have relapsed would not receive adjuvant treatment.

The model was tested further by comparing it to already-validated prognostic methods that employ clinicopathological features of the primary disease. Tumor size, nodal status and RF score all had significant association with recurrence, but on multivariate analysis none remained significantly associated (tumor grade was not included, as all early cancers were grade 3). When compared to Adjuvant! Online in multivariate analysis, only RF score showed statistically significant association with relapse, indicating that the RF score offered prognostic power over and above that offered by Adjuvant! Online in this cohort.

There were some potential confounders in the trial, some of which were accounted for. First, when searching the MSKCC database for patients early disease, only cases with ER-negative disease were selected for the relapse-free cohort, as 5 years follow up was deemed insufficient for ER-positive early breast cancer. No selection for ER status was made on the relapsed cohort or the metastatic cohort. Subsequent analyses showed that ER-positivity could not be predicted from the metabolomic spectra, and the authors concluded that differences in ER status between

the early and the advanced breast cancer cohorts could not explain the observed results. This was further validated by confining the study to ER-negative patients only and repeating the analysis, subsequently achieving similar sensitivity, specificity and accuracy. Second, the time interval between surgery and blood sampling varied from 5 to 80 days, but again further analysis demonstrated that metabolomic spectra could not be used to differentiate early sampling (time interval < 30 days) from late (30–80 days).

Limitations

The first study controlled for a number of variables by confining the patient population to a single institution, and by taking blood samples specifically for metabolomic analysis after an overnight fast and with a diary of the previous day's food intake and medication. This reduces a number of potential confounders, but in doing so also reduces the generalisability. Furthermore it lacked outcome data for its early patients, instead comparing its risk score stratification to standard clinicopathological prediction. But it served as a proof of concept.

The second study again also used serum from a single institution, but here the serum had been stored for a variable length of time, and did not control for fasting state or time of blood collection. Whilst potentially confounding, this may render positive results more robust, as the likely effect of such variation is dilution or disguise of genuine metabolomic profile differences. More importantly, perhaps, a large proportion of the early breast cancer patients went on to receive chemotherapy, undoubtedly influencing the outcome data. Thus its predictive ability here may be limited to identifying those who are likely to relapse *in spite* of chemotherapy.

Other groups have demonstrated the presence of a metabolic signature from breast cancer. A similar study aiming to create a model to differentiate early and metastatic breast cancer using ^{1}H-NMR spectra was performed by Jobard et al. [51], using a training cohort of 46 early and 39 metastatic breast cancer patients, and an independent validation cohort of 61 early and 51 metastatic breast cancer patients. Their model was also reported to have even higher discriminating power. Crucially however, serum samples for the early patients were taken preoperatively, ie with the primary cancer *in situ*. Thus it represents more a discriminator of tumor bulk, rather than tumor presence. Furthermore it did not examine the model against any clinical outcome, and its utility in prognostication or prediction remains unknown. Common to all these trials is the problem of small numbers of participants.

Specific Metabolites

In each of the studies described, certain individual metabolites were identified that showed significant correlation with the presence of metastatic disease (Table 2).

Table 2 The identified discriminating metabolites detected in four metabolomic studies. Note the low rate of concordance between studies. MBC, metastatic breast cancer; NS, not statistically significant

Study	Higher in MBC	Lower in MBC
Oakman	Phenylalanine, glucose, proline, lysine, N-acetyl cysteine	Lipids
Jobard	Phenylalanine, glutamate, N-acetyl cysteine, mannose, pyruvate, glycerol, acetoacetate, lipids (NS)	Histidine, alanine (NS), betaine (NS)
Tenori	Glucose, lactate, tyrosine, lipids	Histidine
Asiago	Tyrosine (NS), lactate (NS)	Histidine formate proline choline, N-acetyl glycine, ketone body

In the Tenori study, reduced serum histidine and increased glucose and lipids were significantly correlated with metastatic disease [50]. In the Jobard study however, nine different metabolites were identified, which included low histidine [51]. Glucose and lipids had a trend to significance. Much greater reproducibility will be needed before any particular metabolite can be used clinically. Moreover, this tends to move away from the unique benefit of metabolomics, ie the consideration of the combined picture of tumor and host response. Many single metabolites, including amino acids, have been shown to correlate with the presence of cancer, yet none have proven discriminatory enough to be clinically meaningful [52, 53].

Further Trials

These exploratory trials give support to the potential of metabolomics in the detection of micrometastatic disease and the prediction of relapse, but require further validation in larger cohorts. A proposed trial by our group aims to repeat the experiment performed by Tenori et al. using a larger data set. Serum samples from some 600 early (post-operative) and metastatic breast cancer patients with documented follow up data from a number of centres will be analysed, a risk score generator created, and prediction of outcome compared to actual clinical outcome. While aiming to achieve similar results to the first study and demonstrate reproducibility, it will also shed light on transferability to other populations.

Metastatic Breast Cancer

Studies of metabolomics within metastatic breast cancer have been less productive. This is likely in part due to the greatly increased mutational load and hetereogeneity in advanced disease, that leads to far more complex, variable and inconsistent metabolic profiles. Another study by Tenori et al. [54] aimed to predict responses to

treatment based on changes in metobolomic profile before and after treatment, but were unable to demonstrate any discriminatory power. In a small subset of HER2-positive patients, metobolomic analysis was able to predict response to lapatinib plus paclitaxel, but the results in this cohort were discouraging.

A proposed investigation will aim to study the serum metabolic profiles of a large cohort of metastatic breast cancer patients over time as part of a much broader prospective longitudinal cohort study, and follow their progress over time. It is hypothesised that metobolomic analyses may demonstrate prognostic or predictive power for response to therapy and disease time course, identify novel biomarkers and help to refine data derived from 'upstream' analysis such as gene expression profiling.

Conclusions

Metobolomic studies in breast cancer have shown that a metabolic signature of cancer exists and can be detected in patient serum. It has the potential to allow early identification of relapsed disease, predict likelihood of relapse, and act as a biomarker of disease activity and response to treatment. It is limited by its complexity, requiring high-cost specialised equipment and analysis, which may hinder its progress into larger patient population studies, while retrospective analysis of completed clinical trials is frequently unfeasible.

It would be ideal, for example, to go back to early placebo controlled trials in the adjuvant treatment of early breast cancer to assess differences between the metabolomic spectra of those who were cured with surgery alone and those who relapse. Unfortunately of course this is not possible for a number of reasons, not least of which is a lack of stored serum. Given this barrier, one may conclude that it will be impossible to develop evidence strong enough to convince clinicians and patients to ignore a traditional 'high-risk' assessment, and forego adjuvant therapy, based on a novel risk score without the backing of a placebo controlled trial, and that such a trial would be ethically impossible. The dream of sparing 'cured' patients adjuvant therapy, at least by metobolomic methods, may indeed be unattainable.

A more achievable goal may be to focus on the lower risk groups who would traditionally forgo adjuvant chemotherapy, and attempt to predict relapse. A prospective study could then assess the benefit of adding adjuvant chemotherapy to those deemed more likely to relapse. For example, future studies might combine genomic risk with metabolomic risk in patients with ER-positive early breast cancer, and observe for differences in outcome between those assessed as low genomic and low metabolomic risk, and those with low genomic but high metabolomic risk, all treated with adjuvant hormone therapy alone. In this way it may be seen if metabolomics offers complementary risk stratification power.

For now, in this field at least, metabolomics remains exploratory, until a robust algorithm for analysing metabolic spectra can be achieved that both accurately predicts the presence of cancer and the clinical outcome, and is resistant to the influ-

ence of the multitude of normal variables that impact the metabolome. Only then can it be prospectively validated as a meaningful tool to aid in risk stratification and decision making about adjuvant therapy.

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Circulating Tumor Cells

Costanza Paoletti and Daniel F. Hayes

Abstract Circulating Tumor Cells (CTC) are shed from primary or secondary tumors. Prior studies have demonstrated that enumeration of CTC is a robust independent prognostic factor of progression free and overall survival in patients with early and metastatic breast cancer. CTC, as well as other circulating tumor markers, have the appealing advantages over tissue biopsy of (1) ease of collection, (2) serial evaluation, and (3) interrogation of the entire tumor burden instead of just a limited part of the tumor. Advances have been recently made in phenotyping and genotyping of CTC, which should provide insights into the predictive role of CTC for sensitivity or resistance to therapies. In addition, CTC phenotypic marker changes during the course of treatment may serve as pharmacodynamic monitoring tools. Therefore, CTC may be considered "liquid biopsies," providing prognostic and predictive clinical information as well as additional understanding of tumor heterogeneity.

Keywords Circulating Tumor Cells (CTC) · Breast cancer · Enumeration · Characterization · Cluster · Prognosis · Prediction

Introduction

The metastatic process accounts for the majority of cancer-related deaths. A major component of the metastatic process involves tumor cell dissemination from primary and metastatic sites through the circulation [1]. However, compared to the billions of erythrocytes and millions of leucocytes in a milliliter of blood, circulating tumor cells (CTC) are very rare events, and isolation, enumeration, and characterization of CTC is technically challenging. The first identification of tumor cells

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in blood was reported in 1869 by Ashworth [2], who reported "The fact of cells identical with those of the cancer itself being seen in the blood may tend to throw some light upon the mode of origin of multiple tumors existing in the same person […]. One thing is certain, that if they came from an existing cancer structure, they must have passed through the greater part of the circulatory system to have arrived at the internal saphena vein of the sound leg."

In the past few decades, several technologies have been developed to isolate and characterize CTC from whole blood (WB). These techniques take advantage of different properties between epithelial CTC and normal constituents of blood. Several studies have demonstrated that enumeration of CTC with one or another of these techniques is a specific, independent predictor of progression-free survival (PFS) and overall survival (OS) in patients with early and metastatic breast cancer [3–6]. However, besides simple enumeration, characterization and phenotyping of CTC might also be of value. In this regard, biopsy of a metastatic site is a standard approach to establish diagnosis and re-evaluate tumor biomarker status to predict response to targeted therapy. However, biopsy is invasive, costly, uncomfortable for patients, and sometimes not feasible, depending on the metastatic site. Therefore, CTC might serve as a "liquid" biopsy, providing a minimally invasive and real time biomarker assessment in the metastatic setting. Further, CTC evaluation might offer a better method to analyze tumor heterogeneity of the underlying cancer than a single biopsy, since CTC interrogate the entire burden of disease. Finally, serial CTC investigation may provide a pharmacodynamic tool to monitor biomarker expression over time to help guide personalized therapeutic management for patients with metastatic breast cancer.

Methods to Isolate and Detect CTC

Many different technologies have been studied to isolate CTC from WB, based on distinguishing the CTC from normal hematopoietic constituents, principally erythrocytes and leukocytes. Investigators have chosen diverse separation strategies that exploit differences between these cellular entities, such as physical properties (size, weight, or density), flow and elasticity characteristics, and differential expression of biological factors, including putative tumor associated antigens (TAAs) or simply markers of epithelial vs. mesenchymal/hematopoietic derivation (Table 1). Regardless, two critical issues must be addressed: (1) enrichment or purification of the rare occurring CTC from WB; and (2) confirming that the enriched or purified "event" is, indeed, a malignant cell.

Table 1 Examples of selected assays for CTC detection in breast cancer

Assay/Company if available/[references]	Enrichment/Capture strategy	Method to distinguish epithelial/cancer from hematopoietic cells	Other characteristics
Positive selection: Immuno-separation			
CellSearch® (Janssen Diagnostics, LLC) [3]	EpCAM-Ab coupled with ferrofluid	+ marker: CK 6, 8, 18, 19 − marker: CD45 Nucleus: DAPI	Semi-automated system; FDA cleared; EpCAM positivity dependent
Magsweeper® [12]	EpCAM-Ab immumagnetic cells separation	Microscope visualization; Gene expression profiling	Automated immunomagnetic separation technology; live cells can be isolated
CTC-chip [13]	EpCAM-Ab coupled to micro-posts	+ marker: CK − marker: CD45 Nucleus: DAPI	Microfluidic microchip technology; high recovery, further molecular investigation at genomic, transcriptional and translational levels
CTC-iChip [85]	Size-based, hydrodynamic cell sorting, and immunomagnatic selection (^{pos}CTC-iChip selection by EpCAM or ^{neg}CTC-iChip selection by CD45)	+ marker: CK − marker: CD45 Nucleus: DAPI	Allows for either positive and negative selection; single cell RNA expression
Adna test, AdnaGen [23, 24]	EpCAM antibodies and MUC1 based using labeled beads	+ marker: EpCAM Cells are detected using a multiplex RT-PCR based expression analysis such as MUC-1, HER2, and GA733-2 for breast cancer	RNA is isolated, followed by multiplex RT-PCR to distinguish specific tumor biomarkers; high sensitivity; not morphologically identifiable
Herringbone-chip or "HB-Chip" [15, 16]	EpCAM-coated microposts and chip surface	+ marker: CK − marker: CD45 Nucleus: DAPI Potential additional tumor-specific markers such as antibody cocktail direct against EpCAM, EGFR, and HER2 can be used	High-throughput microfluidic mixing device that utilizes surface ridges or herringbones in the wall of the devices to maximize collision between CTC and antibody-coated walls

Table 1 (Continued)

Assay/Company if available/[references]	Enrichment/Capture strategy	Method to distinguish epithelial/cancer from hematopoietic cells	Other characteristics
CTC-chip designated "Ephesia" [14]	Immunomagnetic sorting based on advantages of microfluidic cell sorting and flow-activated interaction between cells and antibody-bound magnetic beads	Immunochemistry for cell surface and nuclear marker	Combines micro-fluidic cell sorting; broad range of capture Ab
GO-chip [17]	Antibody against EpCAM coated to a functionalized graphene oxide nanosheet on a patterned gold surface	+ marker: CK	Novel graphene oxide strategy with high yield capture
		− marker: CD45	
		Nucleus: DAPI	
		Potential additional markers such as HER2	
CellCollector™ (Gilupi, Germany) [25]	Functionalized EpCAM-coated medical guide-wire	+ markers: CK; EpCAM	*In vivo* isolation of CTC; screening of large blood volume
		− marker: CD45	
		Nucleus: DAPI	
Negative selection: RBC lysis/Ficoll Gradient/Size/CD45 depletion			
Maintrac (SIMFO Bayreuth GmbH) [86]	Lysis of red blood cells	+ marker: EpCAM	Detection of a single cell
		− marker: CD45	
Ariol system (Genetix USA Inc.) [87]	Lysis of red blood cells; Positive selection CK alone or with EpCAM linked to microbeads	+ marker: CK 8, 18, 19	High detection rate; capable of both bright field and fluorescent imaging; detection of EpCAM+ and EpCAM- CTC
		− marker: CD45	
		Nucleus: DAPI	
RT-PCR Methods [20–22]	Immunomagnetic/Ficoll Gradient centrifugation	A wide variety of mRNA epithelial cell and more specific cancer cells such as CK-19, CK 20, MUC1, mammoglobin, HER2 for breast cancer	CTC cannot be morphologically identified

Table 1 (Continued)

Assay/Company if available/[references]	Enrichment/Capture strategy	Method to distinguish epithelial/cancer from hematopoietic cells	Other characteristics
EPISPOT [55, 88]	Negative selection: Depletion of $CD45^+$	Secretion of proteins shed from CTCs antibodies labeled with fluorochromes: CK-19, Mucin-1, Cathepssin-D (breast cancer)	Isolation of viable cells
CAM (Collagen Adhesion Matrix) [89]	Ficoll density gradient centrifugation	+ marker: CK 4, 5, 6, 8, 10, 13, and 18	CTC differentially invade and ingest fluorescently labeled CAM; isolation of viable cells that allows further molecular characterization
		− marker: CD45	
ISET (Rarecells SAS) [10]	Size	+ marker: CK	Isolation of viable CTC by size via calibrated pores of 8 μm
		Nucleus: Mayer's haematoxylin	
Dielectrophoretic field-flow fractionation (depFFF) [8]	Cell-separation based on density and dielectric proprieties of cells	Immunocytochemistry	Isolation of viable CTC

Abbreviations: *EpCAM* Epithelial Cellular Adhesion Molecule, *Ab* Antibody, *CK* cytokeratin, *DAPI* 4,6-diamidino-2-phenylindole hydrochlorid, *FDA* Food and Drug Administration, *RT-PCR* reverse-transcriptase-polymerase chain reaction, *MUC-1* Mucin 1, *HER2* Human Epidermal Growth Factor-2, *EGFR* Epidermal growth factor

Isolation/Enrichment/Purification/Capture of CTC from Whole Blood

Separation of CTC from Hematopoietic Cells Based on Physical Properties

In general, cancer cells are larger, heavier, and denser, and may have different electro-magnetic charge than erythrocytes or leukocytes. Therefore, several investigators have separated cancer cells by passing WB, or the nucleated component of WB, through either a density gradient or a filter of some sort. For example, standard density gradient centrifugation through the synthetic polymer of sucrose (Ficoll) has been a common method of separation, although it is not very efficient and there is considerable admixing of the malignant and normal cells. Likewise, other methods, such as the OncoQuick® (Greiner Bio One, Munich, Germany), still take advantage of cell separation by density gradient using a porous barrier, but more efficient than standard Ficoll depletion of mononuclear blood, thereby increasing the tumor cell density [7]. Taking advantage of differential cell surface electric charge between malignant and normal cells, dielectrophoretic field-flow fractionation (depFFF) separates the two, first by density and then by dielectric differential proprieties [8]. Yet another approach to enrichment has been based on the observation that CTC have a cellular diameter that is larger than hematopoietic cells (12–25 μ compared to 8–10 μ for leukocytes and 8 μ for erythrocytes). Separation has been achieved by passing WB or buffy coat through membrane micropore filters [9], special filters such as ISET (Isolation by Size of Epithelial/Throphoblastic Tumor cells) with calibrated pores of 8 μm [10].

Separation of CTC from Hematopoietic Cells Based on Biological Expression Differences

Obviously, the genotypes and associated phenotype of malignant and normal cells differ. Thus, several investigators have exploited these differences by attempting to enrich or identify CTC based on expression of TAAs. However, malignant and normal cells are really more alike than different, and because of tumor heterogeneity between and within a single patient, few if any TAAs have been identified that are sufficiently sensitive and specific to be of much value for CTC capture or enumeration. Nonetheless, efforts to isolate and distinguish CTC from hematopoietic cells have been made using immunologic approaches and/or methods to identify and quantitate RNA expression and DNA abnormalities. In contrast, several investigators have taken advantage of the differences between epithelial and hematopoietic cells in general to isolate CTC originating from the common epithelial cancers, including breast.

Regardless, the most common immunologic approach to capture CTC has been to coat some sort of solid state with antibodies against cell surface antigens, be they TAAs or epithelial-related proteins. In this regard, immunocapture of CTC using

antibodies against the epithelial cell adhesion molecule (EpCAM), coated on some sort of solid phase surface, has been frequently employed to enrich CTC from WB. EpCAM is expressed on 80 % of solid cancers such as breast, colorectal, and prostate. The most widely commercially available assay based on EpCAM capture, the CellSearch® system (Janssen Diagnostics, LLC, Raritan, NJ), utilizes ferromagnetic particles coated with an antibody to EpCAM (anti-EpCAM) with subsequent immunomagnetic separation [11].

Another immunomagnetic cell separator, the MagSweeper®, gently enriches target cells using a magnetic rod which attracts cells prelabeled with EpCAM-coated magnetic particles [12]. In addition, the CTC-chip is an anti-EpCAM coated microfluidic device which is composed of carefully constructed microposts that permit smaller cells to pass through freely but divert larger cells into a capture "trap" [13]. Likewise, the Ephesia assay in collaboration with the Nikon Imaging Company Fluigent, combines the advantages of microfluidic cell sorting and flow-activated interaction between cells and a broad range of antibody-bearing beads for immunomagnetic sorting which could include EpCAM [14]. Yet another approach, the herringbone-chip or "HB-Chip," involves flowing blood through a microfluidic mixing device for CTC isolation [15, 16], while other researchers have reported another microfluidic device in which EpCAM antibody has been coated to a functionalized graphene oxide nanosheet on a patterned gold surface [17].

Confirmation that Captured Events are Malignant Cells

Regardless of the capture method, none is truly a CTC-purification strategy. Therefore, one must be able to determine whether the captured "events" are truly cells, and whether they are more likely cancer or normal hematopoietic cells. This step has most commonly been accomplished by staining the captured "event" with DAPI, which binds to double-stranded DNA proving that the event has a nucleus, and with differently labeled antibodies to cytokeratin (CK) (epithelial) and selected leukocyte antigens, usually CD45. Light or fluorescent microscopy or flow cytometry has then been used to characterize the stained events. Obviously, this strategy identifies circulating epithelial cells, which may or may not be malignant. However, chromosomal analyses with random fluorescent *in situ* hybridization (FISH) probes [18] have demonstrated that most if not all epithelial cells captured by CellSearch® in patients with known breast cancer are aneusomic. Coupled with intensive visual inspection in the CellSearch®, EPIC, and other systems suggesting that such cells are indeed malignant, one can be comfortable in the assumption that they are indeed CTC.

Each available system differs in the manner in which events are captured and characterized. However, in the CellSearch® system, each of these steps, including fluorescent scanning of identified events, is automated [11]. The only operator-dependent analysis required is review of the subsequent computer-generated galleries of each event to determine if it is DAPI positive, CK positive, and CD45 negative.

Some systems bypass the enrichment step, using a strategy of simply identifying evidence that CTC are present. For example, the system developed by EPIC Sciences™ involves smearing the nucleated component of WB onto specially-coated slides to which the cells adhere [19]. The slides are subsequently stained with a cocktail of DAPI and fluorescently-labeled antibodies against CK and CD45 and scanned with a whole slide fluorescent scanner for computerized image analysis of cellular morphology and CK and CD45 expression.

Another method to presumably detect CTC is by searching for the presence of transcripts of either epithelial markers or TAA. For example, using real-time polymerase chain reaction (RT-PCR), investigators have reported the presence of mRNA for CK, or for HER2 [20–22]. Since extracellular mRNA is unstable (as opposed to circulating cell free plasma tumor DNA (ptDNA) or miRNA), one can presume that the presence of these transcripts must represent intact CTC. Of course, although such an approach is likely to be quite sensitive, it is limited by the fact that one cannot determine definitively that indeed the transcript does represent the CTC, and further phenotyping or genotyping of the "cell' is impossible. In this regard, the Adna test (AdnaGEn) combines whole cell capture using immunomagnetic techniques (against EpCAM and MUC1) followed by CTC lysis and RT-PCR for whatever gene product is of interest [23, 24].

All of the enrichment and characterization strategies above are performed *ex vivo* from a relatively limited volume of blood (usually 1–30 ml). Novel methods for the *in vivo* isolation of CTC from peripheral blood have been developed in order to interrogate a larger volume of blood [25]. For example, the CellCollector™ (GILUPI Nanomedicine, Berlin), is a stainless steel wire (0.5 mm diameter) that is anti-EpCAM-coated, functionalized gold. The device is inserted intravenously, much like an intravenous catheter, and captures CTC as they circulate. Although this approach is intriguing, substantially more clinical research is required to determine if the CellCollector™ truly provides an advantage over the more traditional *ex vivo* devices.

Superiority of One CTC Assay Over Another

Most if not all of the reports of the devices reviewed above claim superiority in one way or another over other available devices. The majority of the capturing CTC devices may be more sensitive than the only Food and Drug Administration (FDA)-cleared CellSearch® assay by either capturing more CTC per volume of blood, or identifying more patients who have elevated CTC. While increased sensitivity is clearly a laudable goal, one must be cautious about such claims, or about using sensitivity as the only criterion for superiority.

To address the issue of when a biomarker assay should be used to direct patient care, the Evaluation of Genomic Applications in Practice and Prevention (EGAPP) initiative established three principles of evidence-based medicine [26]. Although EGAPP deliberations were directed specifically towards germline genetic testing, their principles apply to considerations whether any tumor biomarker tests should

be used to guide clinical decision-making. The three principles espoused by EGAPP include *analytical validity* (is the test accurate, reproducible, and reliable); *clinical validity* (does the test divide one population into two or more separate groups that have significantly different clinical outcomes); and *clinical utility* (has the test been shown with high levels of evidence to improve patient outcomes compared to those if the test were not used) [26]. More recently, these definitions have been supported and reinforced by the National Academy of Medicine of the United States regarding the generation of tumor biomarker omics-based tests, and definitions of levels of evidence have been proposed for tumor biomarker tests [27, 28].

Analytical validity is key to application of any diagnostic device, either in future clinical research or, more importantly, patient care. Only a few of these devices has been carefully shown to have analytical validity, with precise accuracy, reproducibility, and reliability [11]. Thus, claims of superiority must be tempered with careful scrutiny of data to support analytical validity, and these data are often lacking. Further, clinical validity is also a key element of comparing different assays. For example, there is no question that the presence of CTC as enumerated by CellSearch® is strongly associated with poor prognosis, as discussed below. Assays that appear to be more sensitive, even if this claim is supported with high analytical validation, may not result in improved, or even equivalent, clinical validity, since it is very possible, if not likely, that many of the additional CTC that are identified may not have malignant potential. Thus, head to head studies of sensitivity are not nearly as informative as head to head comparison of prediction of outcomes in the same dataset, and such studies are frankly completely lacking at present.

Clinical Validity and Utility of CTC Assays

Clinical utility requires that the intended "use context" for the marker be defined. In other words, what is the setting in which it will be used, and why will it be used? Intended use contexts for tumor biomarker tests, including CTC, include risk assessment, screening, differential diagnosis, prognosis and prediction of benefit from therapy, and monitoring disease course. Each of these must be placed into the context of the spectrum of breast cancer, from screening or prevention of unaffected subjects to palliative therapy for those with established metastases [29] (Fig. 1). Importantly, prediction of response to therapy can apply to a generic class of agents, such as endocrine therapy or chemotherapy or anti-HER2 therapy, or to specific agents within a class, such as tamoxifen or an aromatase inhibitor or fulvestrant. We agree with guideline recommendations by panels convened by the American Society of Clinical Oncology (ASCO) [30] and National Comprehensive Cancer Network (NCCN) [31, 32] that CTC currently have no clinical utility, or even validity, for risk categorization, differential diagnosis, or screening for new primary tumor. Nonetheless, there is emerging evidence of a potential role of CTC to determine prognosis in early and metastatic disease, as well as for monitoring patients with metastases. Indeed, more than 400 clinical trials using CTC are currently registered, and the majority of them are in the metastatic setting.

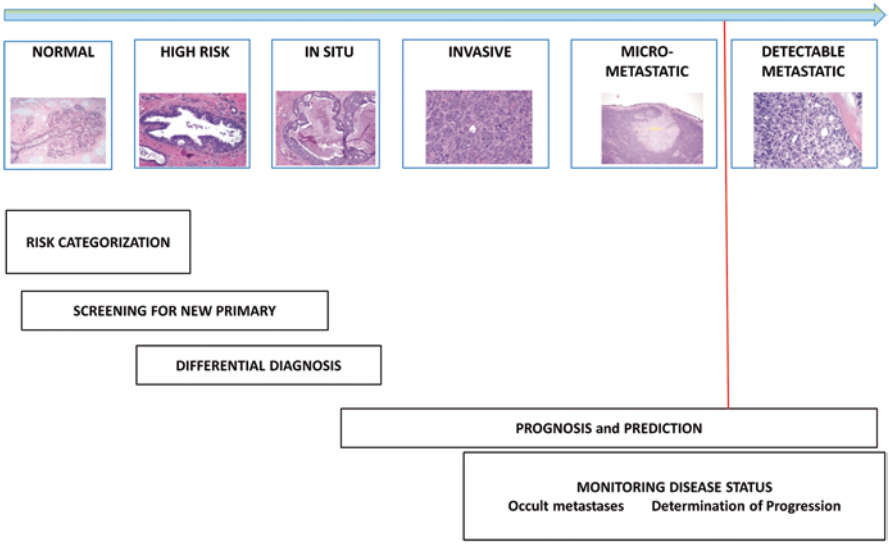

Fig. 1 Clinical spectrum of breast cancer

Prognosis in Early Breast Cancer

In stage 1 or 2 breast cancer, the incidence of CTC is lower than in the metastatic setting. Therefore, detection of CTC in the early setting has been even more challenging. Nonetheless, several studies have, indeed, reported detection of CTC using one of the many available methods described above. The most broadly used assays in the early breast cancer setting have been RT-PCR for CK or whole cell enumeration using CellSearch®. Using RT-PCR-based assay, Greek investigators have reported that approximately 40% of patients have evidence of CTC, and this has been associated with worse prognosis [20, 22]. Likewise, in two separate studies, CTC as enumerated by CellSearch® are elevated in 5–24% of patients with stage 1 or 2 disease (depending on cutoffs and blood volume), and are associated with slightly worse prognosis when compared to patients who do not have elevated levels [33, 34]. Further, in a meta-analysis by Zhang L. et al. [6] of several studies in the early setting, the presence of CTC was significantly associated with shorter disease free survival (DFS) and OS in patients with early breast cancer (DFS: hazard ratio [HR], 2.86; 95% confidence interval [CI], 2.19–3.75; OS: HR, 2.78; 95% CI, 2.22–3.48). Other studies conducted in the neoadjuvant setting have also shown that the presence of CTC, regardless of the assay, were associated with poorer outcomes [35–37].

Taken together, these studies establish the clinical validity, but not clinical utility, of CTC enumeration in this setting. None of these studies directed care according to CTC levels, and indeed, although statistically significant, the magnitude of the difference in outcomes between groups with or without CTC is not large. Thus, one

cannot determine if having low levels of CTC identify patients with a very favorable prognosis who could be spared adjuvant systemic therapy by virtue of having low levels of CTC. Conversely, it is also uncertain whether patients with higher levels of CTC should be given more treatment than patients with lower levels of CTC. These studies have established a principle that needs further support from new interventional, properly controlled trials to determine if CTC have clinical utility as a prognostic factor in early stage breast cancer.

Metastatic Breast Cancer

Over the last decade, the prognostic effect of CTC at baseline and during follow-up in metastatic disease has been well established for several types of cancers, including colorectal, prostate, non-small-cell and small cell lung (SCLC), and breast [3, 38–40]. *Apropos* to this review, at least when performed by the CellSearch® System, enumeration of CTC is a specific, independent predictor of PFS and OS in patients with metastatic breast cancer [3]. These results have been confirmed in a recent pooled analysis including data from 17 centers [5] and a meta-analysis of published reports [6].

However, all of these studies were conducted without the specific intent of generating high levels of evidence to demonstrate clinical utility for a specific context use. In this regard, Smerage et al. [4] have reported a trial conducted by SWOG (SWOG protocol S0500) to test the clinical utility of changing therapy based on "CTC response" in patients with metastatic breast cancer who were starting first line chemotherapy. All patients had CTC levels at baseline and were treated with the chemotherapy deemed best for them by their oncologist. Consistent with prior studies [3, 5], approximately one-half of the patients did not have elevated CTC (≥ 5 cells/7.5 ml WB being considered elevated) at baseline. These patients, designated group A, were followed with no further CTC evaluation. Those patients who did have elevated CTC at baseline (≥ 5 cells/7.5 ml WB) were re-evaluated after one cycle of chemotherapy. Approximately 60 % of these patients experienced a drop in CTC to < 5 cells/7.5 ml WB, presumably reflecting a response to the chemotherapy that they had begun, and this group (Group B) remained on that regimen until classic evidence of progression and survival. The other 40 % ($n = 123$) of patients (Group C) were randomly assigned to either stay on the chemotherapy regimen that they had started (Group C1), or to switch to an alternative chemotherapeutic regimen of their oncologist's choice (Group C2). OS was the primary endpoint of the trial.

Overall the results demonstrated that changing chemotherapy regimens early in a patient's disease course based on failure to reduce CTC to < 5 cells/7.5 ml WB did not change OS (Fig. 2). However, the prognostic role of CTC in this population was remarkable (Fig. 3). In the group of patients who did not have elevated CTC at baseline (Group A), median OS was 35 months, compared to 23 months for Group B and only 13 months for Group C. The prognostic role of CTC at baseline and at first follow-up was similar regardless of intrinsic subtype. The odds of reducing CTC if elevated at baseline did not vary by biological subtype except for HER2

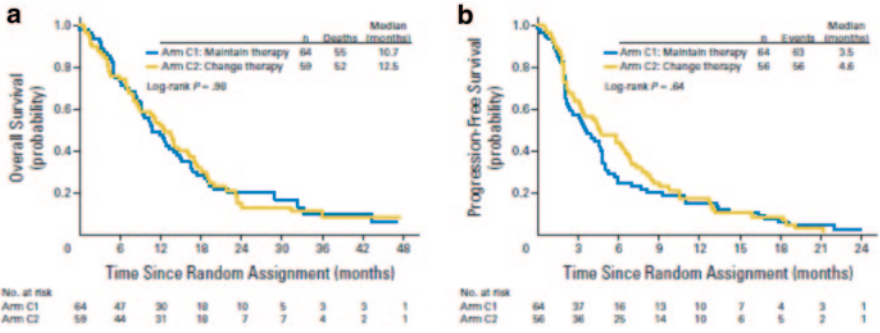

Fig. 2 Results of patients with metastatic breast cancer (MBC) for whom first line chemotherapy failed to reduce CTC at first follow-up in the S0500 clinical trial. **a** Overall survival (OS) and **b** Progression Free Survival (PFS). (From [4] Copyright® 2014 by American Society of Clinical Oncology. All rights reserved)

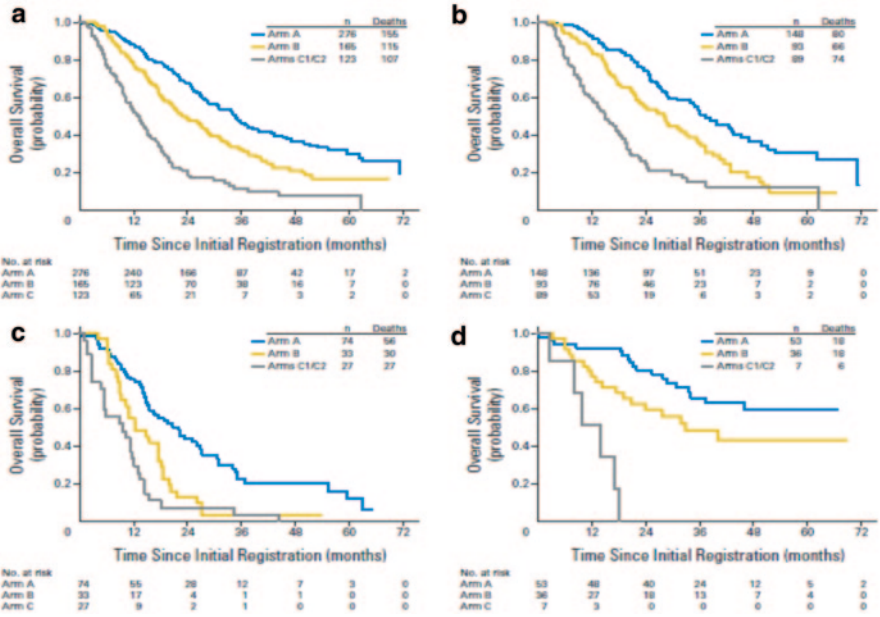

Fig. 3 Prognostic outcomes including overall survival (OS) of patients with metastatic breast cancer according to CTC levels at baseline at first-follow-up who participated in S0500 clinical trial. **Group A**: Baseline CTC < 5 cells/7.5 ml whole blood (WB); **Group B**: CTC ≥ 5 cells/7.5 ml WB at baseline and decreased to < 5 cells at first follow-up after starting first line chemotherapy; **Group C**: ≥ 5 cells/7.5 ml WB at baseline and at first follow-up after starting first line chemotherapy. **Panel A**: All Patients; **Panel B**: Hormone receptor-positive and HER2-negative disease; **Panel C**: triple negative disease; **Panel D**: HER2-positive disease (From [4]. Copyright® 2014 by American Society of Clinical Oncology. All rights reserved)

positivity. Taken together these results suggest that patients that did not clear CTC by first follow-up after starting first line chemotherapy appear to be have chemo-resistant disease, regardless of the regimen chosen. Clearly, a different treatment strategy is required for these patients.

In another attempt to develop clinical utility of CTC for a context use, Giordano A. et al. [41] have generated a prognostic nomogram consisting of baseline CTC levels and a combination of clinical factors (age, disease subtype, visceral metastases, and performance status) to estimate probability of OS at 1, 2, and 5 years and progression-free-survival at 6 months, 1, and 2 years for patients with metastatic breast cancer starting first-line chemotherapy. After development of the nomogram in a test set, they validated it in a subsequent dataset. While this nomogram refines the estimates of prognosis using CTC, it is not clear that differential treatment strategies based on these estimates (such as giving chemotherapy instead of endocrine therapy for patients with estrogen receptor (ER)-positive tumors, or using combination or higher dose chemotherapy instead of standard dose single-agent treatment) will improve patient outcomes. Therefore, it has yet to be accepted for clinical use, but it is an example of how one might incorporate CTC levels with other important prognostic factors to perhaps tailor specific treatment regimens for patients with metastatic breast cancer.

Other clinical trials to address clinical utility of CTC in metastatic breast cancer are ongoing. For example, two interventional trials currently underway are the French “CirCe01” and the “STIC.” The former trial has a design similar to SWOG S0500, but it aims to address early discontinuation of chemotherapies in more heavily treated patients (3rd line of chemotherapy) [42]. On the other hand, the STIC CTC trial evaluates CTC-guided hormone therapy vs. chemotherapy decision as first line treatment for metastatic, hormone-receptor positive, breast cancers (ClinicalTrials.gov Identifier: NCT01710605) [42].

In conclusion, the weight of the evidence strongly demonstrates that the presence of CTC has prognostic significance in patients with metastatic breast cancer. However, it is not clear that patients with elevated CTC prior to starting a new therapy (first or later line) should be treated differently than they would if CTC were not known, and thus no CTC assay has clinical utility in this setting. Further, although it appears that patients who do not experience a “CTC response” at an early follow-up time-point are probably not receiving active therapy, the available data (such as from S0500) do not support changing therapy early. Nonetheless, the data do consistently suggest that a rising CTC level during treatment follow-up is very likely associated with progressive disease, as measured by classic clinical and radiographic evaluation. Thus, one might conclude that if a patient with metastatic breast cancer receiving treatment does not have evidence of progression by clinical indications or rising circulating soluble tumor markers (such as CA15-3/CA27.29 or CEA) or CTC, there is probably no indication for radiographic imaging until one of these suggests the need to do so.

Analyses of CTC Beyond Enumeration

Although enumeration of CTC using the presently applied algorithms is prognostic, none of these assays perfectly identifies patients who are likely to be cured versus those who have a very short, and poor, expected OS (Fig. 3). Therefore, it has been of interest to further characterize, phenotype, and genotype CTC. Moreover, CTC analysis might provide more accurate predictive information for a subsequent therapy under consideration, and finally CTC characterization might improve fundamental understanding of the metastatic process.

Improving CTC Prognostic Information

As noted, clinical data combined with CTC enumeration have been shown to be more accurate in predicting patient outcome than CTC alone [41]. However, clinical features are not terribly reproducible, and therefore, efforts have been made to further refine CTC prognostication. These efforts have been directed either towards capturing CTC that may not express epithelial markers, by reviewing CTC that are commonly captured in currently available CTC assays, or both.

CTC Epithelial to Mesenchymal Transformation As noted, almost all CTC capture strategies only enrich and identify cells that express epithelial markers (EpCAM, CK). A growing body of evidence suggests that malignant epithelial cells undergo an epithelial-mesenchymal transition (EMT), in which expression of EpCAM and CK is down-regulated [43, 44]. Such cells, which may actually be the tumor/metastases-initiating cells of concern, would obviously not be detected in many of the assays described above. Therefore several investigators have developed new enrichment strategies that are independent of epithelial marker expression, or even if capture is based on EpCAM, subsequent characterization for mesenchymal phenotype [16, 45–47]. For example, Yu et al. [16] have reported that dual-colorimetric RNA-*in situ* hybridization (ISH) identified cells captured by the EpCAM-coated CTC-chip that expressed mesenchymal transcripts. Importantly, presence of these mesenchymal-like CTC was associated with disease progression in this small series of breast cancer patients. While it is unclear that detection of these cells will provide more clinically useful information than enumeration of CTC by systems such as CellSearch®, clearly the presence of these cells in the circulation provides insight into the metastatic process.

CTC Clusters Several investigators have now reported the presence of not only single CTC, but also the presence of CTC clusters in patients with lung, renal, prostate, and breast cancer [16, 40, 48–51]. Tumor cell clusters, designated as circulating tumor microemboli (CTM), are defined as a group of CTC containing three or more distinct nuclei with contiguous cytoplasm membranes (Fig. 4). CTM may occur due to many mechanisms, including maintenance of cell-to-cell signals, production of

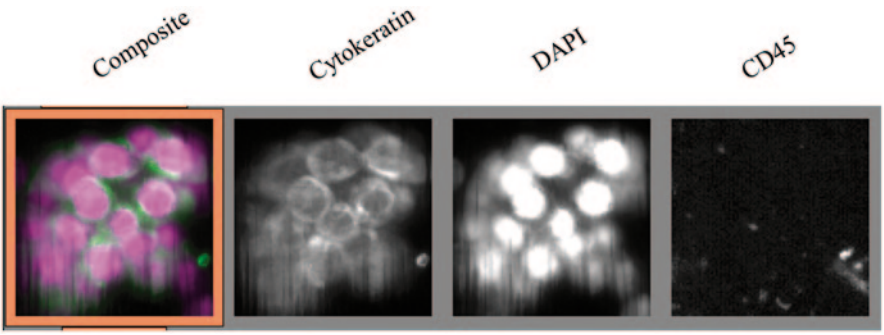

Fig. 4 Example of a CTC-cluster isolated by CellSearch® technology

autocrine pro-migration factors, and protection from immunological response [48]. All of these might suggest a survival advantage of CTM over single CTC. Recently, Hou JM et al. [40, 48] have reported that CTM did not express either proliferative or apoptotic markers, suggesting relative resistance to cytotoxic drugs and protection from anoikis. Indeed, CTM were associated with worse survival in SCLC in this study [40]. Likewise, Yu et al. [16] have shown that CTC clusters were strongly positive for mesenchymal and weakly positive for epithelial markers. They hypothesize that this could be either the result of the transformation of a pre-existing cluster from the EMT or the result of the proliferation of single CTC that underwent EMT within the cluster. Likewise, Aceto et al. [51] have shown that the presence of CTC clusters isolated by a novel microfluidic device in blood from patients with metastatic breast cancer and prostate cancer was associated with shorter PFS. Therefore, quantification of CTM might add additional prognostic information to simple CTC-enumeration, although the evidence to suggest evaluation of CTM should be used to direct care is still lacking.

Phenotyping and Genotyping CTC for Prediction of Response to Targeted Therapy

The era of "precision medicine" promises the use of targeted therapies that are specific to the patient's cancer. To accomplish this, the clinician requires precise and accurate predictive markers, such as ER to direct endocrine therapy and HER2 for anti-HER2 therapies [52, 53]. Primary tumor tissue is routinely stained to determine these markers, but discordance between primary and metastatic tumors has stimulated re-biopsy of metastatic sites [54]. However, this strategy is invasive, and the metastasis is not always easily accessible or cannot be safely approached. Further, a single biopsy site may not reflect the entire heterogeneity of the whole body tumor burden, and finally, serial multiple biopsies at each progression are impractical. Therefore, if CTC reflect the underlying tumor, they could be used as a convenient, safer, and perhaps more informative "liquid biopsy" [55, 56].

Several investigators have reported the ability to phenotype CTC for a variety of biomarkers that might be important in breast cancer management, including ER [57–62], HER2 [36, 63–66], Ki-67 [61], BCL-2 [61, 67], apoptosis (M-30) [67–69], IGFR1 [70], EGFR [71], PI3K protein [72], and gammaH2AX [73]. For example, Paoletti et al. [61] have recently reported development and analytical validity of a CTC-endocrine therapy index (CTC-ETI). The CTC-ETI incorporates a weighted semi-quantitative assessment of relative expression on CTC of ER and BCL2 (both of which predict sensitivity to endocrine therapy) and HER2 and Ki-67 (which predict resistance to endocrine therapy). In a pilot clinical study, this assay has high analytical validation when generated using the CellSearch® system, even among different, trained operators. As expected, the CTC-ETI varied widely among 50 patients with ER-positive metastatic breast cancer (Fig. 5). Therefore, CTC-ETI may identify patients with ER-positive metastatic breast cancer whose cancers are refractory to endocrine therapy and who would be better served by treatment with chemotherapy, as if their cancers were ER negative. A prospective trial to address this issue is now underway in North America (the COMETI trial, ClinicalTrials.gov Identifier: NCT01701050).

Recently, the ability to detect multiple important somatic mutations or other genomic alterations in ptDNA has been reported and these alterations might provide insight into therapeutic resistance or serve as targets for therapy [74–77]. It is unclear if ptDNA and cell-bound CTC-DNA analyses provide identical, similar, or different results. Since ptDNA must come from lysed cells, it may not reflect the genotype of viable tumor, whereas CTC are, at least, intact. However, as noted most CTC capture strategies are enrichment, and not purification, steps, and thus genetic analyses are limited by the presence of contaminating leukocytes. Technologies, such as DEPArray™ (Silicon Biosystems, Italy), have been developed to purify single CTC from blood for analysis on pre-enriched samples [78, 79]. Alternatively, Yu et al. [80] have cultured *ex vivo* CTC isolated from 6/36 patients with metastatic ER-positive breast cancer using the CTC-i-Chip. CTC were screened for mutations in a panel of 1000 annotated cancer genes with a hybrid-capture based next generation sequencing (NGS) platform. They found pre-existing mutations and newly acquired mutations in the *PIK3CA* gene, as well as in *ESR1, TP53, KRAS*, and fibroblast growth factor receptor-2 (*FGFR2*). Although the clinical utility of this technology is far from proven, these advances will permit comparison of genomic analyses of ptDNA vs. CTC-DNA, which may or may not be the same.

CTC representation of Tumor Heterogeneity Tumor heterogeneity has been recognized for more than 150 years, and more recently has been identified as the main driver for treatment resistance [81]. Therefore, a better understanding of an individual's tumor heterogeneity, especially over time in response to treatment, might help improve personalized therapy. As noted, biopsies only permit evaluation of a single site, and serial biopsies are impractical. Thus, CTC phenotyping and genotyping might be a more accurate, and practical, approach to monitor tumor heterogeneity [82].

For example, in our CTC-ETI pilot study, we observed enormous intra-patient CTC-biomarker heterogeneity (Fig. 5) [61]. Patient #4 in Fig. 5 serves as an illus-

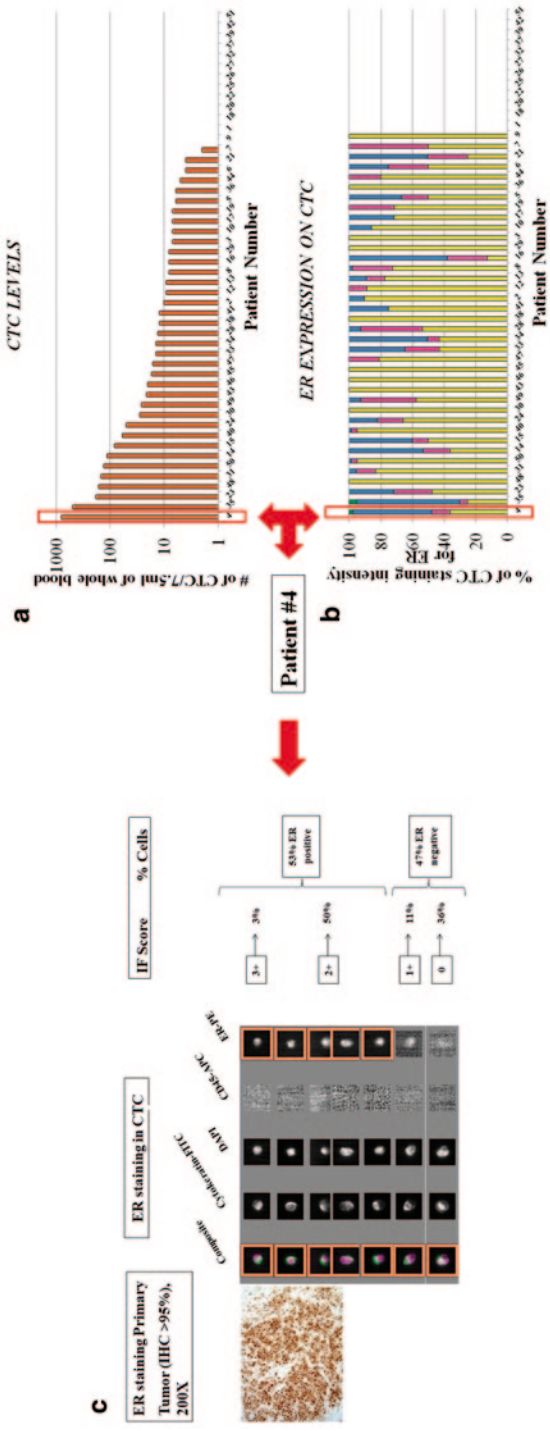

Fig. 5 Heterogeneity of CTC-ER biomarker staining for patients with ER-positive metastatic breast cancer. **Panel A**: *Bars* represent the CTC count/7.5 ml whole blood for each of 50 patients entered into a pilot study of analytical validity. **Panel B**: *Stacked bars* represent the CTC-ER expression for each patient as determined by immunofluorescent staining. *Yellow* = ER 0; *Pink* = ER 1+; *Blue* = ER 2+; *Green* = ER 3+ as determined by visual inspection of CellSearch images. **Panel C**: Immunohistochemistry staining of the primary breast cancer and representative CellSearch images (from ~1000 reviewed) of CTC-ER ranging from 0, 1+, 2+, and 3+ from patient #4, who is represented in Panels A and B as the first bar on the graphs (*red arrow*). Nearly 100% of the cells in this patient's primary tumor stained positive (by IHC), whereas 3, 50, 11, and 36% of the CTC were 3+, 2+, 1+, and 0. By convention, 0, 1+ are considered "negative" and 2, 3+ are considered "positive" for CTC-ER staining. (Adapted with permission from [61]. Copyright © 2014, American Association for Cancer Research. All rights reserved)

tration of this heterogeneity. She had 790 CTC/7.5 ml WB at the time of the blood draw. Nearly one-half (46%) of these CTC were ER-negative, while her primary tumor was originally ≥95% ER-positive. We hypothesize that CTC-biomarker changes might provide insight into whether a patient's treatment should be changed by virtue of emergence of biomarker target disappearance, or other targeted therapies should be added, by virtue of appearance of new biomarker targets.

In a similar manner, a multicenter Phase II trial conducted by Pestrin et al. [83] was designed to evaluate the activity of anti-HER2 therapy in metastatic breast cancer patients with HER2-negative primary tumors and HER2-positive CTC. Seven percent of these patients had positive CTC-HER2 and were treated in a Phase II study with lapatinib, but unfortunately no responses were observed. Two larger, ongoing trials (DETECTIII, ClinicalTrials.gov Identifier: NCT01619111; and CirCEX1 ClinicalTrials.gov Identifier: NCT01975142) are similarly designed to test the efficacy of HER2-targeted therapy in patients with initial HER2-negative metastatic breast cancer but who are found to have HER2-positive CTC [42].

Moreover, CTC biomarker changes, in addition to CTC levels, might be used as an early predictive marker for treatment response. For example, a targeted agent might either downregulate its target or induce other downstream gene expression changes. Although use of CTC for this indication has not been reported, one might be able to use circulating CTC-biomarker changes as a pharmacodynamic tool to determine if the agent is, indeed, hitting the appropriate target.

Finally, a critical issue is whether circulating markers (CTC, ptDNA) truly represent the underling malignant tissue. It is possible that either ptDNA or CTC arise from cells that are either already dead, or are non-viable and have merely detached from the body of the tumor into the circulation. Clearly, some form of CTC must be responsible for subsequent metastases, but it is unknown if the CTC that are captured and characterized by currently available technologies are truly the cells with malignant potential. At the least, CTC identified by RT-PCR or by CellSearch® do represent malignant behavior, by virtue of their proven prognostic effects. However, we do not know if they, themselves, are the "tumor/metastases initiator" cells or merely symbolic of those that are but are not being captured, such as cells undergoing EMT as discussed above. Several investigators have reported considerable discordance between CTC and tissue biomarker results such as ER staining [57–62, 84]. However, these are difficult studies to do since often one is comparing the CTC-phenotype with a primary tissue biopsy, or even with a metastatic biopsy, that was performed months or even years before. Moreover, as noted, the single site biopsy may, in itself, not represent the entire body tumor burden. Therefore, while of interest, it is unlikely that these types of studies will really provide much clinically useful information. Rather, the real proof that a CTC-biomarker has clinical utility will only be generated by correlating the CTC-phenotype/genotype with clinical outcomes in the appropriate use context.

Conclusion

Several studies have now demonstrated analytical and clinical validity, as defined by EGAPP, for prognosis of CTC enumeration in early and metastatic breast cancer. However, the clinical utility of monitoring CTC continues to be controversial. Appealing results in phenotyping and genotyping single-cell CTC have been generated. Nonetheless, the clinical role of these findings is still under investigation. Additional definitive trials are needed to achieve clinical utility in order to be used to help guide treatment.

As noted, genotyping of ptDNA for somatic mutations has recently generated excitement in the field [74–77]. This topic is covered in a separate chapter of this book. However, we believe that CTC as well as ptDNA are complementary approaches. In addition to the possibility that the DNA alterations may not be the same from the two specimen types, CTC characterization permits exploration of expression of the genes, which is likely to be plastic in response to environmental stresses, as opposed to the fixed genetic changes associated with cancer. Ongoing and future studies will need to consider these issues as we move into the era of "liquid biopsies."

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Circulating Plasma Tumor DNA

Heather A. Parsons, Julia A. Beaver and Ben H. Park

Abstract Circulating cell-free DNA (ccfDNA)—first identified in 1947—is "naked" DNA that is free-floating in the blood, and derived from both normal and diseased cells. In the 1970s, scientists observed that patients with cancer had elevated levels of ccfDNA as compared to their healthy, cancer-free counterparts. The maternal fetal medicine community first developed techniques to identify the small fraction of fetal-derived ccfDNA for diagnostic purposes. Similarly, due to the presence of tumor-specific (somatic) variations in all cancers, the fraction of circulating cell-free plasma tumor DNA (ptDNA) in the larger pool of ccfDNA derived from normal cells can serve as extremely specific blood-based biomarkers for a patient's cancer. In theory this "liquid biopsy" can provide a real-time assessment of molecular tumor genotype (qualitative) and existing tumor burden (quantitative). Historically, the major limitation for ptDNA as a biomarker has been related to a low detection rate; however, current and developing techniques have improved sensitivity dramatically. In this chapter, we discuss these methods, including digital polymerase chain reaction and various approaches to tagged next-generation sequencing.

Keywords Circulating cell-free DNA · Plasma tumor DNA · Cancer biomarker · Breast cancer biomarker · Digital PCR · Tagged next-generation sequencing

Background: Circulating Cell-Free DNA

In 1947, Mandel and Métais first identified circulating cell-free DNA (ccfDNA) in human blood [1]. This "naked" DNA is free-floating in the blood and is derived from both normal and diseased cells. Interest in the utility of ccfDNA for medical purposes returned almost five decades later when scientists began to explore uses for ccfDNA in maternal-fetal medicine and oncology. More recently, techniques to

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identify and quantitate ccfDNA have been applied to other disease processes such as sepsis, myocardial infarction, stroke and diabetes [2–5].

Currently, it is thought that ccfDNA enters the circulation by a few mechanisms. Macrophages and other scavenger cells, which phagocytose necrotic and apoptotic cells, can release DNA and cellular debris into the surrounding environment [6]. It has also been suggested that direct secretion of ccfDNA into the plasma is possible [7]. ccfDNA is found in various human secretions, such as whole blood, serum, plasma and urine. These ccfDNA fragments are generally quite small, averaging ~180 base pairs (bp) in length in one study, but can be as large as ~10,000 bp [8, 9]. The half-life of ccfDNA is relatively short, varying from ~15 min to a few hours. It is quickly cleared by the kidney and the liver [8, 10, 11]. Patients with inflammatory conditions such as metastatic cancer, trauma, myocardial infarction and sepsis have on average a higher concentration of overall ccfDNA than normal controls [12–17]. Given these properties, ccfDNA has the potential to play an important role in the diagnosis, prognosis and monitoring of various disease states, including cancer.

The maternal fetal medicine community was the first to pursue ccfDNA as a diagnostic tool. In the 1970s, researchers first identified fetal cells in the maternal circulation, and subsequent work in 1997 demonstrated that a small percentage of ccfDNA originating from the fetus could also be found in the maternal blood [18–20]. The size of fetal-derived ccfDNA was determined by Li et al. to be <300bp, whereas ccfDNA fragments derived from maternal cells are >300bp [21]. The fetal fraction of ccfDNA likely accounts for approximately 3–6% of the total ccfDNA population although studies differ slightly, and as gestation continues the percentage may rise [22, 23].

Given this small percentage of fetal ccfDNA, different technologies with varying sensitivities have been examined to detect and quantify this minority population of DNA. Currently, next generation sequencing (NGS) of maternal plasma DNA is used in the clinic to detect increased representations of chromosomes 21, 18 and 13 in order to identify a fetus harboring these chromosomal abnormalities [24, 25]. In addition, advances in detection of ccfDNA from maternal plasma have allowed for Rhesus D genotyping and the detection of paternally inherited genetic disorders [26, 27]. Thus the feasibility of detecting relatively rare fetal DNA molecules in maternal peripheral blood was established with these earlier studies and set the stage for applications of these technologies for cancer diagnostics.

Applications in Cancer Diagnostics

Leon et al. first showed with radioimmunoassays that, on average, cancer patients had an increased amount of ccfDNA as compared to healthy patients without cancer [28]. However, the range of ccfDNA concentrations in cancer patients varied substantially between 0 and >1000 ng/ml of blood with normal subjects typically exhibiting ccfDNA concentrations between 0 and 100 ng/ml [10, 29–31]. Given the

significant overlap of ccfDNA concentrations in normal and cancer patients, the total quantity of ccfDNA would not prove to be a reliable diagnostic tool.

Therefore similar to its use in fetal DNA detection from maternal blood, the promise of ccfDNA in cancer diagnostics and monitoring comes from the ability to detect the small population of cell-free plasma tumor DNA (ptDNA) from the larger population of normal ccfDNA through the identification of tumor-specific (somatic) variations. It should be noted that the plasma fraction of blood contains *cell-free*, ptDNA and that this is distinct from current efforts studying circulating tumor cells (CTCs) as cancer biomarkers. Studies have demonstrated that for a patient with a tumor containing approximately 3×10^{10} cells, tumor DNA comprises 3.3 % of the ccfDNA found in the blood stream daily [14]. In addition, multiple groups have demonstrated that the size of ptDNA is smaller than that of ccfDNA derived from normal cells, and typically ranges from 70–200 bp [9, 14, 32]. Given the decades of research demonstrating that cancer DNA harbors somatic changes that include epigenetic alterations and mutations, as well as rearrangements resulting from translocations, deletions, and amplifications, ptDNA therefore also harbors these genetic and epigenetic changes. Specifically the ptDNA contains the same mutations and genomic rearrangements in tumor suppressor genes or oncogenes, which are driving the development and progression of the cancer. In addition, so called "passenger" mutations or genetic alterations that are likely the result of genetic instability but not of functional consequence, are still somatic changes, and therefore both driver and passenger mutations/alterations could serve as potential cancer markers through the use of ptDNA [33].

Thus, the ability to use a patient's blood sample to perform a "liquid biopsy" allows for the identification of residual micrometastatic cancer, and for a non-invasive test to query for specific mutations without any surgical intervention. In theory the liquid biopsy would be a real-time assessment of molecular tumor genotype (qualitative) and existing tumor burden (quantitative). The short half-life of ptDNA lends itself as a reliable marker of tumor burden and possibly response to therapies. Therefore, the potential applications for clinical oncology that stem from ptDNA detection are vast. This technology has the capacity to completely change the paradigm of how clinicians make decisions regarding adjuvant systemic therapies as well as therapies for metastatic disease. In the adjuvant setting one could theoretically test each patient post-surgery to determine if there is residual micrometastatic disease in order to make an informed assessment of the need for adjuvant systemic treatment and to prevent the administration of toxic systemic therapies if unnecessary. Additionally the validation of this technique could direct the changing of chemo/hormonal/biologic therapies during adjuvant or metastatic therapies, if a decrease in personalized DNA markers does not occur, suggesting that the current regimen is ineffective. Furthermore, real-time knowledge of the molecular profile of a tumor without the need for a biopsy would also help to drive rational therapies and clinical trial enrollment and to create new surrogate endpoints allowing for a more rapid pace of drug approval. Until recently, the technology to realize these applications had not existed. However, with the introduction, and improvement upon, NGS technologies and digital and emulsion polymerase chain reaction (PCR), there

is now the capacity to identify DNA-based genetic biomarkers that are unique to a patient's cancer and to perform subsequent analysis of the patient's plasma to quantify the amount of residual tumor burden via the measurement of ptDNA.

Investigators have previously shown that mutations in proto-oncogenes and tumor suppressor genes found in tumor tissues can be detected in the corresponding plasma using the above-mentioned technologies. Mutations in *TP53* for example were found in 42.9% of the plasma DNA samples from patients with *TP53* mutations in their tumor [34]. Similarly, the common *V600E BRAF* mutation has been shown to be present in ptDNA from patients and has been used to monitor patient responses for those receiving BRAF-directed therapy [35]. Multiple studies have examined mutant *KRAS* in a primary tumor and identified corresponding *KRAS* mutations in the plasma. However these studies demonstrated varying sensitivities for ptDNA detection ranging from 27 to 100% [36–38].

In 2005, investigators used a digital PCR (dPCR)-based technique termed BEAMing (Beads, Emulsion, Amplification and Magnetics) to identify patients with point mutations in mutant *APC* molecules in both early stage and metastatic colorectal cancer patients [14]. The authors reported a 100% concordance between *APC* mutations in the plasma of six patients with metastatic colorectal cancer and the known *APC* mutations within their solid tumors. They also analyzed 16 patients with early stage colorectal cancer with known *APC* mutations and found that 63% had detectable mutant *APC* DNA in their plasma. On average they described that 11.1% of the total *APC* gene fragments in the plasma of metastatic patients were mutant compared to 0.04–0.9% in early stage patients, explaining the likely reason for decreased sensitivity of detection in the early stage patients. Subsequent work by this group examined the plasma of 18 patients undergoing therapy for colorectal cancer and correlated the amount of ptDNA with tumor burden using BEAMing for four genes (*APC, PIK3CA, TP53* and *KRAS*) [8]. The investigators identified ptDNA in the plasma of all 18 patients, with ptDNA levels varying over a wide range. These patients were followed after surgery with subsequent blood draws and assessment of their ptDNA during chemotherapy and surveillance seemed to correlate with clinical status.

Taniguchi and colleagues recently demonstrated the ability to detect second site T790M epidermal growth factor receptor (*EGFR*) mutations in non-small cell lung cancer patients treated with EGFR kinase inhibitors [39]. Interestingly they also detected the same mutation in a significant fraction of patients that were not treated with these inhibitors suggesting the existence of a minority population of cancer cells that are clonally selected following kinase inhibitor therapies. In addition, two separate studies reported the use of BEAMing to detect the emergence of *KRAS* mutations that conferred resistance to antibody mediated EGFR-targeted therapies [40, 41]. Taken together, these studies suggest that assessment of ptDNA status in treated cancer patients may have a powerful potential to monitor for the emergence of resistant clones with a particular mutation or genotype.

For hematologic malignancies there are established techniques available to detect minimal residual disease in the blood of patients exploiting tumor specific rearrangements in cancer DNA. For example, in Chronic Myeloid Leukemia (CML)

detection of the BCR-ABL fusion transcript by quantitative real-time PCR (qPCR) using PCR primers specific for the fusion transcript has allowed for real-time monitoring of the disease and the ability to follow response to treatment using peripheral blood or bone marrow [42]. The capacity to perform this sort of assay in solid tumor malignancies has not yet come into clinical practice since it is rare to identify a recurrent somatic rearrangement in solid tumor malignancies. In addition, because leukemias are by definition a blood based disease, cells in the peripheral blood and/or bone marrow further facilitate the use of qPCR of fusion transcripts as a reliable measure of disease burden. In contrast, the ability to identify CTCs for most solid malignancies is still hindered by low sensitivity though newer methods for improving capture and therefore sensitivity of isolating CTCs have shown promise [43].

With the understanding that tumor cells "shed" DNA as ccfDNA, and the advent of NGS technologies, several groups have now demonstrated the ability to identify tumor-specific genetic rearrangements that are patient-specific. The technology of "mate paired end" sequencing can identify many of the genomic alterations found in cancers including mutations, translocations, amplifications, deletions, etc. [44, 45]. In 2008, Campbell et al. identified the presence of multiple patient-specific somatic rearrangements in cancer using massively parallel sequencing [44]. To date, hundreds of cancers have been subjected to this form of NGS, with rearrangements found in virtually all samples and the majority of samples containing more than 10 rearrangements. Using a technology termed PARE for Personalized Analysis of Rearranged, investigators from two groups identified somatic rearrangements in primary tumor tissue, designed unique patient-specific PCR markers and were able to detect and quantify these markers in the plasma of five cancer patients [46, 47]. The sensitivity for detecting rearranged DNA was calculated to be one cancer genome equivalent among 390,000 normal genome equivalents. To minimize false negative results both groups recommended the use of multiple somatic rearrangement markers to increase the reliability of detection. Theoretically these markers should be 100 % specific, since each marker is validated to detect only tumor-specific rearrangements.

More recently, further work has verified that genomic rearrangements can be directly identified from the plasma of metastatic cancer patients using NGS and specific bioinformatics criteria [48]. In this work, Leary et al. expand upon the use of NGS by analyzing the copy number of chromosomes found in the plasma of metastatic cancer patients compared to healthy controls. Similar to efforts in fetal medicine using NGS of maternal plasma to query copy number changes of various chromosomes, this study demonstrated a 0.61–1.97-fold copy number increase in the plasma of cancer patients compared to healthy controls. Thus, it is possible to identify patients with metastatic cancer compared to normal controls by assessing copy number alterations present in ccfDNA. The study suggests that this approach is feasible if the percentage of ptDNA compared to ccfDNA is at least 0.75 %, and at this level of ptDNA the assay had a sensitivity of > 90 % and a specificity of > 99 %. However it should be noted that the sensitivity and specificity of this technique is dependent upon the amount of sequencing data collected, which reflects how many molecules of DNA are assayed for each individual.

As alluded to above, the differing levels of sensitivity between these ptDNA detection studies may reflect the number of genome equivalents sampled by the investigators as well as the techniques used. With the knowledge that there is a greater amount of ccfDNA (both tumor and normal derived) in patients with metastatic cancer compared to those with early stage disease, increasing the number of genome equivalents sampled in patients with early stage cancer is likely to improve the sensitivity of these assays [28]. In addition, tumor heterogeneity can result in a low clonal frequency of a given mutation within a solid tumor mass. In this situation, wild-type sequences shed from other tumor cells and normal cells may significantly decrease the amount of ptDNA for the given mutation, and will not be reflective of the overall tumor burden [36, 49, 50]. Similar issues with dilution of ptDNA by total ccfDNA have been hypothesized to cause difficulties in the detection of loss of heterozygosity (LOH) in ptDNA in a number of studies [4, 10, 52, 53]. As discussed above, the use of multiple somatic alterations as markers can mitigate some of these concerns. Investigators have also discussed the possibility of using stool, urine and increased volumes of plasma to improve the sensitivity of detecting rare mutations within ccfDNA [54].

The ratio of long to short DNA fragments (DNA integrity) is also being studied as a possible biomarker of tumor presence and burden. It is technically feasible to detect non-coding repetitive DNA sequences such as ALU sequences in ccfDNA, and the length and ratio of these markers can determine the DNA integrity within ccfDNA. This has led to studies examining whether changes in DNA-based markers are prognostic and/or diagnostic for multiple different cancers [35, 53]. Testing for DNA integrity could be broadly applicable for many cancer subtypes and therefore could also improve sensitivities of current assays. In addition, studies examining epigenetic alterations in the plasma of patients with cancer, specifically detection of promoter hypermethylation by methylation-specific PCR have been performed in various cancer subtypes and hold significant promise as another biomarker of cancer burden [55, 56].

Breast-Cancer Specific Applications

Because of the prevalence of breast cancer and questions about which patients with early stage disease will benefit from further systemic treatment, and which treatments would benefit patients with metastatic disease, ptDNA as a biomarker of disease burden and its use for mutation profiling in breast cancer are of strong interest.

In the early stage breast cancer setting, issues of sensitivity become even more important as patients with a lower burden of disease will tend to have lower levels of ccfDNA. One group recently looked at a prospective cohort of 29 patients with early stage breast cancer [57]. Most of these patients had stage I disease that was predominantly estrogen receptor (ER)-positive and HER2-negative. Blood samples were drawn at the time of enrollment, after diagnosis but before surgery. Focusing on the *PIK3CA* gene, the tumor tissue first underwent Sanger sequencing, and then

droplet digital PCR (ddPCR) with probes directed at the known hotspot mutations H1047R and E545K. Sanger sequencing identified seven *PIK3CA* H1047R mutations and 3 E545K mutations. ddPCR identified these mutations plus an additional two H1047R and one E545K mutations, plus one tumor with both of the mutations. The investigators then performed ddPCR, using probes for both mutations on the matched, preoperative plasma samples. They were able to identify the same mutations in all but one sample, for a sensitivity of 93.3%. Additionally, they had no false positive results, for a specificity of 100%. In the post-operative setting, five patients had detectable ptDNA mutations [57]. One of those patients—who had had extremely aggressive, metaplastic disease at diagnosis as well as the highest mutational allelic fraction in her preoperative plasma sample—developed metastatic disease 26 months following her initial diagnosis. Interestingly, she had had a CT scan in the pre-operative setting that showed no evidence of metastatic disease. These results are exciting and are an important step toward demonstrating that ptDNA may be a valuable biomarker in the early-stage setting, in addition to a marker of metastatic disease. However, the study is small, and larger, longer-term studies are needed to validate these findings.

In the metastatic setting, investigators have looked at frequent or "hotspot" *PIK-3CA* mutations (a gene commonly mutated in breast and other cancers) in metastatic breast cancer patients and their corresponding plasma using BEAMing. In a retrospective study, 49 archival matched tumor and plasma samples were examined for exon 9 and exon 20 hotspot *PIK3CA* mutations using BEAMing of both tumor tissues and plasma. They found 100% concordance between the presence and type of *PIK3CA* mutations in the tumor and plasma from these patient samples. However a subsequent prospective study by the same group identified an approximately 70% concordance of *PIK3CA* mutational status between tumor tissues and peripheral blood. These seemingly disparate results may be related to tumor heterogeneity and clonal evolution, since the prospective study used archived primary cancer tissues and compared them to blood drawn at the time of study entry—up to nine years later—as the source for tumor mutational and ptDNA analyses, respectively. Change in *PIK3CA* status, was only seen in patients whose tumors were harvested 3 or more years prior to blood draw for ptDNA analysis [58]. These results raise concerns regarding the use of archival specimens when assessing mutation status and genetic profiling in cancer patients with metastatic disease, as the mutational and genomic spectrum may differ significantly between primary and metastatic sites of disease as has been recently reported [59].

Another group recently evaluated matched tumor and plasma samples from 17 breast cancer patients with metastatic disease [60]. The patients primarily had ER-positive, HER2-negative breast cancer that had been previously treated. Via a commercially available NGS panel, the Ion AmpliSeq™ Cancer Hotspot Panel v2, the authors first sequenced tumors—both primary and metastatic—to identify mutations. Sixty of the 69 tumors had evaluable results. They performed NGS with the same panel on 31 plasma samples, and had evaluable results from each assay. The investigators identified mutations in tumors from 12 of 17 patients, and in plasma samples from 12 of 17 patients. They confirmed these mutations using another NGS

platform. Interestingly, in two patients, a mutation that was found in the plasma was not also found in the synchronous tumor, arguing that plasma might yield further information than one metastatic biopsy can provide. Though larger, prospectively enrolled studies of ptDNA in metastatic breast cancer are needed, this report provides further clinical evidence that ptDNA is likely to be a valuable biomarker in this setting.

Technologies to Detect ptDNA

ccfDNA can be isolated from blood, plasma, stool and urine of patients. In maternal-fetal medicine most studies have employed the use of column-based extraction methods and/or other automated techniques [61–63]. Once isolated, total ccfDNA can be measured by fluorescence-based methods utilizing PicoGreen staining or UV spectrometry, or by quantitative real-time PCR with detection by intercalating dyes such as SYBR green or with dual labeled fluorescent/quencher probes (for example, TaqMan technology). For the purpose of detecting variant molecules within ccfDNA such as fetal DNA or cancer ptDNA, a number of methods have been developed. They can broadly be grouped into two approaches: methods based on dPCR and on those based on NGS. Both approaches have strengths and limitations, and each may find their place in clinical medicine in the future.

Digital PCR

In 1992, Sykes et al. first described the concept behind digital PCR (dPCR), which assesses individual DNA molecules after serial dilution and/or separation such that the end read-out yields individual reactions with a binary result of either the presence or absence of variant DNA [64]. A few years later, in 1999, Bert Vogelstein and Kenneth Kinzler at Johns Hopkins University coined the term, and demonstrated dPCR's ability to detect rare mutations in colon cancer patients [65]. Because ptDNA represents only a small fraction of total ccfDNA, dPCR is particularly well suited to its detection.

BEAMing—or Beads, Emulsion, Amplification, and Magnetics—is a first-generation dPCR technology developed to identify and quantitate rare genetic molecules found in a larger population of normal –or wild-type—DNA molecules [8, 66–68]. In BEAMing, single molecule PCRs are performed on magnetic beads in water-in-oil emulsions. Genetic variants are then subsequently quantified by flow cytometry. Subsequent dPCR platforms build on the strengths of BEAMing, but rely primarily on water-oil emulsion technology and the use of fluorescence-labeled TaqMan probes. Early in development are microfluidics-based platform that rely on the same underlying principles of dPCR, but we will not discuss these here.

Droplet Digital PCR

Droplet Digital PCR (ddPCR) is a variation of emulsion-based dPCR based technology. In ddPCR, the target sample is separated into tens of thousands of partitions of single molecules that individually undergo PCR. ddPCR technologies use specialized capillary equipment to generate emulsions that are in the nanoliter to picoliter size range, allowing for quite sensitive quantification of nucleic acid variants. In terms of strengths of this approach in evaluating ptDNA, ddPCR is extremely sensitive—able to detect one mutant event in 100,000 wild-type events. ddPCR is also relatively inexpensive and does not require complicated bioinformatics to evaluate the data, as compared to NGS. On the other hand, currently, ddPCR is only able to evaluate a limited number of base pair alterations within a single assay. Therefore, ddPCR currently can only be used for hotspot mutation detection, and cannot be applied to mutation discovery.

Step 1. Sample Preparation and Droplet Generation.

In this first step, PCR reactions are prepared using TaqMan hydrolysis probes in addition to the standard primers. After the samples are prepared, droplets are generated using the proprietary droplet generator and appropriate emulsion oil. This step divides each sample into tens of thousands of individual partitions with the goal of only one molecule of DNA per partition. This will allow for analysis via Poisson statistics.

Step 2. PCR Amplification of Sample.

Next, the sample undergoes traditional PCR thermal cycling.

Step 3. Droplet Reading and Analysis.

In the third step, the sample is placed in the droplet reader. If a droplet has a positive event—the molecule of interest is present and the hydrolysis probe binds and is then released—a fluorescent signal will be given off and detected by the reader. If it does not, no signal will be recorded and this will be considered a "negative" event.

Tagged Next-Generation Sequencing

Next-generation sequencing (NGS), the sequential identification of bases of small fragments of DNA massively and in parallel, has already made enormous contributions to disease research. It has also made its way in to the oncology clinic, with many cancer centers offering NGS analysis of patients' tumors, though this practice has not yet been prospectively validated in most clinical situations. However, some aspects of NGS that are acceptable in sequencing tumors prove problematic when moving into the realm of ptDNA. Because of the low frequency of ptDNA as compared to ccfDNA derived from normal cells, sensitivity of NGS is an issue that must be addressed in evaluating ptDNA. Though significantly more sensitive than the first generation sequencing technology of Sanger-sequencing, which has a detection sensitivity on the order of 20 %, because of PCR errors in library preparation and sequencing errors, NGS has a detection sensitivity of ~ 1 %. Though this is more

than adequate for tumor tissue mutation detection, this sensitivity is not enough for ptDNA, where allelic frequencies might easily be less than 1 %. The approaches discussed below aim to address this issue and make NGS an option for detection of ptDNA. The first technique, Tagged Amplicon Deep Sequencing addresses the issue of sensitivity by adding a targeted amplification step. It does not address the issues of PCR- and sequencing-introduced errors. The following two techniques, the Safe-Sequencing System and Duplex Sequencing, address both problems of low allelic frequency and PCR- and sequencing-related errors with methods that we describe below.

TAm-Seq: Tagged Amplicon Deep Sequencing

In 2012, in response to the problem of sensitivity of traditional NGS approaches when applied to ptDNA, Forshew et al. described a method for deep sequencing of ptDNA that they termed "TAm-Seq," or tagged-amplicon deep sequencing that amplifies and sequences regions from even very low allelic frequency ptDNA (Fig. 1) [69]. They found that this method enriched the sample for the sequence of interest, and so increasing the sensitivity as compared to standard NGS.

Step 1. Pre-Amplification of Regions of Interest.

In the first step, ccfDNA is isolated from the plasma and purified using standard protocols. Then, using pooled, target-specific primer pairs, multiple regions of the fragmented, ccfDNA genome are amplified in parallel. For instance, in their original paper, Forshew et al. pre-amplified coding regions of *TP53* and *PTEN*, and selected regions in *EGFR, BRAF, KRAS*, and *PIK3CA*.

Step 2. Target-Specific Amplification.

After step 1 is complete, in order to exclude non-specific products, that sample is then selectively amplified in multiple, single-plex PCRs. This step also serves to isolate each amplicon so that it can be tagged with a sample-specific barcode in the following step.

Step 3. Addition of Sequencing Adaptors and Barcodes.

Next, sequencing adaptors and sample-specific barcodes are attached to the amplicons using another round of PCR.

Step 4. Sequencing.

After step 3, the samples are pooled and purified. That pooled sample is then quantified and undergoes Illumina cluster generation, followed by single-end sequencing of 100 bases, and then a 10-base barcode (indexing) read.

Step 5. Analysis of Sequencing Data.

Once sequenced, data are aligned to the reference human genome. Using bioinformatics and other computational algorithms, putative genetic aberrations can be identified.

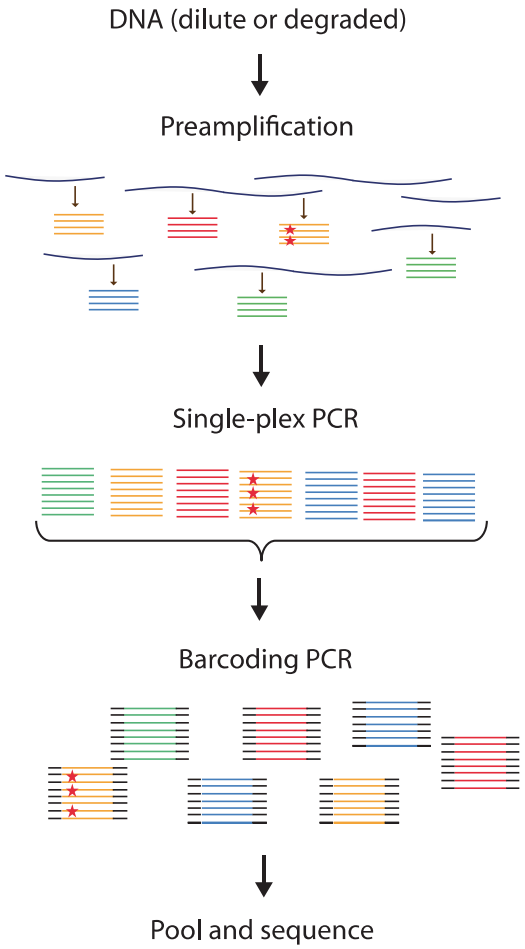

Fig. 1 Workflow overview. Multiple regions were amplified in parallel. An initial pre-amplification step was performed for 15 cycles using a pool of the target-specific primer pairs to pre- serve representation of all alleles in the template material. The schematic diagram shows DNA molecules that carry mutations (*red stars*) being amplified alongside wild-type molecules. Regions of interest in the pre-amplified material were then selectively amplified in individual (single-plex) PCR, thus excluding nonspecific products. Finally, sequencing adaptors and sample-specific barcodes were attached to the harvested amplicons in a further PCR [69]

SAFESeqS

In 2011, Kinde et al. described an approach to NGS that aimed to overcome the limitations brought on by the inherent NGS error rate of ~ 1 % [70]. In SAFESeqS, the authors assign a unique identifier—termed "UID"—to each template molecule, which then undergoes amplification, creating a "UID family." The sample is then sequenced redundantly and data is analyzed to identify true genetic variants (Fig. 2).

Step 1. Addition of Unique Identifiers.

In the first step, ccfDNA is isolated from the plasma according to standard protocols. Using a set of gene-specific primers selected to target the gene(s) of interest, this DNA is amplified via PCR. During this round, a UID is added to the amplified DNA as a random sequence of 14 DNA bases. Because each region of interest has two separate, complementary sequences—a coding and non-coding strand—each

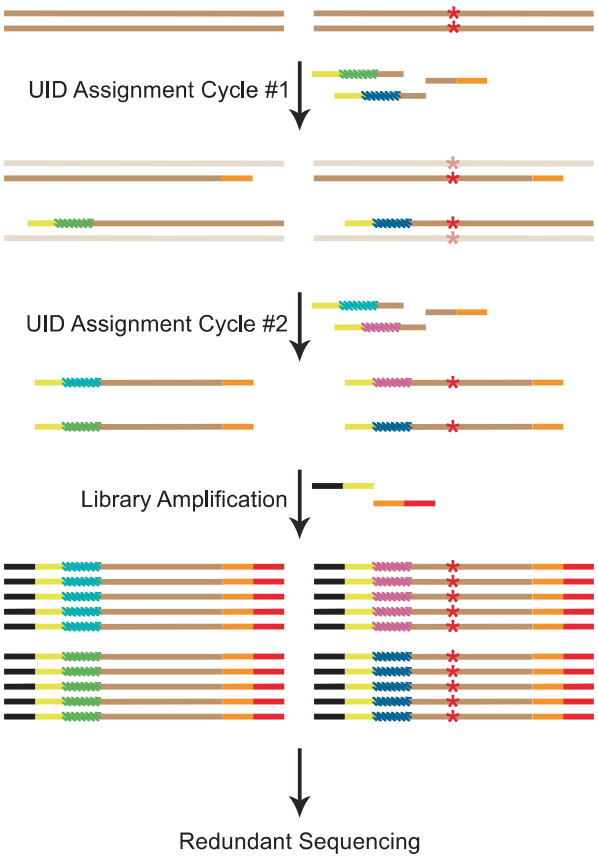

Fig. 2 Safe-SeqS with exogenous UIDs. DNA (sheared or unsheared) is amplified with a set of gene-specific primers. One of the primers has a random DNA sequence (e.g., a set of 14 Ns) that forms the unique identifier (UID) (variously colored bars), located 5′ to its gene-specific sequence, and both have sequences that permit universal amplification in the next step (*yellow* and *orange bars*). Two UID assignment cycles produce two fragments—each with a different UID—from each double-stranded template molecule, as shown. Subsequent PCR with universal primers, which also contain "grafting" sequences (*black* and *red bars*), produces UID families that are directly sequenced [70]

region will produce two complementary strands with two, distinct UIDs. During this first step, amplification also adds universal sequencing sites to each gene-specific sequence.

Step 2. Amplification Of UID Families And Addition of "Grafting" Sequences.

In the second step, the sample from Step 1 undergoes another round of PCR with universal primers, this time adding what the authors term "grafting" sequences. These enable binding of the sequences to be analyzed to the flow cell of the next-generation sequencer. This step amplifies the products from Step 1, producing UID families.

Step 3. Sequencing and Bioinformatic Analysis: Identification of "Supermutants."

In the third step, products from Step 2 then undergo sequencing using the available NGS platform. Sequencing data then undergoes analysis for identification of "supermutants." A supermutant is a UID family in which ≥95 % of family members have the same mutation. This approach, in combination with the most stringent base-calling algorithms, yields an error frequency of $\sim 1.4 \times 10^{-5}$, much improved over standard NGS approaches.

Duplex Sequencing

In 2012, in response to the same problem of NGS error rates, a group at the University of Washington led by Lawrence Loeb described their tagged-NGS approach, termed "Duplex Sequencing" [71]. In this method, Schmitt et al. take advantage of the double-stranded nature of DNA, independently tagging and sequencing each strand. True mutations should show up in each amplified copy of each strand; single copies of mutations are reflective of errors introduced by PCR or sequencing. This approach theoretically reduces the error rate to 5×10^{-8}. Though very promising, this approach has so far not been tested using ptDNA. The original paper used sheared DNA from a model system bacteriophage, and subsequent publications have examined its use in mitochondrial DNA [72] (Fig. 3).

Step 1. Adapter synthesis.

In step 1, duplex tags are synthesized from two complementary, overlapping oligonucleotides, which contain a random sequence of 12 bases, akin to the UID in SAFESeqS, as well as adapter sequences to enable the sample to bind to the NGS platform (figure TKTK). Duplex tags then undergo A-tailing, and prepared DNA undergoes T-tailing.

Step 2. Ligation.

In step 2, enabled by the A- and T-tailing of the tags and DNA of interest in step 1, tags are ligated to the DNA sample.

Step 3. PCR amplification with Addition of Flow-Cell Sequence Adapters.

In step 3, the duplex-tag-DNA-of-interest complexes undergo amplification via PCR. During this step, sequences that will bind the complex to the NGS platform's flow cell adaptor are also added. After this step, each original piece of DNA will be represented in two identifiably related, but distinct amplicons.

Step 4. Sequencing and Data Analysis.

Next, in step 4, the samples undergo sequencing on whatever NGS platform is chosen. The sequencing data is then analyzed. First, sequence reads that share a unique pair of tags are grouped into families. Again, each pair represents amplification of one double-stranded DNA fragment. True mutations will show up in both strands of the DNA and in all members of a particular family. Mutations showing up in only one of the pairs originated early in the process, during the first round of amplification. Mutations showing up in one or a few of the family members originated later—in sequencing or in later amplification.

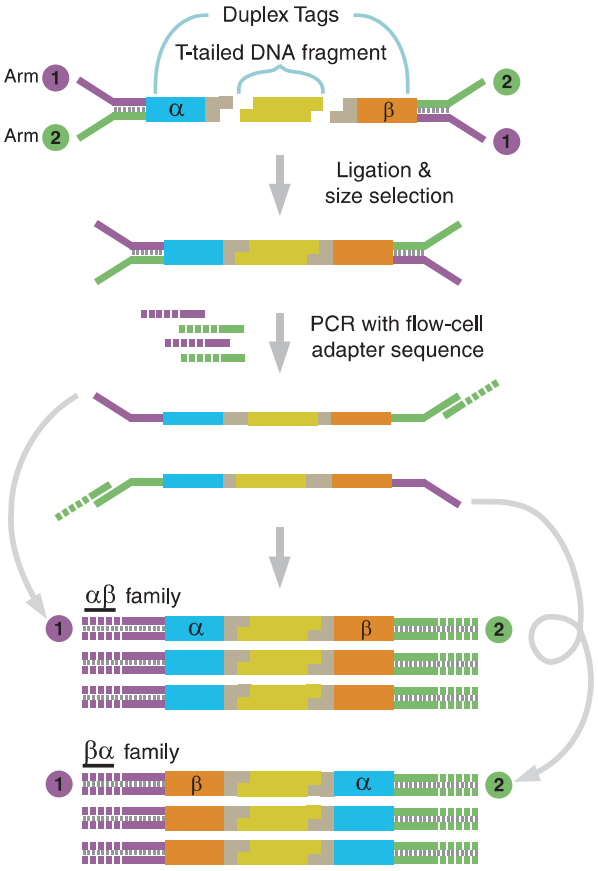

Fig. 3 Duplex Sequencing workflow. Sheared, T-tailed double-stranded DNA is ligated to A-tailed adapters. Because every adapter contains a Duplex Tag on each end, every DNA fragment becomes labeled with two distinct tag sequences (arbitrarily designated α and ß in the single fragment shown). PCR amplification with primers containing Illumina flow-cell–compatible tails is carried out to generate families of PCR duplicates. Two types of PCR products are produced from each DNA fragment. Those derived from one strand will have the α tag sequence adjacent to flow cell sequence 1 and the ß tag sequence adjacent to flow cell sequence 2. PCR products originating from the complementary strand are labeled reciprocally [71]

Future Directions and Conclusions

Future directions for the field of plasma tumor DNA as a cancer diagnostic include continued improvements in technology, potential new assays to measure circulating nucleic acids from body fluids and tissues, and verification of analytic validity, clinical validity and clinical utility. Although great progress has been made in exploiting ccfDNA for cancer and other disease states, there is much room for further discovery and progress. A current critical barrier is the difficulties in detecting a relatively small percentage of mutant or variant molecules within the vast majority

of normal/wild-type ccfDNA. Although in this chapter we described some of the current methods for quantification and detection of these small populations of ccfDNA, there is currently no industry standard or widespread clinical acceptance for the use of ccfDNA, nor a uniformly agreed upon platform. Future studies are needed to establish the best techniques to quantify, detect, and monitor ccfDNA, and appropriate criteria for ccfDNA surveillance will need to be validated with prospective clinical trials. Through the standardization and improved technologies to detect ccfDNA, the future holds great promise in developing and implementing clinical assays that will enable ccfDNA to help clinicians and their patients make better and more informed therapeutic decisions.

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